



EFFICACY OF SYNBIOTIC DIET INCORPORATING *Lactobacillus plantarum* L20 AND *Sargassum polycystum* FOR *Penaeus monodon* AGAINST ACUTE HEPATOPANCREATIC NECROSIS DISEASE

By

CHIN YONG KIT

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in
fulfilment of the requirement for the degree of Doctor of Philosophy

**EFFICACY OF SYNBIOTIC DIET INCORPORATING *Lactobacillus*
plantarum L20 AND *Sargassum polycystum* FOR *Penaeus monodon*
AGAINST ACUTE HEPATOPANCREATIC NECROSIS DISEASE**

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December 2024

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Acute hepatopancreatic necrosis disease (AHPND) has inflicted massive economic losses and posed serious threat to the development of penaeid shrimp aquaculture in Malaysia. Although antibiotics were applied for combating AHPND, but they caused antibiotic resistance emergence which directly led to regulate antibiotic restriction for aquaculture in Malaysia. Thus, the alternatives, including *Lactobacillus* species as probiotic, *Sargassum polycystum* as prebiotic and their combination as synbiotic were introduced against AHPND outbreak. In the initial study, several *Lactobacillus* strains that possessed *in vitro* anti-*Vibrio* properties were identified and screened from lactic acid bacteria (LAB) isolates of different marketed food fish using biochemical characterization, 16S ribosomal RNA gene identification and *Vibrio* antagonistic analyses. Then, the 10^8 CFU/mL of these single *Lactobacillus* strains supplemented diets were used for 35 days of feeding trial using *Penaeus monodon* postlarvae, followed by immersion challenge using 6

$\times 10^5$ CFU/mL of AHPND-causing *Vibrio parahaemolyticus* S2-4. An effective *Lactobacillus* strain that possessed *in vivo* anti-AHPND was chosen upon analyses of growth performance, survival, bacterial enumeration and histopathology. In the second study, the effective 10^8 CFU/mL of *L. plantarum* L20 (probiotic), 2 % of *S. polycystum* (prebiotic) and their combination (synbiotic) supplemented diets were used for the feeding trial and challenge test using same regime of the initial study. The most effective supplemented diet was chosen upon analyses of growth performance, survival, bacterial enumeration, immune gene expression and histopathology. For the final study, the effective synbiotic diet fed shrimp was subjected to gastrointestinal microbiota analyses of high throughput 16S rRNA amplicon sequencing and histopathology. In the result of initial study, *L. plantarum* L20 showed significant ($p < 0.05$) highest of *in vitro* anti-*Vibrio* activities, high shrimp growth rate (5.32 ± 0.29 %), high L20 abdomen colonization ($23.676 \pm 0.193 \log_{10}$ CFU/mL), high post-challenge survival (80 ± 4.71 %), lowest S2-4 cephalothorax colonization ($4.504 \pm 0.203 \log_{10}$ CFU/mL) and hepatocytes with epithelial cells protection. Thus, L20 was chosen as an effective probiotic among other strains for subsequent second study. In the result of second study, synbiotic diet showed significant ($p < 0.05$) highest shrimp growth rate (3.94 ± 0.33 %), high L20 abdomen colonization ($3.676 \pm 0.193 \log_{10}$ CFU/mL), high post-challenge survival (76.67 ± 4.71 %), lowest S2-4 cephalothorax colonization ($4.504 \pm 0.203 \log_{10}$ CFU/mL), hepatocytes with epithelial cells protection, improve shrimp cephalothorax immunity of feeding trial including cell-adhesive signaling (at 2 fold up-regulation), prophenoloxidase system (at 3 fold up-regulation) with lysozyme secretion (at 5 fold up-regulation) and challenge test

including prophenoloxidase system (at 2 fold up-regulation), toll-like signaling (at 2 fold up-regulation) with penaeidin secretion (at 3 fold up-regulation). Thus, the synbiotic diet was considered as the best supplement diet compared with probiotic and prebiotic diets. In result of last study, the synbiotic diet could improve shrimp gastrointestinal microbiota as significant ($p<0.05$) increased Lactobacillaceae, Mycoplasmataceae, and Devosiaceae with decreased Vibrionaceae, enriched potential microbial glycolysis and gastrointestinal tissue protection. This study suggested the synbiotic improve growth performance, immune responses, gastrointestinal microbiota and disease resistance of shrimp. Therefore, it can be applied a sustainable solution to prevent substantial production losses in shrimp aquaculture.

Keywords: Acute hepatopancreatic necrosis disease (AHPND), *Vibrio parahaemolyticus*, *Lactobacillus plantarum*, *Sargassum polycystum*, *Penaeus monodon*

SDG: GOAL 15: Life below water

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**KEBERKESANAN MAKANAN SINBIOTIK YANG MENGANDUNGI
Lactobacillus plantarum L20 DAN *Sargassum polycystum* UNTUK
Penaeus monodon TERHADAP PENYAKIT NEKROSIS
HEPATOPANKREATIK AKUT**

Oleh

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Penyakit nekrosis hepatopankreatik akut (AHPND) telah menyebabkan kerugian ekonomi besar dan menimbulkan ancaman serius terhadap pembangunan akuakultur udang penaeid Malaysia. Antibiotik digunakan untuk merawat AHPND. Akibatnya, kemunculan rintangan antibiotik dan pelaksanaan pengawalan sekatan antibiotik untuk akuakultur di Malaysia. Oleh itu, pelbagai alternatif termasuk spesies *Lactobacillus* sebagai probiotik, *Sargassum polycystum* sebagai prebiotik dan gabungannya sebagai sinbiotik telah diperkenalkan untuk pencegahan wabak AHPND. Dalam kajian awal, strain-strain *Lactobacillus* yang mempunyai sifat anti-*Vibrio in vitro* telah dikenalpasti dan disaring antara isolat-isolat bakteria asid laktik (LAB) pelbagai ikan makanan berdasarkan pencirian biokimia, pengenalpastian gen RNA ribosom 16S dan analisis antagonis *Vibrio*. Kemudian, pelbagai makanan yang ditambah 10^8 CFU/mL strain *Lactobacillus* masing-masing tersebut digunakan

untuk ujian makanan yang selama 35 hari dengan menggunakan postlarva *Penaeus monodon*, diikuti dengan ujian cabaran rendaman dengan menggunakan 6×10^5 CFU/mL *Vibrio parahaemolyticus* S2-4 penyebab AHPND. Satu strain *Lactobacillus* terkesan yang mempunyai anti-AHPND *in vivo* telah dipilih berdasarkan analisis prestasi pertumbuhan, kemandirian, penghitungan bakteria dan histopatologi. Dalam kajian kedua, pelbagai makanan yang ditambahkan 10^8 CFU/mL *L. plantarum* L20 berkesan (probiotik), 2% *S. polycystum* (prebiotik) dan gabungannya (sinbiotik) masing-masing telah digunakan untuk ujian percubaan pemakanan dan ujian cabaran menggunakan rejim yang sama seperti kajian awal. Makanan pertambahan terkesan dipilih dengan berdasarkan analisis prestasi pertumbuhan, kemandirian, penghitungan bakteria, ekspresi gen-gen imun dan histopatologi. Untuk kajian akhir, udang-udang yang diberi makan diet sinbiotik terkesan telah tertakluk kepada analisis mikrobiota gastrousus bagi penjujukan ampikon 16S rRNA truput tinggi dan histopatologi. Dalam hasil kajian awal, *L. plantarum* L20 menunjuk ketara ($p < 0.05$) bagi aktiviti anti-*Vibrio in vitro* yang tertinggi, kadar pertumbuhan udang tinggi (5.32 ± 0.29 %), kolonisasi L20 tinggi dalam abdomen ($23.676 \pm 0.193 \log_{10}$ CFU/mL), kemandirian lepas cabaran tinggi (80 ± 4.71 %), kolonisasi S2-4 terendah dalam sefalotorak ($4.504 \pm 0.203 \log_{10}$ CFU/mL) dan perlindungan hepatosit dengan sel epitelium. Oleh itu, L20 dipilih sebagai probiotik yang berkesan dalam strain-strain lain untuk kajian kedua seterusnya. Dalam hasil kajian kedua, makanan sinbiotik menunjuk ketara ($p < 0.05$) bagi kadar pertumbuhan udang tertinggi (3.94 ± 0.33 %), kolonisasi L20 tinggi dalam abdomen ($3.676 \pm 0.193 \log_{10}$ CFU/mL), kemandirian tinggi lepas cabaran (76.67 ± 4.71 %), kolonisasi S2-4

terendah dalam sefalotorak ($4.504 \pm 0.203 \text{ Log}_{10} \text{ CFU/mL}$), perlindungan hepatosit dengan sel epitelium, meningkatkan imuniti cephalothorax udang termasuk isyarat pelekat sel (kenaikan 2 kali ganda), sistem prophenoloxidase (kenaikan 3 kali ganda) dengan perembesan lisozim (kenaikan 5 kali ganda) dalam ujian makanan dan sistem prophenoloxidase (kenaikan 2 kali ganda), isyarat tol (kenaikan 2 kali ganda) dengan perembesan penaeidin (kenaikan 3 kali ganda) dalam ujian cabaran. Oleh itu, makanan sinbiotik adalah makanan tambahan terbaik berbanding makanan probiotik dan prebiotic tersebut. Hasil kajian terakhir, makanan sinbiotik boleh menambah baik mikrobiota gastrousus udang yang ketara ($p < 0.05$) bagi peningkatan Lactobacillaceae, Mycoplasmataceae, dan Devosiaceae dengan penurunan Vibrionaceae, memperkaya potensi glikolisis mikrob dan perlindungan tisu gastrousus. Kajian ini mencadangkan sinbiotik ini dapat meningkatkan prestasi pertumbuhan, tindak balas imun, mikrobiota gastrousus dan rintangan penyakit udang. Oleh itu, ia boleh digunakan sebagai penyelesaian mampan untuk mengelakkan kerugian besar dalam bidang akuakultur udang.

Kata Kunci: Penyakit nekrosis hepatopankreatik akut (AHPND), *Vibrio parahaemolyticus*, *Lactobacillus plantarum*, *Sargassum polycystum*, *Penaeus monodon*

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I certify that a Thesis Examination Committee has met on 13 December 2024 to conduct the final examination of Chin Yong Kit on his thesis entitled "Efficacy of Synbiotic Diet Incorporating *Lactobacillus plantarum* L20 and *Sargassum polycystum* for *Penaeus monodon* Against Acute Hepatopancreatic Necrosis Disease" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

UPM Universiti Putra Malaysia

°C Degree Celsius

μL Microliter

s Second

Min Minute

h hour



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CHAPTER 1

INTRODUCTION

1.1. Background of study

Penaeid shrimp aquaculture is fast growing global aquaculture sector, as highly expanding of farm scales and numbers, as well as increase of shrimp production in last five years which driven by the increasing demand for fish and seafood proteins worldwide (González-Rivas & Tapia-Silva, 2023). Their high consumption value due to favorable their flavor, nutritional benefits, and commonly used for different cuisines worldwide due to (Sheu et al., 2020). In fact, the annual global penaeid shrimp production was exponential increased with annual growth rate of 37.6% from 6.05 million tonnes in the year 2018 to 7.93 million tonnes in the year 2022 (FAO, 2024). The shrimp production was mainly contributed by countries of Latin America, East Asia, and Southeast Asia through semi-intensive culture and intensive culture using high stocking density of cultured pond for supplying fresh shrimp products including Pacific white shrimp (*Penaeus vannamei*) and Black tiger shrimp (*Penaeus monodon*) to global market niche (FAO, 2022). United States is largest shrimp import country in the world as import values increased from 6.4 billion USD in year 2018 to 8.4 billion USD in year 2022 (N'Souvi et al., 2024). Although *P. vannamei* is more preferred in global market, the average marketed price of *P. monodon* (11.49 USD/kg) was 40% higher than that for *P. vannamei* (8.23 USD/kg) in year 2020 (Asmild et al., 2024). Thus, the many farmers in Southeast Asia countries gradually switched to *P. monodon* cultures recently (Hukom et al., 2020). Malaysia is also known as one of penaeid shrimp

production countries, which mainly produce *P. vannamei* and *P. monodon* to supply market niche of own country and other countries for contributing economic income (Ismail & Abdullah 2013).

However, global warming is recent global climate change favors the opportunistic *Vibrio* spp. proliferation of marine microbial communities and suppress the shrimp immune system, which lead to outbreak of shrimp vibriosis in aquaculture (Sanches-Fernandes et al., 2022). Acute hepatopancreatic necrosis disease (AHPND), previously known as early mortality syndrome (EMS) is one of vibriosis that causes major shrimp production loss, which badly impact penaeid shrimp industries (Elias et al., 2023). AHPND causes sudden mass mortalities of up to 100% within the initial 30–35 days after stocking of penaeid shrimp postlarvae (Peña et al., 2015). The clinical symptoms of AHPND show pale hepatopancreas in shrimp cephalothorax and empty intestine line in shrimp abdomen (López-Cervantes et al., 2021). The AHPND caused significant economic losses for countries of shrimp producers including China (USD 11.0 billion from 2009 to 2016), Vietnam (USD 2.5 billion from 2010 to 2016), Malaysia (USD 1.2 billion from 2010 to 2016), Thailand 2012 (USD 7.9 billion from 2010 to 2016), and Mexico (USD 0.7 billion from 2012 to 2016) (Estrada-Perez et al., 2020). Penaeid shrimp, including *P. vannamei* and *P. monodon* depend on their innate immune system against infectious pathogen because lack of adaptive immune system, thus, immune efficacy of penaeid shrimp more insufficient (Zhang et al., 2021). AHPND damages shrimp hepatopancreas, result in hepatopancreatic tissue failure and morbidity, as well as increase diseases susceptibility of the infected

shrimp to other infectious pathogen which cause co-infection with other shrimp diseases, including *Enterocytozoon hepatopenaei* and White spot syndrome virus (Aranguren et al., 2017; Han et al., 2019). *Vibrio parahaemolyticus* is the first recognized etiological agent that cause prevalent of AHPND in global shrimp aquaculture, which carried crucial toxin genes as virulence factor that produced the extracellular toxins that homologous to two *Photobacterium* insect related (*pir*) toxin A and B which carried in a plasmid named pVA1. Consequently, AHPND-causing *V. parahaemolyticus* uses their adhesion factors to colonize on gastrointestinal tract and releasing different of their extracellular binary *pirAB* toxins to cause cell cytotoxicity of hepatic cells (Wang et al. 2015).

1.2. Problem statement

The antibiotic class, including tetracyclines, sulfonamides and quinolones were widely used for tackling shrimp diseases in penaeid shrimp aquaculture of Malaysia (Thiang et al., 2021, Devadas et al., 2024). Although antibiotic administration shows promising results, the overuse of antibiotics can pollute aquaculture environments to become antibiotic resistant reservoirs, which cause environmental selective pressure and bacterial horizontal gene transfer that favors the emergence of antibiotic resistance bacteria (Schar et al. 2020).

The global antibiotic resistance emergences cause infectious diseases for humans and animals, as well as microbiota imbalance for environment, which become an inevitable challenge for “One Health”, which is known as united and integrated approach that attempts to sustain balance and optimize the health of humans, animals and ecosystems (WHO, 2014; Mariappan et al.,

2021). Thus, most countries, including China, Thailand, Vietnam, Malaysia, Brazil, Chile, Bangladesh, Norway, Philippines, and India banned antibiotic application in aquaculture farming and restricted importation of antibiotic-treated aquaculture products to erase any possible factor of antibiotic resistance bacteria (Ferri et al., 2022). However, antibiotic resistance genes were found in shrimp farms of peninsular Malaysia despite farmers claimed that they did not apply antibiotic for their shrimp farm since antibiotic administration was banned by Malaysia government (Haifa-Haryani et al., 2022). In this situation, antibiotic illegal application may be possible in some of the farms and antibiotic residues with their resistance genes might be moved from other nearby farms sites or antibiotic polluted waterbodies through ocean water circulation (Thiang et al., 2021; Ferri et al., 2022). Therefore, other alternatives, including vaccine and feed additives can overcome the issues of antibiotic resistance and against aquaculture diseases (Ringø et al., 2018). Vaccine, known as one of the alternatives which enhances the adaptive immune system of host, contributed limited success against infectious diseases in penaeid shrimp aquaculture (Kumar et al., 2021). In fact, penaeid shrimp, including *P. vannamei* and *P. monodon* depend on cellular and humoral responses of their innate immune system against infectious pathogen because lack of adaptive immune system as similar as other species of crustacean (Zhang et al., 2021). Fortunately, the efficacy of probiotic, prebiotic and synbiotics were discovered on their applications to enhance shrimp immunity and reduce the shrimp mortalities caused by vibriosis, including AHPND (Abdel-Latif et al., 2022). Besides, *P. monodon* requires a longer culture period about 160 days compared with *P. vannamei* requires short

culture period about 90 days for achieving marketable size because earlier shrimp maturation of *P. vannamei* (Nisar et al., 2021). Therefore, *P. monodon* has higher feed conversion ratio than *P. vannamei*, which indicates more amount of feed are needed, led higher cost are required for *P. monodon* production (Amit et al., 2022). Therefore, the probiotic, prebiotic and synbiotic are preferred as growth enhancers because they have additional nutrients and improve digestibility for enhancing growth performance in the shrimp aquaculture directly (Norman et al., 2024). The probiotic, including several strains of *Lactobacillus* spp., *Bacillus* spp. and prebiotic, including deviates of Fructooligosaccharide, Galactooligosaccharide are commonly applied for shrimp aquaculture (Khanjani et al., 2023; Wee et al., 2024). However, viability and beneficial effects of probiotic with indigenous beneficial microorganisms during probiotic, prebiotic and synbiotic administration for shrimp is still questionable (Akhter et al., 2015). Therefore, application of probiotic, prebiotic and synbiotic consider several strategies, including 1) host health states, as specific clinical tried probiotic strain and/or prebiotic compound for disease therapeutic; 2) host characteristics, as suitability of specific probiotics and prebiotics for diet type, life stage, environment, species; 3) digestive microbiota composition, as promoting beneficial bacteria and reducing opportunistic pathogen; 4) digestive microbiota function, as provision of prebiotic substrates and/or probiotics with specific metabolic capacity, as well as reduction of pathogen toxins (Cunningham et al., 2021).

1.3. Justification of study

Nowadays, one of key strategies for addressing global antibiotic resistance issues, known as effective animal feed additives, including probiotic, prebiotic and synbiotic, which their research and development (R&D) are encouraged and rapidly conducted by institutes and organizations, implemented as the “One Health” approach (Velazquez-Meza et al., 2022). Therefore, this study focused on newly introduced probiotic, prebiotic and their combination, synbiotic as alternative of antibiotic and innovative solution for penaeid shrimp against infectious diseases. In this study, probiotics are beneficial microorganism that applied as alternatives of antibiotic, which capable to proliferate and persist the digestive systems of aquatic animals to confer beneficial effects for the aquatic animal hosts (Giatsis et al. 2016). Lactic acid bacteria are used as probiotics to improve their growth performance, immune response and disease resistance for aquaculture (Ringø et al., 2018). Among the other species of lactic acid bacteria, *Lactobacillus* spp. is commonly applied for aquaculture industries because their broad-ranged environmental adaptation, long life cycle, host immunity stimulation and enzymatic activity regulation (Amit et al., 2022). Therefore, different *Lactobacillus* spp. was screened as probiotic in this study. Prebiotics are also mainly applied as an alternative of antibiotic to improve host immune system and gastrointestinal health against infectious diseases of aquatic farming animal species (Amenyogbe et al. 2020). Prebiotics are usually applied as feed additives to improve aquafeed for targeted aquaculture farming species in aquaculture (Ganguly et al. 2012). Administration of combined multiple typical prebiotics or feed additives, including Inulin, mannanoligosaccharide (MOS),

Fructooligosaccharide (FOS) perform better promising results in improving growth, enhancing immune response and disease resistance of farmed aquatic animals compared with single typical prebiotic (Wee et al., 2024). Hence, the seaweeds show prebiotic effects, which can be utilized as prebiotic or feed additive for farmed aquatic animals because consist multiple various type of complex carbohydrates with prebiotic properties including alginates, fucoidans, mannitol and laminarin (Makkar et al., 2016). Among seaweeds, *Sargassum* spp. are brown macroalgae which have nutritious contents (vitamins, dietary fibers, proteins, and minerals) and many types of bioactive compounds (terpenoids, flavonoids, sterols, sulfated polysaccharides, polyphenols, sargaquinoic acids, sargachromenol, pheophytine) which exhibit diverse biological activities (anti-inflammatory, antioxidant, anti-microbial, immune-modulatory and hepatoprotective activities) (Yende et al., 2014; Yap-Dejeto et al., 2022). Dietary Supplementation of *S. polycystum* improved feed consumption and growth performance, immune response and cold-tolerance of *P. vannamei* (Abdel-Rahim et al., 2021). Therefore, *S. polycystum* was chosen as prebiotic in this study.

Synbiotics are mainly applied as alternative of antibiotic for improving immune system and maintaining gastrointestinal health against infectious diseases of aquatic farming animal species which similar with probiotics and prebiotics in aquaculture (Amenyogbe et al. 2020). Thus, probiotic (*L. plantarum*) and prebiotic (*S. polycystum*) were combined to form synbiotic as dietary Supplementation for *P. monodon* in this study. AHPND-causing *V. parahaemolyticus* S2-4 that carried two Photorhabdus insect related (*pir*) toxin

A and B was previously isolated from diseased shrimp by Wan Haifa-Haryani et al. (2022). The *V. parahaemolyticus* S2-4 was selected for the immersion challenge test to determine disease resistance of *P. monodon*. Moreover, no study was conducted to investigate the synergistic potential of *L. plantarum* and *S. polycystum* as an effective synbiotic for improving *P. monodon*'s growth performance, immune responses, gastrointestinal bacterial community and disease resistance against infection of Malaysia-prevalent AHPND-causing *V. parahaemolyticus* with associated dysbiosis. Therefore, this study aims to investigate the dynamic modulation of growth, immune responses, gastrointestinal microbiota and survival in *P. monodon* fed with the synbiotic supplemented diet, focusing on its protective efficacy against AHPND-causing *Vibrio parahaemolyticus* infection.

1.4. Objectives

Research framework of this study based on objectives through chapter 3, 4 and 5 subsequently were shown in Table 1.1.

1. To identify and screen *Lactobacillus* spp. that possessing growth promoting and anti-AHPND properties as dietary probiotic supplement for *P. monodon*.
2. To determine growth performance, immune response, and bacterial profile of *P. monodon* upon dietary supplementation of synbiotic comprising *L. plantarum* L20 and *S. polycystum*.

3. To evaluate immune responses, bacterial profile, diseases resistance of *P. monodon* upon dietary supplementation of synbiotic comprising *L. plantarum* L20 and *S. polycystum* against AHPND-causing *V. parahaemolyticus* S2-4.
4. To investigate gastrointestinal microbiota composition, function and dysbiosis resistance of *P. monodon* upon dietary supplementation of synbiotic (*L. plantarum* L20 with *S. polycystum*) against AHPND-causing *V. parahaemolyticus* S2-4.

Table 1.1: Research framework of this study based on objectives through chapter 3, 4 and 5.

	Experimental chapter 3	Experimental chapter 4		Experimental chapter 5
Objective	To identify and screen <i>Lactobacillus</i> spp. that possessing growth promoting and anti-AHPND properties as dietary probiotic supplement for <i>P. monodon</i> .	To determine growth performance, immune responses and bacterial profile of <i>P. monodon</i> upon dietary supplementation of Synbiotic comprising <i>L. plantarum</i> L20 and <i>S. polycystum</i> .	To evaluate immune responses, bacterial profile, diseases resistance of <i>P. monodon</i> upon dietary supplementation of synbiotic comprising <i>L. plantarum</i> L20 and <i>S. polycystum</i> against AHPND-causing <i>V. parahaemolyticus</i> S2-4.	To investigate gastrointestinal microbiota composition, function and dysbiosis resistance of <i>P. monodon</i> upon dietary supplementation of synbiotic (<i>L. plantarum</i> L20 with <i>S. polycystum</i>)
Experiment design	<i>Lactobacillus</i> spp. isolation from fresh marine fish in wet market. <i>Vibrio</i> inhibition test. - Feeding trial and AHPND challenge test. - Bacterial enumeration. - Histological analysis.	- Feeding trial of the Probiotic, Prebiotic and Synbiotic groups. - Bacterial enumeration. - Immune gene expression - Histological analysis.	- AHPND-causing <i>V. parahaemolyticus</i> S2-4 challenge test of the Probiotic, Prebiotic and Synbiotic groups. - Bacterial enumeration - Immune gene expression - Histological analysis.	- High throughput 16S ribosomal RNA Amplicon sequencing analysis for Synbiotic group. - Histological analysis.
Output	Effective <i>L. plantarum</i> L20 was isolated and screened as probiotic	Growth performance, survival, immune response and AHPND resistance of the synbiotic was evaluated.		Gastrointestinal microbiota composition of was determined.
Outcome	The provided data will demonstrate the promising effectiveness of Synbiotic (<i>L. plantarum</i> L20 & <i>S. polycystum</i>) for shrimp health and against adverse effects of AHPND in <i>P. monodon</i> .			

CHAPTER 2

LITERATURE REVIEW

2.1. Penaeid shrimp aquaculture and industry

The common species of cultured penaeid shrimp for aquaculture included Pacific white shrimp (*P. vannamei*), Black tiger shrimp (*P. monodon*), Brown tiger shrimp (*P. esculentus*), Kuruma shrimp (*Marsupenaeus japonicus*), Chinese white shrimp (*Fenneropenaeus chinensis*), Redtail shrimp (*P. penicillatus*), Banana shrimp (*P. merguensis*) and Greasyback shrimp (*Metapenaeus ensis*) (Biao & Kaijin 2007). *Penaeus vannamei*, and *P. monodon* were shown in Figure 2.1. Among these species, Pacific white shrimp (*P. vannamei*) is the most economically valuable cultured shrimp in the world because exhibits rapid growth rate and tolerance to a wide range of environmental conditions including temperature and salinity (Ye et al., 2023). Asian countries more favored *P. vannamei* over *P. monodon* for cultivation because of the availability of unique pathogen-free brood stock, its low protein requirements and production costs (El-Saadony et al., 2022).

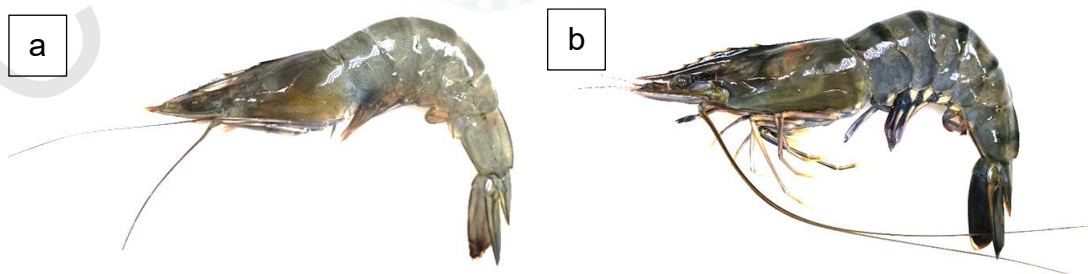


Figure 2.1: Important penaeid shrimp species in global shrimp production. The important species, especially, (a) *P. vannamei* and (b) *P. monodon*.

The penaeid shrimp aquaculture consists of two stages of shrimp production systems, including nursery phase (larviculture) that operated by hatcheries and grow-out phase that managed by farmers (Wang & Leiman 2000). In the nursery phase, shrimp broodstock are reared in breeding tank of shrimp hatchery with density of 6 broodstock/m² which (3 female to 2 male ratio) after they are selected for eyestalk ablation to produce fertilized eggs (Racotta et al., 2004). After hatching from eggs, the nauplii are cultured in circular tank of the hatchery with density 120 nauplii/L and feed them using cultured microalgae (Álvarez et al., 2004). After the nauplii grows into postlarvae 1 (PL1), the PL 1 is cultured in new hatchery circular tank with density of 20 PL/L and feed them using *Artemia* spp. nauplii until grow into postlarvae 15 (PL15) (Álvarez et al., 2004). Nusery phase of penaeid shrimp in commercial farming was shown in Figure 2.2.

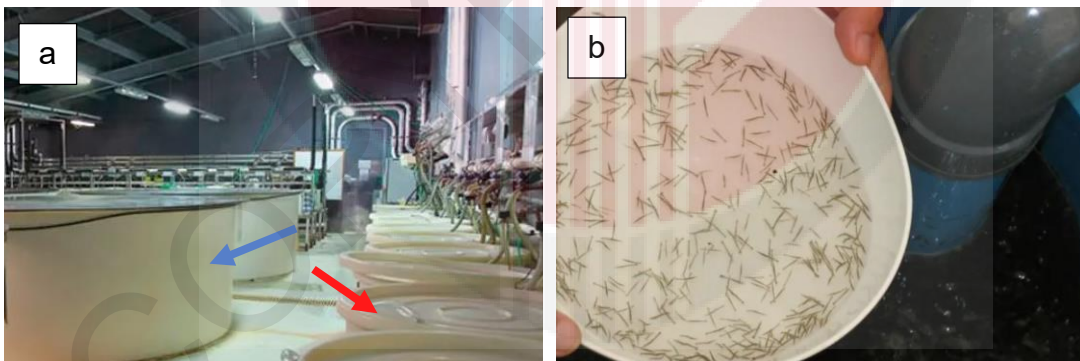


Figure 2.2: Nursery phase of penaeid shrimp in commercial farming (GAA 2007). (a) Spawning tanks (→) for broodstock to produce egg and hatching tanks (→) for egg hatching into nauplii in shrimp hatchery. (b) Larval rearing tanks for rearing nauplii to become postlarvae (PL15) in shrimp hatchery.

In the grow-out phase, there are two typical types of shrimp stocking system such as recirculating aquaculture system and pond system are widely used by shrimp production industry for grow-out culture of shrimp postlarvae (Sang et

al., 2020). The recirculating system, or known as recirculating aquaculture system (RAS) uses lower volume of water supply and utilizes treatment of effluent culture water through a series of consecutive processes, as the first process is physical, mechanical filtration and biofiltration for removing organic matters, possible toxic substances and toxic nitrogenous chemical compounds, the second process is disinfection of pathogenic microorganism, the last process is treated water oxygenation before being reused for the rearing tank of the system (Suantika et al., 2018). However, the high cost is required for construction, equipment, operations and management of the RAS (Widiasa et al., 2023).

The pond system utilizes higher volume of direct water supply from sea or brackish river and remove effluent culture water through the nearby rivers or canals, which yield higher shrimp production with lower input cost for construction and management (Ahmed et al., 2023; Widiasa et al., 2023). Thus, this system is commonly constructed in subtropical-tropical mangrove coverage of the under-develop and developing countries (Ahmed et al., 2023). For grow-out stage, there are three types of penaeid shrimp production system which include extensive culture, semi-intensive culture and intensive culture depending on farmer capability and facility (Hoa et al., 2011) and shown in Figure 2.3. In the shrimp stocking of grow-out culture, extensive culture using small-scale ponds (area, 250 m²; depth, 1 m) with low stocking density of average 6 PL/m² (Hari et al., 2004). The semi-intensive culture using medium-scale pond (area, 50000 m²; depth, 1 m) with standard stocking density of average 15 PL/m² (Casillas-Hernández et al., 2007). The intensive culture

using large-scale pond (area, 80000 m²; depth, 1 m) with high stocking density of average 80 PL/m² (Ruiz-Velazco et al., 2010). The number of installed paddlewheels or aerators depend on the stocking intensity of shrimp pond practice, rearing period, water salinity and types of aerators used for ensuring sufficient water movement and dissolved oxygen (Marappan et al., 2020). Typically, the crumble-form feed is used to feed PLs with 2 times per day for first month, then feed pellet is used for the shrimp with 3 times per day onwards until harvest (Chaikaew et al., 2019). In shrimp production, the extensive system can produce below 400 kg/hectare/year for *P. monodon* or below 700 kg/hectare/year for *P. vannamei* through multiple harvest and stocking; the semi-intensive system can produce up to 3.5 tons/hectare/cycle for 2 cycles per year of *P. vannamei* cultivation or below 3 tons/hectare/cycle for 1 cycle per year for *P. monodon* cultivation; the intensive system can produce above 4 to 9 tons/hectare/cycle for 2 cycles per year of *P. vannamei* and *P. monodon* cultivation (Joffre et al., 2018). Commonly, the intensive culture of *P. vannamei* is preferred by many large-scale farms because high survival rate and low feed conversion ratio (FCR) to produce high quantity of small and medium sized shrimp for supplying increasing demand in global markets (Asmild et al., 2024). During shrimp harvest, the grading is performed based shrimp body weight and size for evaluating shrimp price, as large size is perceived as highly palatable and attractive in perspective of customer (Putri et al., 2022). High production of *P. vannamei* due to early shrimp maturation and require short culture period about 90 days for achieving marketable size but *P. monodon* require extended culture period about 160 days for achieving marketable size with higher and better prices (Nisar et al., 2021).

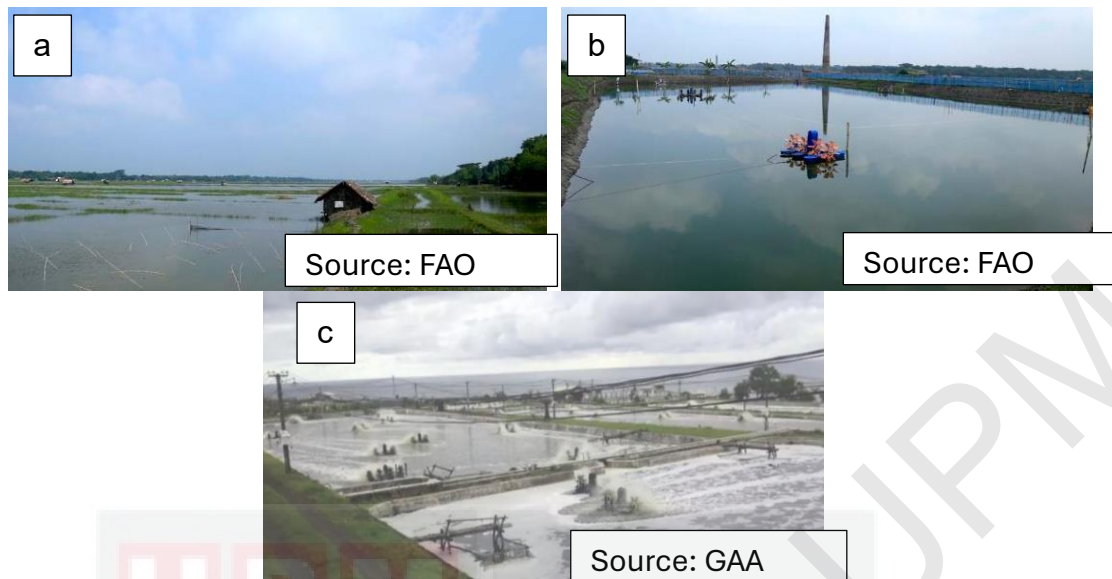


Figure 2.3: Grow-out phase of penaeid shrimp in commercial farming ponds (FAO 2017; GAA 2018). The figure shows (a) extensive pond, which has no aeration device in the small-scale pond; (b) semi-intensive pond, which has two paddlewheels in the medium-scale pond (c) intensive pond, which have multiple paddlewheels in the large-scale pond (Marappan et al., 2020).

2.1.1. Global economics impact of penaeid shrimp production

Shrimp aquaculture is one of the important seafood production sectors that rapidly contributed income for low and lower-middle income countries because high demand of shrimp is required by some of upper-middle and high income countries in the world (Kumar et al., 2016a). The penaeid shrimp farming can improve the social-economic status of coastal communities in developing and underdeveloped countries by increasing their income level about 26 % to 36 % compared with other agricultural cultivation (Ray et al., 2021). The penaeid shrimp aquaculture industry in the countries of Latin America, East Asia, and Southeast Asia are the main contributors for yielding total penaeid shrimp production of 7.9 million tonnes, valued at USD 26.27 billion among other crustacean species at year 2022 (FAO, 2024). United States is largest shrimp import country in the as import values increased from 6.4 billion USD in year

2018 to 8.4 billion USD in year 2022 (N'Souvi et al., 2024). Moreover, *P. vannamei* is more preferred by consumers in United States (El-Saadony et al., 2022). Consequently, China is largest shrimp production country but also second largest shrimp import country, as shrimp import values of China increased from 1.81 billion USD in year 2018 to 6.29 billion USD in year 2022 (Boyd et al., 2021; N'Souvi et al., 2024). The *Penaeus* spp. were highest production among crustacean species in Global production of year 2022 (Figure 2.4).

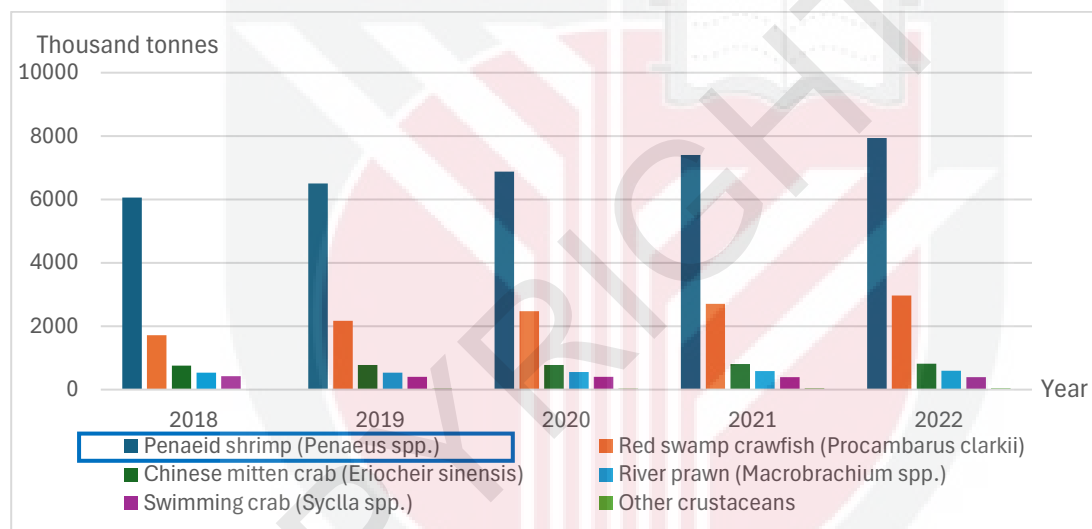


Figure 2.4: Global production of different crustacean species from the year 2018 to 2022 (data adapted from FAO, 2024).

2.1.2. Penaeid shrimp production of Malaysia

According to the study of Goh et al., (2021), 32.7 % and 20.2 % of Malaysian population that residing at high populated area (Kuala Lumpur with Selangor area) consumed *P. vannamei* and *P. monodon* respective in year 2016. This shows most of Malaysians considered shrimp as their favorite seafood. In early of last decade, Malaysia imports a high amount low-quality or cheaper shrimp

product for supplying local consumption but exported high-quality or high price of fresh shrimp for selling to foreign market (Ismail & Abdullah 2013). In this circumstance, high production of *P. vannamei* and *P. monodon* are required for Malaysia. In past decades of Malaysia, the shrimp aquaculture more focused on *P. monodon* production because its high market price for exportation in year 1980 to 1990, but the *P. vannamei* became major cultivated species due to white spot syndrome virus (WSSV) outbreak of *P. monodon* farming in year 2000 to 2010 (Islam et al., 2014; Asche et al., 2021). Local penaeid shrimp culture practice primarily involved *P. vannamei* (80%) and *P. monodon* (15%) between the years 2017 and 2021, these two species contributed about 70% (approximately 85000 tonnes) of total aquaculture production worldwide (FAO, 2023). In example, 36007 tonnes (203.82 million USD) of *P. vannamei* and 9906 tonnes (70.19 million USD) of *P. monodon* were produced from Malaysia in year 2018 (Waiho et al., 2020). Furthermore, the five largest *P. vannamei* producing states of Malaysia including Sabah (12937.40 tonnes), Johor (5748.67 tonnes), Pahang (4275.14 tonnes), Sarawak (3449.74 tonnes) and Kedah (2,793 tonnes) which supplying local and foreign market niches in year 2018 (Lee et al., 2018). Figure shows examples of intensive shrimp ponds in Malaysia. The two examples of famous intensive culture shrimp farms were shown in Figure 2.5.

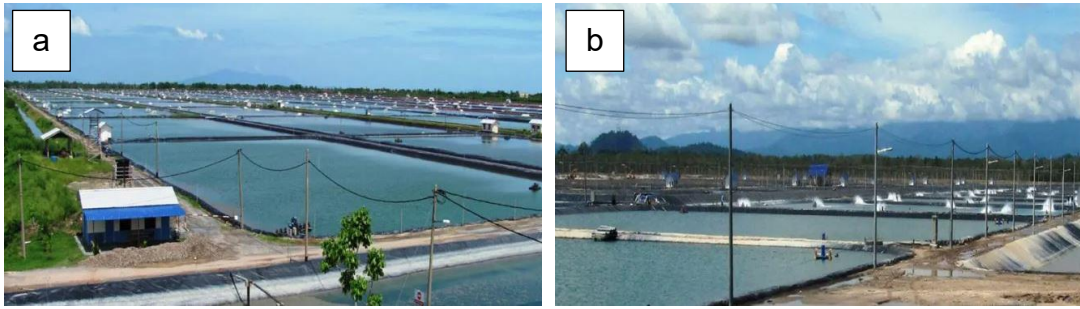


Figure 2.5: Intensive shrimp ponds in Malaysia (GAA 2018). (a) Blue Archipelago's Arca Biru Sdn. Bhd. shrimp farm at Ayer Hitam, Kedah. (b) Integrated shrimp Aquaculture Park (iSHARP) at Setiu, Kuala Terengganu, Terengganu.

Fortunately, few domesticated *P. monodon* strains that resist white spot syndrome virus were developed by Asia countries recently by using selective breeding and PCR method (Debnath et al., 2014). In result, production of *P. monodon* was raised again recently. Therefore, many farmers of shrimp-producing Southeast Asia countries included Malaysia gradually switched to *P. monodon* cultures from *P. vannamei* cultures recently because the higher selling price of the *P. monodon* in local and worldwide markets (Hukom et al., 2020). The average marketed price of *P. monodon* (11.49 USD/kg) was 40% higher than that for *P. vannamei* (8.23 USD/kg) in 2020 because higher market value of *P. monodon*, as larger sized *P. monodon* can be sold with a luxury price and medium sized *P. monodon* are sold in the same market as *P. vannamei* in international market (Asmild et al., 2024). The price of *P. monodon* was higher than *P. vannamei* as shown in Figure 2.6.



Figure 2.6: The price of marketed marine penaeid shrimp in Malaysia in 2024. (a) Pacific white shrimp (*P. vannamei*) and (b) Black tiger shrimp (*P. monodon*) (Photo by Chin, Y.K. 2024).

2.2. Black tiger shrimp (*Penaeus monodon*)

The Black tiger shrimp, known as *P. monodon*, which shown in Figure 2.7 has a natural population distributed from ocean area range of East Africa to the South Pacific Islands included Indo-Pacific Ocean, Indian Ocean and Australasia (Waqairatu et al., 2012). In natural condition, adult of *P. monodon* inhabit and reproduce in shallow ocean water tropical marine environment located at continental shelves (up to 110 m depth) while its larval stages included the juvenile, adolescent and sub-adult stages grow in estuaries, coastal lagoons, and mangroves environment (Khafage et al., 2019). *Penaeus monodon* can live in wide range salinity of water environment including brackish water and marine water, which prefers a salinity of 10 to 35 ppt for its whole life-history stage (Karan et al., 2020). Normally, the colour of *P. monodon* is dark coloured, with black and white band transversely on carapace and abdomen but shrimp colour can be influenced by several factors include

carotenoid availability of shrimp diet, background colour, photoperiod, light intensity and water temperature (Wade et al., 2012).

Penaeid shrimps are popular seafood that are commonly used for different cuisines worldwide because their flavor and nutritional benefits (Sheu et al., 2020). In terms of nutrient value, meats of *P. monodon* and *P. vannamei* have rich in essential amino acids (leucine, isoleucine), but *P. monodon* have higher abundant contents of good fatty acid (DHA 22:6/EPA 20:5) and mineral (Mg, Ca, Fe) than *P. vannamei* (Sriket et al., 2007). In terms of flavour value, both cooked meats of *P. vannamei* and *P. monodon* had favorable texture for consuming because high compact fiber arrangement with the shrinkage of shrimp sarcomere, but the high redness bioactive agent (astaxanthin) of cooked meat of *P. monodon* is contributed as more attractive colour compared with *P. vannamei* (Benjakul et al., 2008). Meat of *P. monodon* also has higher of umami taste amino acids (glutamate, aspartic acid) and sweetness taste amino acids (glycine, glutamate) than *P. vannamei* (Sriket et al., 2007; Hsieh et al., 2021). Overall, the consumption value of *P. monodon* was higher than *P. vannamei*.



Figure 2.7: Live marketed size of *P. monodon* subadult shrimp (Photo by Chin, Y.K. 2024).

2.2.1. Morphological characteristics of *P. monodon*

The shrimp sheds and molts its exoskeleton occasionally to allow body growth. As shown in Figure 2.8., the shrimp head is called the cephalothorax, and the tail is called abdomen which have six segments. The last abdominal segment with telson which allows escape reflex of the by jumping backwards. The cephalothorax has a spine called the rostrum, one pair of eyes, two pairs of antennae, three pairs of maxillipeds for feeding and five pairs of pereopods (walking legs). The abdomen has six abdominal segments except the telson has a pair of fins called pleopods on the ventral side. Shrimp use the pleopods for forward swimming. The morphological features most widely used in taxonomic differentiation of members for the Superfamily Penaeoidea are the rostrum, carapace, antennular flagella, scaphocerite, third maxilliped, pereopods, abdomen, telson and the male and female genitalia, as well as the appendix masculina in the male (FAO, 2023b). In body colouration, ranging from brown to blue color or red, and alternating dark and light transverse cross bands on the back of the abdomen (Khafage et al., 2019). However, different

colouration of *P. monodon* is influenced by several factors, including types of dietary diet, genetic variant and background colour of living environment (Hasan et al., 2022).

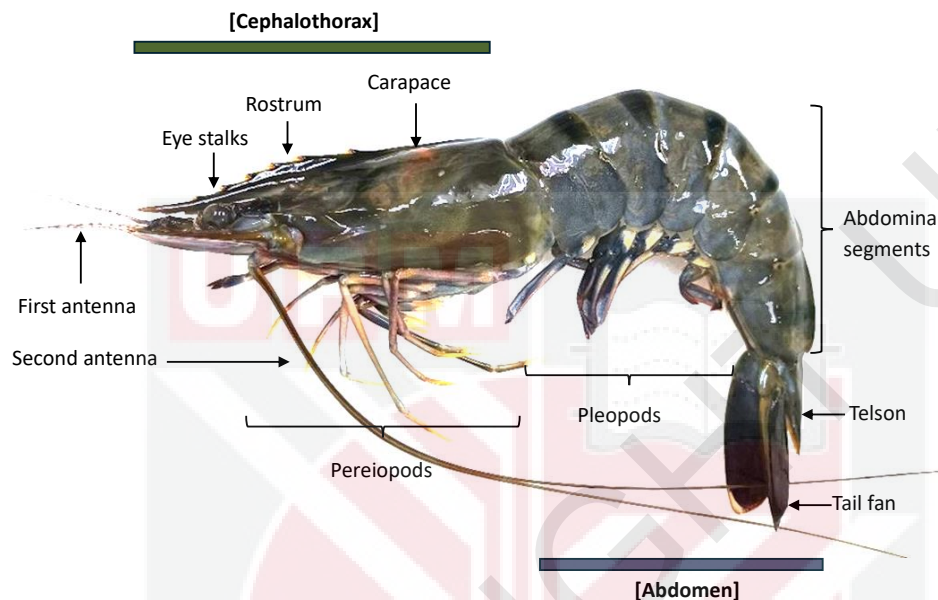


Figure 2.8. External morphology of *P. monodon* (Photo by Chin, Y.K.).

2.2.2. Life cycle of *P. monodon*

The early development of *Penaeus monodon* consists of four sequential larval stages, including nauplius, zoea, mysis and postlarva as shown in Figure 2.9. The planktonic shrimp nauplii hatches from the shrimp egg about 10 -12 h. The nauplii is utilized its egg yolk reserve since its digestive tract not yet developed. The nauplii undergoes metamorphosis for six molting stages (N1- N6) about 1-2 days to develop into planktonic of zoea stage. The wild zoea feed on phytoplankton in the ocean but domesticated zoea feed on cultured microalgae or diatom as food in the hatchery since its mouth and digestive tract are developed. The zoea stage undergoes metamorphosis for three molting stages

(Z1 – Z3) about 3 - 5 days to develop into planktonic Mysis stage. The wild mysis feeds on phytoplankton and zooplankton as food in the ocean but domesticated mysis feed on cultured microalgae and *Artemia* nauplii. The Mysis stage undergoes metamorphosis for three molting stages (M1 – M3) about 4 - 5 days to develop into free-swimming pelagic 5-day-old postlarva (PL5), then develop into benthic swimming of 15-day-old postlarva after 9-10 days. The wild postlarva continuously feed on the phytoplankton, zooplankton and debris in the ocean but the domesticated postlarva feed on particle-sized commercial micro-feed in hatchery. The postlarva stage undergoes growth and molting (PL15- PL45) for 30 days to develop into Juvenile stage. The wild juvenile act as feeding behavior as scavenger or detritus feeder in benthic ocean but the domesticated juvenile feed on commercial pellet feed in grow-out farming. The juvenile stage undergoes growth and molting (J1- J5) for 150 days (5 months) to grow into subadult stage which reached marketed size. The shrimp can be caught from ocean or harvested from the grow-out pond. The shrimp undergoes gonad maturation at 8 -12 months-old which ready for coupling and egg spawning. (Robinson et al., 2005; Jiravanichpaisal et al., 2007; Treerattrakool et al., 2010; Rungrassamee et al., 2013; Angthong et al., 2020).

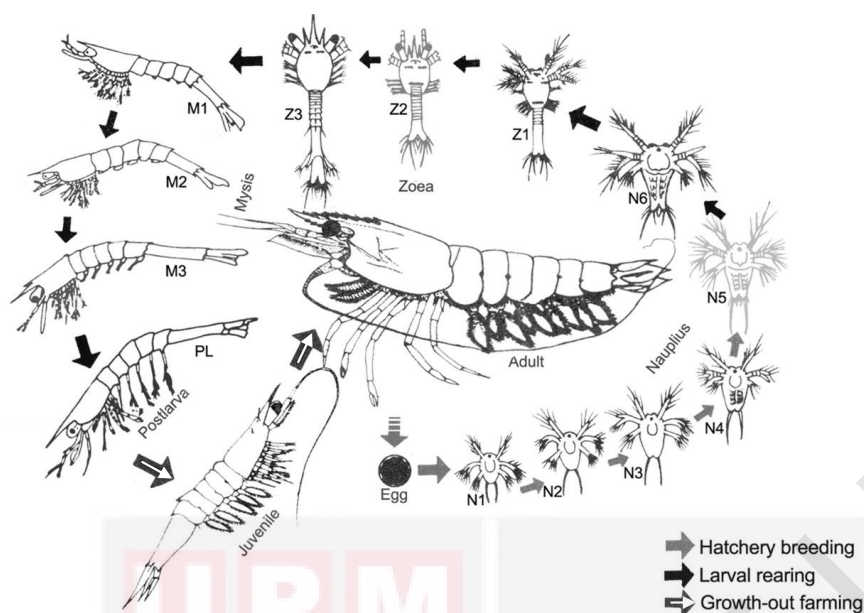


Figure 2.9. Life cycle of *P. monodon* in aquaculture industry (modified from Wang et al., 2020). N1 to N6 indicates six of Nauplii stage; Z1 to Z3 indicates three of Zoea stage; M1 to M3 indicates three of Mysis stage; PL indicate Postlarva stage.

2.3. Diseases associated in shrimp aquaculture

There are few major groups of various diseases including viral, parasitic, bacterial diseases, which cause mass shrimp mortality that impact badly on global economics and aquaculture industries (Yu et al., 2022). In the year 2000 to 2005, the major shrimp diseases were viral diseases outbreak which caused the high loss and mortality, as white spot syndrome virus (WSSV) and yellow head virus (YHV) were known as high lethal shrimp viral pathogens (Flegel 2012). FAO estimated the economic losses from shrimp diseases to be over US\$ 9 billion per year. Subsequently, the *Vibrio* spp.-caused diseases, known as vibriosis in general, caused loss US\$ 4 billion during the period from 2009 until 2018 (Abdel-Latif et al., 2022). The serious impact of shrimp vibriosis was advocated in Figure 2.11. Shrimp vibriosis is highly devastating bacterial disease is caused by multiple *Vibrio* spp. including *V. harveyi*, *V. parahaemolyticus*, *V.*

alginoliticus, *V. campbellii*, *V. vulnificus*, *V. anguillarum*, and *V. splendidus* which affects a wide range of shrimp species (Haifa-haryani et al., 2022), as well as shown in Table 2.1. In spite of shrimp vibriosis is “classical” term refer to *Vibrio* spp. -caused diseases, the shrimp vibriosis is identified to have different forms of diseases recently as different infected shrimp gross signs and different *Vibrio* spp as causative agents, which including luminous vibriosis, Acute hepatopancreatic necrosis disease, septic hepatopancreatic necrosis disease, and white gut disease (Abdel-Latif et al., 2022; Elias et al., 2023).

From year 2009 onwards, Acute hepatopancreas necrotic disease (AHPND) and parasitic diseases, which hepatopancreatic microsporidiosis (HPM) became most dominant and lethal diseases outbreak could badly impact the penaeid shrimp aquaculture (Thitamadee et al., 2016). Although development of various shrimp breeding lines that are certified free of specific pathogens (Specific Pathogen Free, SPF), resistant of specific pathogen (Specific Pathogen Resistant, SPR) and tolerant of specific pathogen (Specific Pathogen Tolerant, SPT) to prevent the shrimp diseases. However, these SPF, SPR and SPT strains of shrimp may be infected with different existing pathogens that were not specific pathogens (Moss et al., 2012).

Table 2.1: Examples of shrimp diseases which occurred in aquaculture

Disease	Causative agent	Reported growth stages	Mortality and symptoms	References
White spot disease (WSD)	Single-stranded DNA virus, White spot disease virus (WSSV)	Juvenile, subadult and adult (broodstock)	100 % mortality within few days Lethargy, reduced appetite, reddish colouration and white spots on the exoskeleton	Peinado-Guevara & López-Meyer (2006)
Yellow head disease (YHD)	Double-stranded RNA virus, Yellow head virus (YHV)	Early-to-late juvenile	100 % mortality within few days Pale yellow coloration of cephalothorax and gills	Kiatpathomchai et al., (2004)
Covert mortality disease (CMD)	Single-stranded RNA virus, Covert mortality nodavirus (CMNV)	Larvae, postlarvae and juvenile	80-90 % mortality within few days Lethargy, whitish muscle, hepatopancreatic atrophy with color fading	Liu et al., (2018)
Taura syndrome (TS)	Single-stranded RNA virus, Taura syndrome virus (TSV)	Postlarvae, juvenile, subadult	100 % mortality within few days Lethargy, expansion of red chromatophores, moult mortalities, red pleopods and tail	Stentiford et al., (2009)
Hepatopancreatic necrosis disease (AHPND)	Gram-negative bacteria, <i>V. parahaemolyticus</i> with positive pirAB	Postlarvae and juvenile	100 % mortality within few days Lethargy, pale hepatopancreas and empty gut	Tran et al., (2013)
Zoea II syndrome	Gram-negative bacteria, <i>V. alginolyticus</i>	Larval	30–100% mortality within few days Lethargy, empty stomach, and absence of molting	Kumar et al., (2017)
Septic hepatopancreatic necrosis (SHPN)	Gram-negative bacteria, <i>V. alginolyticus</i>	Larval and postlarvae	100 % mortality within few days Reddish antennae, uropods and telson, opaque and whitish muscle	Liu et al., (2004)
Luminescent vibriosis	Gram-negative bacteria, <i>V. harveyi</i> , <i>Vibrio campbellii</i>	Larval and postlarvae	100% mortality within few days Lethargy, empty stomach, bright green color luminescence	Soto-Rodriguez et al., (2006)
Hepatopancreatic microsporidiosis (HPM)	Microsporidian parasite, <i>Enterocytozoon hepatopenaei</i> (EHP)	Postlarvae, juvenile, subadult, adult stage of penaeid shrimp	Chronic mortality Growth retarded, soft shells, lethargy, reduced appetite, and empty gut	Aranguren et al., (2017)
White feces syndrome (WFS)	Coinfection of Gram-negative bacteria, <i>Vibrio</i> spp. & Microsporidian parasite, <i>Enterocytozoon hepatopenaei</i> (EHP)	Postlarvae, juvenile, subadult, adult stage of penaeid shrimp	Chronic mortalities Poor growth rate, size disparity, yellowish to whitish discoloration of gut, softshell	Aranguren-Caro et al., (2021)

2.3.1. Acute hepatopancreatic necrosis disease (AHPND)

Acute hepatopancreatic necrosis disease (AHPND), previously known as early mortality syndrome (EMS) which first outbreak at Island of Hainan in China in 2009, consequently spread to Vietnam in 2010, Malaysia in 2011, Thailand in 2012, Mexico in 2013, Philippines in 2015 and South America 2016 (Kumar et al., 2021). This AHPND can cause sudden mass mortalities of up to 100% within the initial 30–35 days after stocking of penaeid shrimp postlarvae (Peña et al., 2015). As they progress to the late-stage juveniles, mortalities can still reach 40–60% within 56–94 stocking days (Peña et al., 2015). The clinical symptoms of AHPND shows pale hepatopancreas in shrimp cephalothorax and empty intestine line in shrimp abdomen (López-Cervantes et al., 2021) as shown in Figure 2.10. AHPND caused significant economic losses for countries of shrimp producers including China (USD 11.0 billion from 2009 to 2016), Vietnam (USD 2.5 billion from 2010 to 2016), Malaysia (USD 1.2 billion from 2010 to 2016), Thailand 2012 (USD 7.9 billion from 2010 to 2016), and Mexico (USD 0.7 billion from 2012 to 2016) (Estrada-Perez et al., 2020). The occurrence of AHPND lead farmers to harvest smaller size shrimp early, which lowering marketed shrimp price and decrease profit that gained by shrimp industry (Asche et al., 2021). In 2013, *Vibrio parahaemolyticus* was first identified as the causative agent of AHPND (Tran et al., 2013). Currently, the etiological agents of AHPND were AHPND-causing *V. parahaemolyticus* and *V. harveyi* strains that prevalent in several Southeast Asian countries such as Malaysia, Thailand and Vietnam (Guzman et al., 2022); AHPND-causing *V. parahaemolyticus*, *V. owensii* and *V. campbellii* that prevalent in mainland China (Dong et al., 2019); AHPND-causing *V. parahaemolyticus* and *V.*

punensis that prevalent in Mexico and other countries of Latin America (Restrepo et al., 2018).

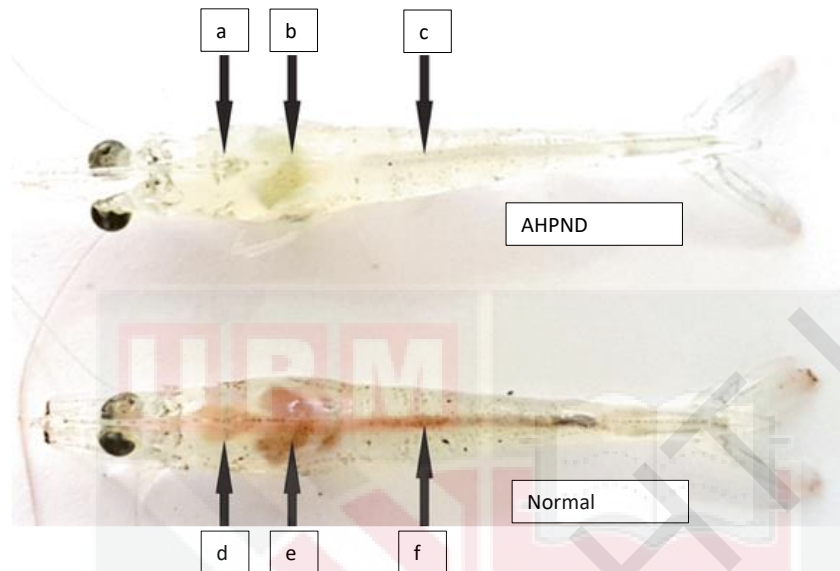


Figure 2.10: Clinical signs of AHPND compared with normal sign in Pacific white shrimp (*P. monodon*) postlarvae. (a) indicates empty stomach, (b) indicates pale hepatopancreas, (c) indicates empty gut, (d) indicates normal stomach, (e) indicates normal hepatopancreas, (f) indicates normal gut (López-Cervantes et al., 2021).

2.3.2. AHPND-causing *Vibrio parahaemolyticus* as etiological agents

Vibrio parahaemolyticus is the first recognized etiological agent that cause prevalent of AHPND in global shrimp aquaculture despite recent reports showed that a *V. harveyi*, *V. owensii*, *V. campbellii*, and *V. punensis* cause the AHPND gross symptoms (Liu et al., 2021). The AHPND strain of *V. parahaemolyticus* commonly carry few crucial toxin genes as virulence factor that produced the extracellular toxins that homologous to two Photorhabdus insect related (*pir*) toxin A and B which carried in a plasmid named pVA1, which can transfer horizontally among *V. parahaemolyticus* strains and other *Vibrio* spp. strains (Dong et al., 2019; Han et al., 2020). *Vibrio parahaemolyticus*

known as a species of non-spore forming, curved rod-shaped and gram-negative halophilic bacteria as shown in Figure 2.13, the bacteria prevalence also in water environment in marine, estuarine and coastal region (Letchumanan et al. 2014). This bacteria species has two different forms based on moving mechanisms, which have long single polar flagella and multiple lateral flagella (Broberg et al. 2011). *Vibrio parahaemolyticus* are bacterioplankton in the seawater, associated with surface and digestive tract of marine aquatic animals (Tan et al. 2020). Importantly, the survival and growth rates of all strain *V. parahaemolyticus* depends on environmental factors included pH, salinity and temperature, it prefers an alkaline pH of 7.9 to 8.6, 3% NaCl of salinity and 35 to 37 °C of favored temperatures (Ndraha et al. 2020). Similar with other *Vibrio* spp., *V. parahaemolyticus* have consists of two types of DNA molecules included chromosomal DNA and extrachromosomal DNA, known as plasmid DNA (Kirkup et al. 2010). Difference virulence genes that found in both chromosomal DNA and plasmid DNA of *V. parahaemolyticus* that can manipulate their pathogenicity for different stains (Loyola et al. 2015). However, non-pathogenic *V. parahaemolyticus* usually does not carry any of the toxin genes (Singhapol & Tinrat 2020). The toxin genes are *pirA* gene and *pirB* gene that involve production of binary *pirAB* complex toxins respectively (Aranguren-Caro et al. 2020). Researchers implicated also that vibriosis-related genes are not responsible for AHPND strain of *V. parahaemolyticus* that causing mortality for penaeid shrimps (Shaheer et al. 2019). The *pirA* and *pirB* genes are 284 bp and 392 bp respectively located in AHPND causing plasmid, pVA1 (Dong et al. 2019).

2.3.3. Pathogenesis of AHPND-causing *V. parahaemolyticus*

In natural bacterial infection, oral route of infection is commonly used by waterborne bacteria, especially *V. parahaemolyticus* enter host body (Jiao et al. 2020). Moreover, the open wound of host can be infection route for *V. parahaemolyticus* to enter host body (Tan et al. 2020). Then, *V. parahaemolyticus* uses their adhesion factors to bind on fibronectin and phosphatidic acid on the host cell, thus releasing different of their extracellular toxins into the host cytoplasm to cause cytotoxicity and serious toxin-dependent diseases (Wang et al. 2015). After proliferation in open wound or infection sites, the bacteria proliferate in many regions of the host hemolymph circulatory vessel and other body parts (Zhang et al. 2017). Thus, cause the systematic infection on the host. Aquaculture bacterial pathogenesis depends on the major virulence factor which are toxins, especially extracellular toxins that crucially decide outcome of infection (Sarkar et al. 2021). In putative mechanism of binary *pirAB* toxin according mainstream theory, the *pirA* of binary *pirAB* complex recognized glycosaminoglycan molecules and the facilitates target-specific recognition by binding certain ligands of receptor located on host cell membrane, then, *pirB* of *pirAB* complex cause host cell membrane oligomerization to cause enhance pore formation, then *pirB* enter host cell through damaged membrane which cause cellular damage, cell morphology changes and cell lysis, subsequently led to cell death (Kumar et al., 2019; Luangtrakul et al., 2021) as shown as Figure 2.11. In result, the binary *pirAB* toxins causes putative damage to the shrimp stomach epithelium would then allow the toxin proteins and the *Vibrio* spp. themselves to spread from the stomach to the shrimp hepatopancreas (Lai et al., 2015).

Subsequently, the binary *pirAB* toxins cause massive sloughing of shrimp hepatopancreatic tubule epithelial cells, which cause shrimp mortality directly (Tran et al., 2013).

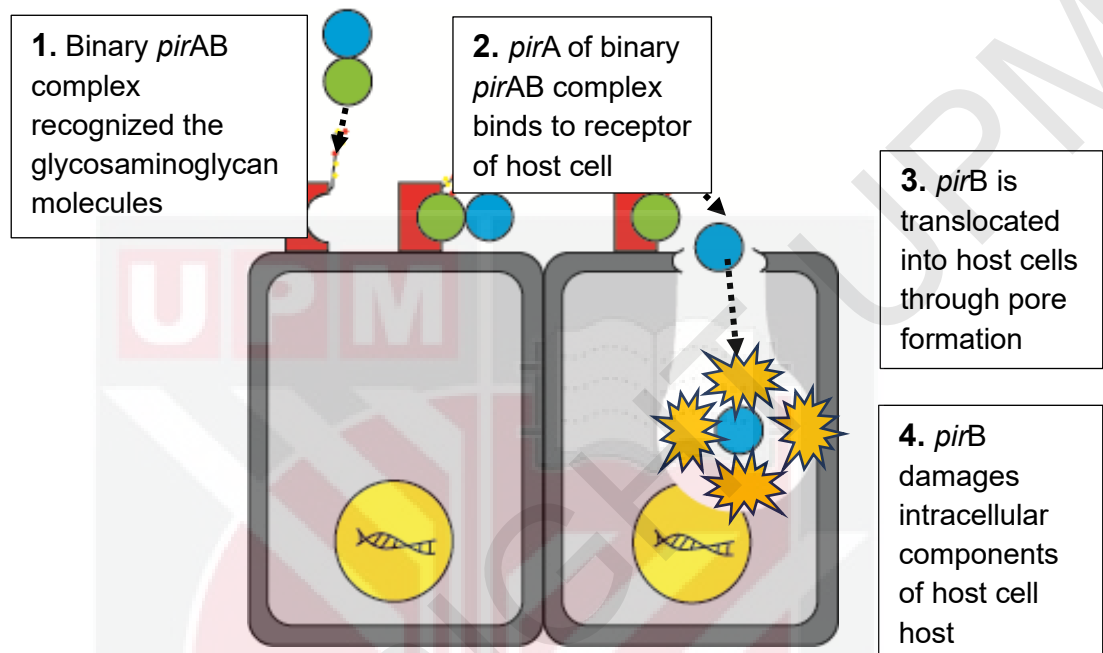


Figure 2.11: Putative mechanism of binary *pirAB* toxins on shrimp hepatopancreatic hepatic cells in result of AHPND.

2.4. Antibiotic application in shrimp aquaculture

Antibiotics are known as chemotherapeutic agents that is specifically used for aquatic bacterial disease treatment of farmed aquaculture species in the past decades (Done et al. 2015; Kumar and Roy 2017). In aspect of global aquaculture, the common antibiotics that applied are tetracycline, oxytetracycline, oxolinic acid, flumequine, sarafloxacin, enrofloxacin, amoxicillin, erythromycin, sulfadimethoxine, ormetoprim and florfenicol against vibriosis of fish and crustacean species (FAO 2019). Especially, several major antibiotic classes, including fluoroquinolone, tetracyclines, quinolones,

chloramphenicol, nitrofurans and sulfonamides are commonly applied for penaeid shrimp aquaculture (Devadas et al., 2024).

Nowadays, antibiotics are still the farmers' choice that are being employed prophylactically and therapeutically in aquaculture farming because their wide broad spectrum of antibacterial effects enhance fish survival and market value, despite their side effects on the environment and human health issues (Okocha et al. 2018; Rosado et al. 2019). Moreover, antibiotic administration has promising advantages that have become the first consideration of farmers including reasonable costs, the low labor efforts required and good farmed species survival improvements (Henriksson et al. 2018). Therefore, farmers of low- or middle- income countries that have limited regulation, knowledge, practices and resources still relied on antibiotic application against aquatic diseases (Topp et al. 2017). However, the frequent of antibiotic application is increased by farmers if increasing incidents of farmed aquatic animal mortalities and unknown diseases outbreaks even the effectiveness is uncertain (Reverter et al. 2020). The farmers of Asia countries, such as Vietnam, India, China, Thailand, Bangladesh and Indonesia mainly depend on antibiotic usage for marine penaeid shrimp production (Luthman et al., 2024). However, antibiotic applications for aquaculture were affected by critical challenges including the emergence of antibiotic-resistance pathogens and global antibiotic restriction laws (Brunton et al. 2019).

2.4.1 Antibiotic resistance

The antibiotic abuse of aquaculture industries causes antibiotic residue pollution of their surrounding water environments through discharged wastewater or effluent (Reverter et al. 2020). Especially, antibiotics application of shrimp feed-based oral administration and direct immersion for shrimp culture in hatchery and grow-out pond (Haifa-Haryani et al., 2022). This antibiotic residue pollution changes aquatic environmental microbiome composition and their ecological function through selective pressure, which favors the emergence of antibiotic resistance bacteria directly (Schar et al. 2020). Moreover, the other bacteria acquire the antibiotic resistance also through horizontal gene transfer which involved transformation, conjugation, transduction and fusion for exchange of genetic materials including antibiotic resistance genes between bacterial species, thus also cause rapid emergence of antibiotic resistance bacteria (Das et al., 2020). Pathogenic bacteria with antibiotic resistance may cause worse infection of human and animal diseases, such as prolongs sickness period, increases spread risk, requires more costly advanced treatments, and raises fatality and mortality rates (Ahmed et al., 2024). Apart from acquired resistance, intrinsic antibiotic resistance of penicillin, sulfonamides and cephalosporins in marine *Vibrio* spp. is also involved in dissemination of antimicrobial resistance in particular environments (Nadella et al., 2025). A meta-analysis for studies of isolated pathogenic *Vibrio* spp. in global shrimp aquaculture from year 2020 to 2022, which found that most prevalent resistance of antibiotic classes, including aminoglycosides, beta-lactams, sulfonamides, glycopeptides, macrolides, nitrofurans, amphenicols, polypeptides, quinolones and tetracyclines (Thaotumpitak et al.,

2024). Table 2.2 shows antibiotic class and their resistance genes with mechanism for shrimp aquaculture.

“One health” is an approach that connect multiple sectors together through communication and cooperation for establishing and executing policies, legislation, programs, research and development to accomplish health of human, animals and ecosystems, particularly related with control of food safety, zoonotic diseases, antibiotic resistance microbes (WHO, 2014). Hence, the global antibiotic resistance emergences cause infectious diseases for humans and animals, as well as microbiota imbalance for environment, which become an inevitable challenge for “One Health” (Mariappan et al., 2021). Therefore, Awareness and interests of the global publics, economic sectors and organizations involved in animal, human, and environmental health are needed to support “One Health” approach for addressing the global antibiotic resistance problems (Velazquez-Meza et al., 2022). Currently, global collaboration of governments, international organizations, and stakeholders was established and integrated for achieving regulatory management, exchanging optimal methodologies, and synchronize efforts to effectively combat antibiotic resistance (Ahmed et al., 2024). In Malaysia, government departments and organizations supported the “One Health” approach, which published an action plan, known as Malaysian Action Plan on Antimicrobial Resistance (MyAP-AMR) through a collaborative effort between the Ministry of Health (MOH) and Ministry of Agriculture and Agro-Based Industry (MOA) Malaysia (Mariappan et al., 2021). In circumferences, the aquaculture related national act was enforced, which no antibiotic is allowed for farmed aquatic

animals through aquafeed incorporation, direct administration into animal bodies and living aquatic environment (Feed Act, 2009). Besides, the Malaysia government established programs, including Programs Fish Disease Surveillance Program, Aquaculture Residue Monitoring Program (ARMP), Sanitary and Phytosanitary Aquaculture, and Malaysia Good Agricultural Practices (myGAP) Certification Program which monitor and control antibiotic application in aquaculture industries of Malaysia (Noordin et al., 2020).



Table 2.2: Antibiotic class and their resistance genes with mechanism for shrimp aquaculture.

Antibiotic class	Resistance genes and mechanism	References
Beta lactam	1. Beta-lactamase (tem1, tem2, shv1) hydrolyse beta-lactam ring for deactivating beta-lactam against beta-lactam class antibiotics.	Bajpai et al. (2017)
Sulfonamide	1. Dihydropteroate synthase (Sul1, Sul2, Sul3, Sul4) disrupt Sulfonamide-dihydropteroate synthase binding.	Sánchez-Osuna et al. (2019)
Tetracycline	1. Tetracycline efflux pumps (tetA, tetC, tetE, tetG, tetH) expel tetracycline from the bacterial cell.	Hong et al. (2014)
Aminoglycoside	1. Aminoglycoside acetyltransferase (aac), nucleotidyl-transferase (ant), adenyl-transferase (aad), phosphotransferase (aph) for inactivating aminoglycoside.	Abo-State et al. (2018)
Macrolide	1. Major facilitator macrolide efflux pump (mef), ATP-binding cassette macrolide efflux pump (msr) to expel out macrolide from the bacterial cell. 2. GTP-dependent macrolide kinases (mph) for inactivating macrolide.	Iannelli et al. (2018); Pawlowski et al. (2018)
Quinolone	1. Proteins (qnrA, qnrB, qnrC, qnrD, qnrS, qnrVC) protect bacterial DNA topoisomerase. 2. Quinolone efflux pump (qepA, oqxAB) expel quinolone from the bacterial cell.	Rahman et al. (2017)
Amphenicol	1. Chloramphenicol acetyltransferase (cat) to modify amphenicol except florfenicol 2. Major facilitator efflux pump (floR) expels florfenicol.	Roberts & Schwarz (2016)
Rifamycin	1. Rifamycin ADP-ribosyltransferases (arr) modify rifamycin into functionless substances.	Shin et al. (2018)
Nitrofuram	1. Nitroreductases (nfsA, nfsB) inactivate nitrofuram into functionless substances.	Ho et al. (2015)
Glycopeptide	1. Peptide d-Ala-d-Lac and d-Ala-d-Ala ligases (van) against glycopeptide binding on the bacteria peptidoglycan.	Yushchuk et al., (2020)

2.4.2 Other prophylaxis as antibiotic alternative

The AHPND outbreaks still occur during shrimp farming despite the antibiotic application being banned by governments or abandoned by shrimp farmers. In this “post-antibiotic era”, environmentally friendly solutions or strategies should be figured out as alternatives to prevent and treat bacterial diseases are crucial for the sustainability of aquaculture (Lubis et al., 2024). However, the global shrimp aquaculture industry still commonly applied antibiotics for treating common infections such as hepatopancreatic necrosis disease (AHPND) because absence of vaccines as effective preventive approach or appropriate health management (Farías et al., 2024). Therefore, other prophylaxis are suggested for overcoming AHPND outbreak instead of antibiotic application.

Bacteriophages, known as virus, can be one of the alternatives to antibiotics against AHPND-causing *V. parahaemolyticus* in aquaculture because host specificity, easy delivery by immersion, self-replication through host, no adverse side effects reported for shrimp and water environment (Hu et al., 2021). Application of bacteriophages require identification of their genomic and biological characteristics before their use in phage therapy which demonstrate strictly lytic activity against the target pathogen, and not contains virulence and antibiotic resistance genes in their genome (Orozco-Ochoa et al., 2023). Vibriophage was known as one of bacteriophage that specific on *Vibrio* spp. including AHPND-causing *V. parahaemolyticus* as host for self-replicate and lysis them (González-Gómez et al., 2022). In other *in vivo* study, vibriophages vB_VpaS_AL-2 not significantly reduced 4 % of mortality *P. vannamei* shrimp

juvenile upon AHPND-causing *V. parahaemolyticus* infection but delayed gross symptoms (González-Gómez et al., 2023).

Quorum sensing (QS) is the regulation of gene expression upon cell-cell communication between same or other bacterial species through secreted autoinducers to regulate a diverse array of physiological activities including symbiosis, virulence, pathogenesis, competence, conjugation, motility, and biofilm formation (Miller & Bassler 2001). QS system modulate virulence of AHPND-causing *V. parahaemolyticus* by regulating expression of the *pirA* and *pirB* genes through AphBvp (Lin et al., 2022). In this concept, various natural and synthetic QS inhibitors (QSIs) as alternatives to antibiotics have been developed to interfere with the QS system which can reduce bacterial pathogenesis. Thus, the QSIs can be applied as alternatives for disease prevention in aquaculture (Naga et al., 2023). In examples, the Cyclo-(L-Leu-L-Pro), and Cyclo-(L-Phe-L-Pro) that secreted by *V. alginolyticus* as natural QSIs showed significant anti-QS activity and inhibited extracellular protein production of the AHPND-causing *V. parahaemolyticus* (Paopradi et al., 2021). the bacterial catecholamine receptor antagonist LED209 as synthetic QSIs shows growth and motility inhibition of the AHPND-causing *V. parahaemolyticus* (Yang et al., 2020). Leaf extracts of *Piper betle* L. as natural QSI showed QS interruption by modulating autoinducer activities of AHPND-causing *V. harveyi* (Guzman et al., 2022).

The application of probiotics, prebiotics and synbiotics as functional feed additive and alternatives to antibiotic has been proved to stimulate immune

responses and defense the gut microbiota against pathogenic bacteria and reduce inflammation of farmed aquatic animal species in aquaculture (Valle et al., 2023). In current trend, the efficacy of probiotic, prebiotic and synbiotics has been assessed in much research studies, as most current knowledge was discovered on their applications to enhance shrimp immunity and reduce the shrimp mortalities caused by vibriosis, including AHPND (Abdel-Latif et al., 2022).

2.5 Probiotic in shrimp aquaculture

Probiotics are beneficial microorganisms that can enhance the dynamic balance of intestinal microbiota in targeted host (Chandran et al. 2013). International Scientific Association of Probiotics and Prebiotics (ISAPP) defined “live microorganisms which when administered in adequate amounts contribute health benefit for the host” (Hill et al. 2014). Probiotics are usually administrated through oral (feed) and immersion for aquatic farming species in aquaculture practices (Hoseinifar et al. 2018). Importantly, probiotics should have capability to proliferate and persist the crucial condition of gastrointestinal tract including nutrient availability, pH and digestive enzymes in the aquatic animals (Giatsis et al. 2016). There are two important characteristics of probiotic, such as indigenous bacteria from same host species, known as autochthonous strains and foreign bacteria from other host species, known as allochthonous strains (Seghouani et al. 2017). Generally, the autochthonous strains have specific host intestinal mucosa-adhere properties but allochthonous strains transient properties on host intestine (Rimoldi et al. 2020). However, the colonization ability and persistence of the allochthonous

strains in host's gastrointestinal tract still remain in highly debate among experts (Suez et al. 2019). Probiotics that are commonly used for shrimp aquaculture, including Lactic acid bacteria (especially, genera of *Lactobacillus*) and genera of *Bacillus* (Ringø et al. 2020).

According to regulations of Malaysia, the criteria of microorganisms can be known as probiotic for human, including the common characteristic of probiotic cultures as (a) safety and health benefits in human studies; (b) gastric acidity resistance; (c) bile acid resistance; (d) mucus and/or human epithelial cells adhesion (e) bile salts hydrolysis (f) long history of consumption; and (g) clear taxonomy identification, viable with not less than 10^6 CFU/mL or CFU/g during the shelf life, without transmissible antibiotic-resistant genes and information of probiotic must be mentioned on the package of the supplemented food prior marketing (Food regulations, 1985). However, legal provision in law and regulation of most countries emphasize probiotic application are different between human and animal, as well as human probiotic is still defined as "probiotic", but animal probiotic is defined as "microbial feed additive" in the provision because different purpose, duration, lifestyle, species and strain specificities (Bernardeau & Vernoux 2013). Similarly, as referred to probiotic definition of the ISAPP, animal probiotic is considered as one of feed additive, which known as added ingredient including harmless microorganism that affects the characteristics of feed according Malaysia act (Feed act, 2009).

2.5.1 Lactic acid bacteria (order Lactobacillales)

Lactic acid bacteria are bacteria that classified into order Lactobacillales, which can be further classified into four families and seven genera, including family Lactobacillaceae (genera *Lactobacillus* and *Pediococcus*), family Leuconostocaceae (genera *Oenococcus* and *Leuconostoc*), family Enterococcaceae (genus *Enterococcus*) and family Streptococcaceae (genera *Lactococcus* and *Streptococcus*) (Zhang et al., 2011). Generally, lactic acid bacteria have characteristics of Gram-positive, catalase-negative, non-spore-forming, non-respiring and aerotolerant (Ayivi et al., 2020). Commonly, lactic acid bacteria metabolize glucose through glycolysis (Embden–Meyerhof–Parnas pathway; EMPP) and the pentose phosphate pathway (PPP) to produce lactic acid as end product during fermentation (Salvetti et al., 2013). However, different species or variant of lactic acid bacteria have various nutrient metabolisms, such as metabolisms of different monosaccharides (fructose, glucose and galactose) and/or plant lignocellulosic material (xylose); metabolism of proteins, lipids, starch and/or small oligosaccharides; but shared deficient of cellulolytically and/or hemicellulolytically metabolism (Lübeck and Lübeck, 2019). In fact, lactic acid bacteria involved the organic acid production (especially lactic acid), protein hydrolyzation, viscous exopolysaccharides secretion and bacteria inhibition (Wang et al., 2021).

Among the other species of lactic acid bacteria, *Lactobacillus* spp. are commonly applied for aquaculture industries because their broad-ranged environmental adaptation, long life cycle, host immunity stimulation and enzymatic activity regulation (Amit et al., 2022). *Lactobacillus* spp. are divided

by referring their different carbohydrate metabolism into three groups including obligate homofermentative group which produce lactic acid only (*L. acidophilus* and *L. salivarius*), facultatively heterofermentative group which produce lactic acid, ethanol, acetic acid and carbon dioxide depends on certain conditions (*L. casei* and *L. plantarum*) and obligately heterofermentative group which always produce lactic acid, ethanol, acetic acid and carbon dioxide (*L. reuteri* and *L. fermentum*) (Dempsey and Corr 2022). Some studies show *Lactobacillus* spp. are used as probiotic to improve growth performance, immune responses and disease resistance for finfish and shellfish species through dietary supplementation, including *L. rhamnosus* for Nile tilapia (*O. niloticus*), *L. sakei* for Kelp grouper (*Epinephelus bruneus*), *L. paracasei* for Pacific white shrimp (*P. vannamei*) and *L. acidophilus* for marron (*Cherax cainii*) (Harikrishnan et al., 2010; Foysal et al., 2020; Huang et al., 2022; Noshair et al., 2023). The application of lactic acid bacteria, include *Lactobacillus* spp. as effective probiotics against diseases in shrimp studies as shown in Table 2.3.

2.5.2 *Lactobacillus plantarum*

Lactobacillus plantarum is a Gram positive, short-rod, micro-aerophilic, acid-tolerant, non-spore forming, non-respiring, low G + C content, non-spore forming lactic acid bacteria (Arasu et al., 2016). The heterofermentative of *L. plantarum* can use the phosphogluconate pathway and phosphoketolase pathway for carbohydrates (hexose and pentose sugars) metabolism to produce lactic acid, organic acid deviates and carbon dioxide (Abedi & Hashemi et al., 2020). The *L. plantarum* can be found in varieties of fermented food, brewed beverage, dairy products and host gastrointestinal tract (Behera

et al., 2018). Moreover, some studies show *L. plantarum* can be isolated from gastrointestinal tract of aquatic animal including yellow shrimp (*Metapenaeus brevicornis*), banana shrimp (*Penaeus merguensis*) (Kongnum & Hongpattarakere 2012). In aquaculture industry, studies show *L. plantarum* supplemented feed can enhanced the growth performance, immune response and disease resistance of farmed crustacean including giant freshwater prawn (*Macrobrachium rosenbergii*), freshwater crayfish (*Cherax cainii*), Pacific white shrimp (*P. vannamei*) (Dash et al., 2014; Foysal et al., 2021; Tseng et al., 2023). The plantaricin, plantaricyclin, plantacyclin are the bacteriocins that produced by *L. plantarum* strains exclusively exert their killing effect through membrane destabilization, pore formation and subsequent cytoplasmic leakage for eliminating infectious pathogen in host intestine (Golneshin et al., 2020; Rocchetti et al., 2021). The application of *L. plantarum* strains as effective probiotics against diseases in shrimp studies as shown in Table 2.3.

Table 2.3: Application of effective probiotic against diseases in shrimp studies.

Effective Probiotic	Sources	Targeted host	Effective administration and regime	Impact and effect	Reference
<i>Lactobacillus plantarum</i>	Wild-type shrimps (<i>Metapenaeus brevicornis</i> , <i>P. merguensis</i>) guts	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 4 × 10 ⁸ CFU/g of probiotic diet for 4 times daily in 42 days of feeding trial	- Improved growth performance - high survival after <i>Vibrio harveyi</i> challenge via 10⁷ CFU/ml of intramuscular injection and 10⁶ CFU/ml of immersion for 2 hours.	Kongnum & Hongpattarakorn (2012)
<i>Lactobacillus pentosus</i>	Pigeon (<i>Columba livia</i>) feces	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 10 ¹⁰ CFU/g of probiotic diet for 2 times daily in 60 days of feeding trial and challenge (7 days).	- Stimulated immune responses - Increased the intestinal beneficial bacteria and reduced the abundance of harmful pathogenic bacteria. - High survival after <i>Vibrio alginolyticus</i> via 10⁵ CFU/ml intramuscular injection, followed by <i>Vibrio parahaemolyticus</i> oral feeding.	Chiu et al., (2021)
<i>Pediococcus pentosaceus</i>	Pacific shrimp (<i>P. vannamei</i>) gut	Pacific White shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 10 ⁸ CFU/g of probiotic diet for 3 times daily in 56 days feeding trial and challenge test (14 days)	- Increased the total haemocyte counts and lysozyme activity. - Improved growth performance, digestive enzyme activity, intestinal microbiota - High survival after <i>V. anguillarum</i> via 10⁶ CFU/ml immersion	Adel et al., (2017)
<i>Lactococcus lactis Subsp. lactis</i>	Pacific shrimp (<i>P. vannamei</i>) gut	Pacific White shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 10 ⁸ CFU/g of probiotic diet for 3 times daily in 56 days of feeding trial	- Increased growth performance, digestive enzyme activity, intestinal microbial microbiota. - Increased the total haemocyte counts and lysozyme activity. - High survival after <i>V. anguillarum</i> via 10⁶ CFU/ml immersion.	Adel et al., (2016)

2.6 Prebiotic for shrimp aquaculture

Prebiotics are nutritious substances that improve growth targeted host and commensal microflora of host intestine (Bassetti et al. 2020). International Scientific Association of Probiotics and Prebiotics (ISAPP) defined dietary prebiotics as “a selectively fermented ingredient that cause specific changes in the composition and/or activity of the host’s gastrointestinal microbiota to contribute advantage(s) for host health” in November of 2008 (Gibson et al. 2010). Since that year, the carbohydrates (dietary fiber) which classified as disaccharides, oligosaccharides and polysaccharides commonly found in edible plant species were known as prebiotics (Markowiak and Śliżewska 2017). The common carbohydrate type of prebiotic can be categorized as digestible and non-digestible, as the digestible carbohydrate can be degraded into simple monosaccharide (sugar) by host’s digestive enzymes while non-digestible carbohydrate can be fermented into benefit substances by host gastrointestinal microbiota which then absorbed by host intestine to improve host health (Navarro et al. 2019). The common prebiotics that used for aquaculture farming mostly belongs to non-digestible oligosaccharides class included fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), mannanoligosaccharides (MOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxyloligosaccharides (AXOS), isomaltooligosaccharides (IMO) (Ringø et al. 2010). The non-digestible polysaccharides are cellulose, hemicellulose, Beta-glucan, pectin, mucilage, gums, lignin and resistant starch (Zhang et al. 2018). Some intestinal microflora can secrete cellulase for assisting enzymatic activity of the crustacean species to hydrolyze ingested cellulose in their gastrointestinal

tract (Tzeng et al. 2015). The application of polysaccharides as effective prebiotics against diseases in shrimp studies as shown in Table 2.4.

Consequently, the definition of prebiotics was re-defined as diverse categories of substrates, including non-carbohydrate based, which are selectively consumed by the inhabiting microbiota to contribute benefit(s) for host's health upon latest updated by ISAPP in December of year 2016 which last until now (Gibson et al. 2017). For instance, raw natural complex compound, honey can be prebiotic for aquaculture farming species because it contains non-digestible disaccharides and polysaccharides (Aryati et al. 2020). Prebiotics are also mainly applied as alternatives of antibiotic to improve host immune system and gastrointestinal health against infectious diseases of aquatic farming animal species (Amenyogbe et al. 2020). Prebiotics are usually applied as feed additives to improve aquafeed for targeted aquaculture farming species in aquaculture (Ganguly et al. 2012). Prebiotic against pathogen usually including nutrient competition by promoted beneficial intestinal microflora, virulent factor inhibition; pathogen quorum sensing disruption, host intestine acidification, host health maintenance (Akhter et al. 2015; Guerreiro et al. 2017). Moreover, some prebiotics might consist of other bioactive compounds which have antipathogenic effects and immune response induction for aquaculture farming species (Caporgno and Mathys 2018). For instance, seaweed, known as marine macroalgae and their crude extracts can be considered as prebiotics because have great dietary components and selectively stimulate growth of intestinal microbiota for host health benefits (Charoensiddhi et al., 2020).

The consensus statement of prebiotic is different or not probably recognized in regulations of other countries because expanding range of prebiotic categories, which include dietary supplements, medicals, cosmetic or other for human (Gibson et al., 2017). Currently, there is no legislation that limits the application of raw, dried and crude extracts of seaweed as prebiotic for aquaculture in American and European countries (Wan et al., 2018). According to regulations of Malaysia, prebiotic is known as one of “added nutrient”, which includes single, or combination of any verified mineral, vitamin, amino acid, fatty acid, nucleotide and/or other food components improve the nutritional value of the food for human consumption (Food regulations, 1985). As referred to current prebiotic definition of the ISAPP, animal probiotic is considered as one of feed additive, which known as added harmless ingredient that affects the characteristics of feed according Malaysia act (Feed act, 2009).

2.6.1 Brown macroalgae (Family Phaeophyceae)

The Asia countries contributed major cultivated seaweed production, including China and Indonesia, as the total cultivated seaweed production achieved 36.5 million tonnes, which valued at USD 16.95 billion in year 2022 (FAO, 2024). These seaweeds, also known as marine macroalgae are classified into three higher taxa according to their pigmentation, including brown macroalgae (Phaeophyceae), green macroalgae (Chlorophyceae), and red macroalgae (Rhodophyceae) (Vilar et al., 2020). For instance, major pigments of Chlorophyta is chlorophyll pigments, major pigments of Phaeophyta are fucoxanthin pigments, as well as major pigments of Rhodophyta are phycocyanin and phycoerythrin pigments (Wirawan et al., 2022). There are few

genera of brown macroalgae that exhibit prebiotic effects, including *Ecklonia* spp., *Sargassum* spp., *Laminaria* spp., *Ascophyllum* spp., *Fucus* spp., *Undaria* spp., *Saccorhiza* spp. and *Porphyra* spp. for improving gastrointestinal microbiota associated with human and animal health (López-Santamarina et al., 2020). The exclusively bioactive compounds were found in brown seaweeds, including polysaccharides (alginate and fucoidan), proteins (phycobiliproteins), polyphenols (phlorotannins), carotenoids (fucoxanthin), phytosterols (fucosterol) and n-3 long-chain polyunsaturated fatty acids (eicosapentaenoic acid) (Olsthorn et al., 2021). Alginates and fucoidans are the most abundant main cell wall components, but cellulose is minor amounts which found in cell wall of the brown macroalgae (Rhein-Knudsen et al., 2023). The raw and extract of brown macroalgae are highly recommended as aquatic animal prebiotic because suitable nutritional components, highly digestible properties, limited antinutritional factor effects for farmed aquatic animals' species (Wan et al., 2018).

2.6.2 Diversity of *Sargassum* spp.

Among brown macroalgae (Phaeophyceae), genera of *Sargassum* spp. shared common morphological characteristics, including a highly branched thallus, hollow berry-like vesicle (pneumatocysts), and numerous small, toothed edges with leaf-like of fronds (Marcin et al., 2024). Most of *Sargassum* spp. are benthic seaweed species that spend their life cycle by attaching themselves on the ocean floor substrate and forms ocean underwater canopies, except *S. natans* and *S. fluitans* are only holopelagic species that remain in a drifting free-floating state on the sea surface for their entire life

cycle (Carral-Murrieta et al., 2023). Although 25 species of *Sargassum* was reported, there are 7 *Sargassum* spp. were well documented, which distributed at intertidal zone of Malaysia ocean such as *S. baccularia*, *S. binderi*, *S. myriocystum*, *S. oligocystum*, *S. siliquosum*, *S. polycystum*, *S. cristaefolium*, *S. ilicifolium* and *S. asperifolium* (Asmida et al., 2017). Beside morphological observation based on axis (stem), leaf shape, leaf margin, vesicle (pneumatocysts) size, and vesicle (pneumatocysts) shape of branched thallus, molecular analysis also can be applied by targeting on specific genes, including Internally transcribed Spacer (ITS), and Large Subunit of Ribulose 1,5 Bisphosphate Carboxylase (rbcL) and 18S ribosomal DNA (18S) to identify species of *Sargassum* spp. (Yip et al., 2018). Figure 2.12 shows benthic and holopelagic species of genera *Sargassum*.

Commonly, *Sargassum* spp. have nutritious contents (vitamins, dietary fibers, proteins, and minerals) and many types of bioactive compounds (terpenoids, flavonoids, sterols, sulfated polysaccharides, polyphenols, sargaquinoic acids, sargachromenol, pheophytine) which exhibit diverse biological activities (anti-inflammatory, antioxidant, neuroprotective, anti-microbial, anti-tumor, fibrinolytic, immune-modulatory, anti-coagulant and hepatoprotective activities) (Yende et al., 2014; Yap-Dejeto et al., 2022). Nutritional composition of *Sargassum* genera can be vary which depend on species, growth stage, environmental factors, and geographic distribution (Lee et al., 2023). In South Korea and China, some of *Sargassum* spp. including *S. thunbergii*, *S. fulvellum*, *S. naozhouense*, *S. horneri*, *S. aquifolium*, *S. macrocarpum* are edible algae with high nutritional value, which being cultivated through artificially for human

consumption and animal farming (Kavale et al., 2021). The raw natural complex compound, *Sargassum* spp. can be applied as prebiotic directly because consists of fermentable dietary polysaccharides (alginates, laminarins, fucoidans) for human and animal consumption (Wan et al. 2018). The application of brown seaweed as effective prebiotics against diseases in shrimp studies as shown in Table 2.4.



Figure 2.12: Benthic and holopelagic species of genera *Sargassum* The benthic species included (a) *S. horneri*, (b) *S. muticum*, (c). *S. horridum* while holopelagic species included (d) *S. fluitans*, (e) *S. natans*. (Carraal-Murrieta et al., 2023).

2.6.3 *Sargassum polycystum*

Sargassum polycystum are large canopy-forming benthic seaweeds which are widely distributed in the intertidal and shallow subtidal zones of tropical Indo-Pacific Asia Ocean as their habitat (Zhang et al., 2022). The specific

morphological characteristics of *S. polycystum*' branched thallus consists of (i) cylindrical and heavily muricate with stolon-like stem (axis); (ii) lanceolate shape of leaf-like fronds; (iii) irregularly and coarsely dentate margin of leaf-like fronds; (iv) globular and mucronate shape of vesicles; (v) 5–12 mm size of vesicles (Yip et al., 2018) as shown in Figure 2.13. In aquaculture, the mass production of *S. polycystum* using its stoloniferous branches as a cultivated material that attach on artificial substrata for its vegetative propagules because it can reproduce asexually with new main axes growing out from its stoloniferous branches (Kavale et al., 2021). In nutritional value, *S. polycystum* that found in Port Dickson, Negeri Sembilan, Malaysia has approximately 9% of protein, 3.5% of lipid, 35% of carbohydrate and 3% of total dietary fibre (Nazarudin et al., 2021). As members of the brown macroalgae, the *S. polycystum* have wide range of bioactive compounds which accomplishes a broad spectrum of the biological activities (Palanisamy et al., 2017). A study demonstrated fucoidan fraction-2 (Fu-F2) of *S. polycystum* has therapeutic potential because its antibacterial activity against the different pathogens (Palanisamy et al., 2019). Another study demonstrated that higher abundance of flavonoid contents compared with phenol contents were found in *S. polycystum* upon various extraction (Johnson et al., 2019). *S. polycystum* is one of edible species that have indigestible polysaccharides, including fucoidans and alginates which known as prebiotic components to improve host gastrointestinal health for human consumption and aquaculture (Nazarudin et al. 2020; Abdel-Rahim et al. 2021; Nazarudin et al., 2021). The application of *S. polycystum* as effective prebiotics against diseases in shrimp studies as shown in Table 2.4.

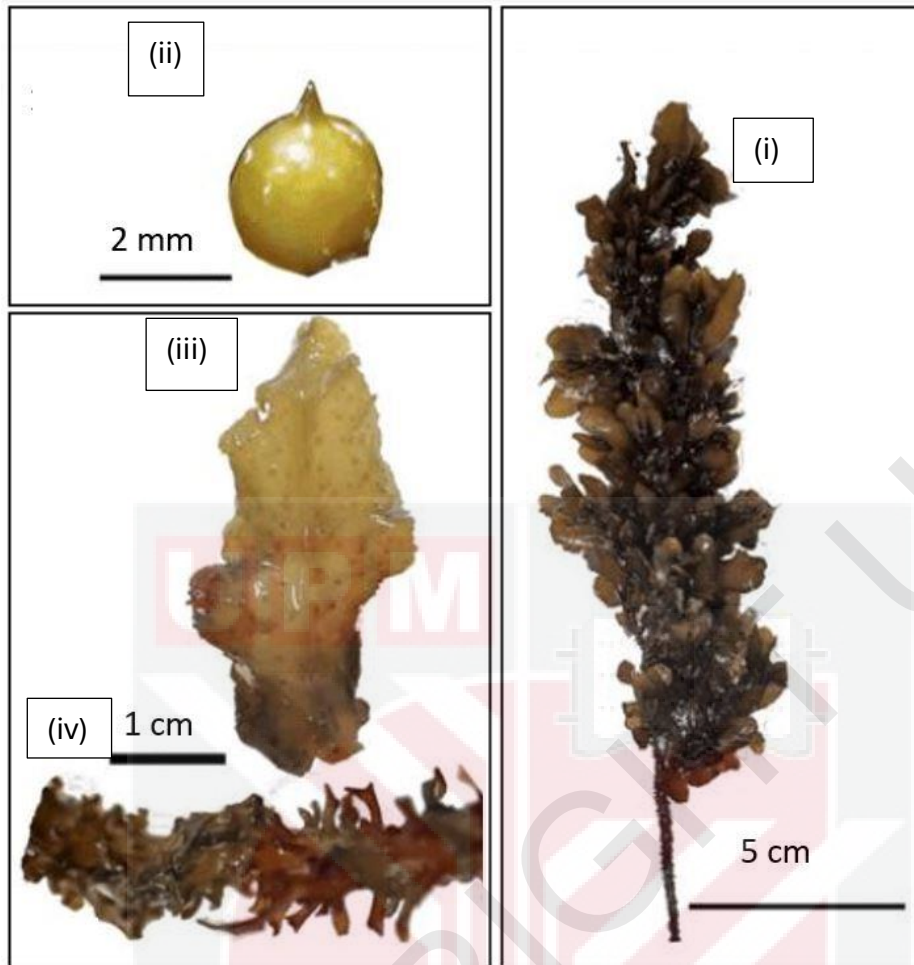


Figure 2.13: Morphological identification of *S. polycystum*. The branched thallus (i) consists of leaf-like frond (ii), vesicle (pneumatocysts) (iii) and stem (iv) (Yip et al., 2018).

Table 2.4: Application of effective prebiotic against diseases in shrimp studies.

Effective prebiotic	Sources	Targeted host	Effective administration and regime	Impact and effect	References
Inulin	Chicory (<i>Cichorium intybus</i>)	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 0.4% of prebiotic diet for 3 times daily in 56 days of feeding trial.	• High body weight gain, body ash and specific growth rate. • High superoxide dismutase, acid phosphate, phenol oxidase and catalase activities. • Increased Firmicutes phylum and <i>Bacillus</i> genus of bacteria.	Zhou et al. (2019)
Mannan oligosaccharide (MOS)	Yeast (<i>Saccharomyces cerevisiae</i>)	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 4.0 g/kg of prebiotic diet for 4 times daily in 56 days of feeding trial.	• High body weight gain and specific growth rate. • Increased microvilli length. • High phenoloxidase, superoxide dismutase activities.	Zhang et al. (2012)
Brown seaweeds powder	<i>Sargassum filipendula</i> and <i>Undaria pinnatifida</i>	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 0.5 % <i>S. filipendula</i> and 2 % <i>U. pinnatifida</i> of prebiotic diet for 4 times daily in 21 days of feeding trial.	• Improved membrane fluidity, lipid and energy metabolism, immune response, apoptosis and antioxidant capacity. • Modulated gastrointestinal microbiota. • High survival of oral administration after 200 μL of White spot syndrome virus inoculum (3.7×10^8 particles μL⁻¹) viral challenge by gavage orally.	Schleder et al., (2020)
Brown seaweed meal (Nutrikelp)	Brown seaweed (<i>Macrocystis</i> spp. and <i>Lessonia</i> spp.)	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with 8 % of prebiotic diet for 4 times daily in 21 days of feeding trial.	• Improve shrimp digestibility and growth performance.	Cárdenas et al., (2015)

2.7 Synbiotic for aquaculture

Synbiotics are combination of probiotic (beneficial microbe) with prebiotic (nutritious substrate) known as promising mediation in the comprehensive nutritional approach for targeting the host gastrointestinal microflora (Sergeev et al. 2020). The “Synergy” term refers to a process of substances have collaborated each other to achieve a combined effect or greater than sum of their separate effects (Pezzani et al. 2019). Therefore, Synbiotics have a synergistic effect by inhibiting the growth of pathogenic bacteria and enhancing the growth of beneficial organisms in the host's gastrointestinal tract (Parian et al. 2018)., International Scientific Association of Probiotics and Prebiotics (ISAPP) defined "A mixture comprising live microorganisms and substrate(s) selectively applied by host microorganisms that contribute health benefit(s) for host" in May of year 2019 (Swanson et al. 2020). Usually, Synbiotics are administrated orally for aquatic farming species in aquaculture practices (Cavalcante et al. 2020).

Synbiotics are mainly applied as alternative of antibiotic for improving immune system and maintaining gastrointestinal health against infectious diseases of aquatic farming animal species which similar with probiotics and prebiotics in aquaculture (Amenyogbe et al. 2020). Ideally, prebiotic substrate in synbiotic mixture improve introduced probiotic of the synbiotic and host autochthonous intestinal microbiota in the targeted (Kearney & Gibbons 2018). In fact, there are two categories of synbiotics including complementary synbiotic and synergistic synbiotic, which complementary synbiotic is non-dependent functional combination of prebiotic and probiotic while synergistic synbiotic is

co-dependent functional combination of prebiotic and probiotic, both synbiotics improve host gastrointestinal microflora differently to contribute specific desired beneficial effects for host (Kolida and Gibson 2011; Swanson et al. 2020). Synbiotic against pathogen usually are combined effects of prebiotics and probiotics, including nutritional competition, gut epithelial surface space competition, antimicrobial compounds synthesis, microbial inhibitory compounds, immunostimulatory effects for host (Huynh et al. 2017). Synbiotics contribute beneficially effects for the hosts by improving survival, implantation and selectively stimulating growth or metabolism of probiotic as well as improve microbial composition in the host's gastrointestinal tract (Gyawali et al. 2019). The application of effective synbiotics against diseases in shrimp studies as shown in Table 2.5.

According to regulations of Malaysia, Synbiotic is combination of “probiotic” and “added nutrient”, which known as the chosen beneficial microorganism is following criteria of microorganisms as mentioned in section 2.5 above and any of verified nutritional food components for human consumption (Food regulations, 1985). As referred to current prebiotic definition of the ISAPP, animal synbiotic is considered also as one of feed additive, which known as combination of added harmless ingredients that affects the characteristics of feed according Malaysia act (Feed act, 2009).

Table 2.5: Application of effective synbiotic against diseases in shrimp studies.

Effective synbiotic	Sources	Targeted host	Effective administration and regime	Impact and effect	References
<i>Pseudoalteromonas piscicida</i> (Probiotic) with Mannan oligosaccharides (Prebiotic)	Probiotic from Pacific white shrimp (<i>P. vannamei</i>) gut; Prebiotic from <i>Saccharomyces cerevisiae</i> cell walls	Pacific white shrimp (<i>P. vannamei</i>) mysis	Oral feeding with combination: 10 ⁶ CFU/mL of probiotic and 12 mg/L of prebiotic encapsulated <i>Artemia</i> spp. for 5 times daily in 13 days of feeding trial	- Improved immune responses, - Increased Total hemocyte count, phenoloxidase activity and respiratory burst activity. - High survival after 3 × 10⁷ CFU/mL of <i>Vibrio harveyi</i> challenge by immersion.	Hamsah et al., (2019).
<i>Lactobacillus acidophilus</i> (Probiotic) with moringa leaf extract (Prebiotic)	Probiotic from laboratory collection, National Ocean University, Taiwan; Prebiotic from <i>Moringa oleifera</i>	Pacific white shrimp (<i>P. vannamei</i>) juvenile	Oral feeding with combination: 1 × 10 ⁷ CFU/g of probiotic and 5.0 g/kg of prebiotic of feed for 3 times daily for 60 days feeding trial and 7 days of challenge	- Improved growth performance, enzymatic activities and intestinal structure. - Improved the gastrointestinal microbiota. - Stimulated immune responses. - High survival after 1 × 10⁷ CFU/mL of <i>V. parahaemolyticus</i> and 1 × 10⁷ of <i>V. alginolyticus</i> challenge by ventral sinus injection. .	Abidin et al., (2022).
<i>Bacillus</i> spp. (Probiotic) with Red seaweed dried powder (Prebiotic)	Probiotic from redtail shrimp (<i>Fenneropenaeus penicillatus</i>); Prebiotic from <i>Gracilaria</i> spp.	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with combination: 5 × 10 ¹⁰ CFU/g of probiotic and 1 mg/g of prebiotic of feed for 2 times daily for 21 days feeding trial and 15 days of challenge	High survival after 1 × 10⁶ CFU/mL of <i>V. parahaemolyticus</i> AHPND challenge by immersion.	Lim et al., (2020).
<i>Halomonas alkaliphila</i> (Probiotic) with <i>Kappaphycus alvarezii</i> powder (Prebiotic I) & <i>Spirulina</i> sp. (Prebiotic II)	Probiotic from Pacific white shrimp (<i>P. vannamei</i>) gut; Prebiotic 1 from Helos®, Indonesia; Prebiotic 2 from CERES®, Indonesia	Pacific white shrimp (<i>P. vannamei</i>) postlarvae	Oral feeding with combination: 1 × 10 ⁹ CFU/g of probiotic, 0.375 % of prebiotic-I and 0.125 % of prebiotic-II for 4 times daily for 14 days feeding trial	- Improved growth performance - Improved the gastrointestinal microbiota - High survival after 1 × 10⁶ CFU/mL of <i>V. harveyi</i> challenge by immersion.	Situmorang et al., (2021).

2.8 Innate immune system of shrimp

Penaeid shrimp depends on their innate immune system against infectious pathogen because lack of adaptive immune system as similar as other species of crustacean (Zhang et al., 2021). Circulating hemocytes and lymphoid organ of the penaeid shrimp are responsible for its innate immune system which involved cellular and humoral immune responses to eradicate the invasive pathogens (Wang et al., 2021). Hemocytes are produced and differentiated by hematopoietic tissue before releasing into hemolymph (Liang et al., 2015). There are three types of mature hemocytes in penaeid shrimp, which are hyalinocytes, semi-granulocytes and granulocytes (Sun et al., 2020). All hemocytes were circulated throughout whole body of shrimp with hemolymph by open circulatory system (Maftuch et al., 2013). Therefore, immune responses of shrimp hepatopancreas and gastrointestinal tract strongly depend on hemocyte infiltration in epithelium which the hemocytes are migrated from circulating hemolymph of shrimp circulatory system (Silveira et al., 2018). In penaeid shrimp, hemolymph is transported by heart contractions into a lymphoid organ for filtering pathogen and foreign material as it moves around the open circulatory system and accumulating it, where immune cells of lymphoid organ immobilize circulating pathogens, phagocytose pathogen and foreign materials, form spheroid cells for viral degradation, and release humoral immune factors into hemolymph circulation (Rusaini and Owens 2010; Wang et al., 2021).

2.8.1 Pattern-recognition proteins

Pathogen-associated molecular pattern (PAMPs) are the components of pathogens including Lipopolysaccharides, peptidoglycan and β -1,3-glucan which are recognized by the appropriate pattern-recognition proteins (PRPs) of shrimp hemocytes, including extracellular proteins which are lipopolysaccharides & β -1,3-glucan-binding protein (LGBP), peptidoglycan-binding protein (PGBP) and β -1,3-glucan-binding protein (β GBP) respectively (Amparyup et al., 2013). Therefore, the LGBP recognizes lipopolysaccharides from outer layer membrane of Gram-negative bacteria (Phupet et al., 2018). The peptidoglycan-binding protein (PGBP) recognizes lysin-motif of peptidoglycan from bacterial cell wall of Gram-positive bacteria (Shi et al., 2016). The β GBP recognizes β -1,3-glucan from fungi cell wall of infectious fungi-like parasites (Goncalves et al., 2012). These activated PRPs of hemocytes activate signal transduction proteins, peroxinectin and trigger a series of downstream immune signaling pathways that mediate the activation of cellular and humoral immune responses (Cai et al., 2020; Zhang et al., 2021). Moreover, the extracellular PRRs bind to PAMPs for triggering serine proteinase-dependent signaling cascade which the cascade is crosstalk between the proPO cascade pathway and the Toll signaling pathway respectively (Clark and Greenwood, 2016). The Toll signaling pathway rely on toll receptor which type I transmembrane receptor (Liu et al., 2018). However, Immune deficiency (IMD) signaling pathways rely on its transmembrane receptor only act as PRR for its downstream signaling (Tassanakajon et al., 2018).

2.8.2 Cellular immune responses

The cellular immune responses of penaeid shrimp included the apoptosis, phagocytosis, encapsulation and nodule formation (Xu et al., 2014). Peroxinectin is crucial protein that have integrin binding motif KGD (Lys-Gly-Asp) binds to integrin of hemocytes for inducing a series of cellular immune activities such as hemocyte adhesion and hemocyte migration (Cai et al., 2020). Hyalinocytes are major phagocytic hemocytes which responsible for engulfing pathogen and foreign substances individually (Sun et al., 2020). Phagocytosis is function of hemocytes (hyalinocytes) which engulfed pathogen and foreign material is digested by hydrolytic enzymes (Liu et al., 2020). Nodulation formation is function of multiple semi-granulocytes and granulocytes aggregate multiple smaller size of pathogen by using peroxinectin to form nodule (Shakweer et al., 2023). Encapsulation is function of multiple semi-granulocytes bind on larger size of pathogen by using peroxinectin to form fibrinogen-related protein capsule (Senghoi et al., 2019). Apoptosis functions as a kind of innate immune responses which granule-containing hemocytes (semi-granulocytes and granulocytes) initiate self-destructive process upon infected by virus using caspases, apoptosis inducing factor (AIF) and cytochrome c (Cyt-c) to prevent the virus proliferation and spreading (Tang et al., 2019).

2.8.3 Humoral immune responses

The humoral immune responses of penaeid shrimp included the prophenoloxidase system, the clotting cascade, and a wide array of antimicrobial peptides (Xu et al., 2014). Semi-granulocytes are hemocytes that regulating humoral immunity signaling pathways for antimicrobial peptides synthesis; granulocytes are hemocytes that regulating proPO system activation for pathogen melanization (Sun et al., 2020). There are two signaling pathways of antimicrobial peptide synthesis that responsible for eliminating pathogenic bacteria, which are Toll signaling pathway (specifies for Gram-positive bacteria and Gram-negative bacteria) and IMD signaling pathway (specifies for Gram-negative bacteria only) (Laohawutthichai et al., 2023). The occurrence of melanization can pathogenic bacteria elimination, cuticle sclerotization, wound healing, and pathogen killing (Boonchuen et al., 2021).

2.8.4 Prophenoloxidase (proPO) system pathway

The serine proteinase cascade which consists of prophenoloxidase-activating enzyme is stimulated upon recognition of PAMPs by PRRs (Noble et al., 2023). The stimulated serine protease activates the prophenoloxidase-activating enzyme (PPAE) (Jearaphunt et al., 2015). The PPAE cleaves a kind of zymogen, prophenoloxidase (proPO) into active phenoloxidase (PO) (Amparyup et al., 2013). The PO catalyzes the phenolic compounds to produce reactive intermediate quinones and melanin for eliminating entrapped pathogens from nodule formation and encapsulation of shrimp hemocytes (Noothuan et al., 2017).

2.8.5 Toll signaling pathway

The serine proteinase cascade is stimulated upon recognition of PAMPs by extracellular PRRs (Noble et al., 2023). The stimulated serine protease cascade cleaves ProSpätzle into active Spätzle for activating toll-like receptor (Boonrawd et al., 2017). Then, the myeloid differentiation factor 88 (MyD88) is recruited by the cytoplasmic interleukin-1 receptor (TIR) domain of the activated toll receptor which type I transmembrane receptor (Liu et al., 2018). Consequently, it triggers the activation of Tube (homolog of IL-1 receptor-associated kinase 4, IRAK4), Pelle (homolog of IL-1 receptor-associated kinase 1, IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) (Liu et al., 2016). The Pelle (known as IRAK1/IKK α) and Tube (IRAK4) phosphorylated and degraded Cactus (homolog of NF- κ B inhibitor) of Cactus-dorsal complex (Wang et al., 2013). Then, the Dorsal (homolog of NF- κ B transcription factor) is released from Cactus-dorsal complex and translocated from hemocyte cytoplasm into the nucleus to induce expression of antimicrobial peptides (Sun et al., 2016).

2.8.6 Immune deficiency (IMD) signaling pathway

Recent study shows the scavenger receptor class B2 (SRB2) which is type III transmembrane receptor of hemocyte act as PRRs recognizes PAMPs to recruit and activate the adaptor protein IMD for recruiting transforming growth factor β -activated kinase 1 (TAK1) and TAK1-binding-protein (TAB2) (Tassanakajon et al., 2018; Shi et al., 2023). Upon the TAK1 is activated by TAB1, the activated TAK1 phosphorylates IKK β (Wang et L., 2017; Wang et

al., 2022). Then, IKK β phosphorylates Relish through proteolytic cleavage on the Relish's ankyrin repeat (homolog of Cactus), and translocated into the nucleus, which induce the expression of immune-related genes, such as antimicrobial peptide genes (Wang et al., 2013; Tassanakajon et al., 2018).

2.9 Gastrointestinal tract of digestive system in shrimp

Gastrointestinal tract of *Penaeus monodon* is divided into three main parts, including foregut, midgut and hindgut as shown in Figure 2.14. The foregut consist of esophagus and stomach; The midgut consist of anterior midgut cecum, hepatopancreas and midgut trunk; The hindgut consist of posterior midgut cecum and hindgut (Muhammad et al., 2012). The shrimp ingested food particles are passed into esophagus, then reach stomach for mechanical digestion. Shrimp hepatopancreas and midgut trunk are crucial organs that responsible for chemical digestion and nutrient absorption but only hepatopancreas take part for nutrient storage and innate immunity regulation. After nutrient absorption, hindgut take part for compressing the undigested substances before being evacuated as feces through shrimp anus (Soonthornchai et al., 2010; Alexandre et al., 2014; Garibay-Valdez et al., 2021).

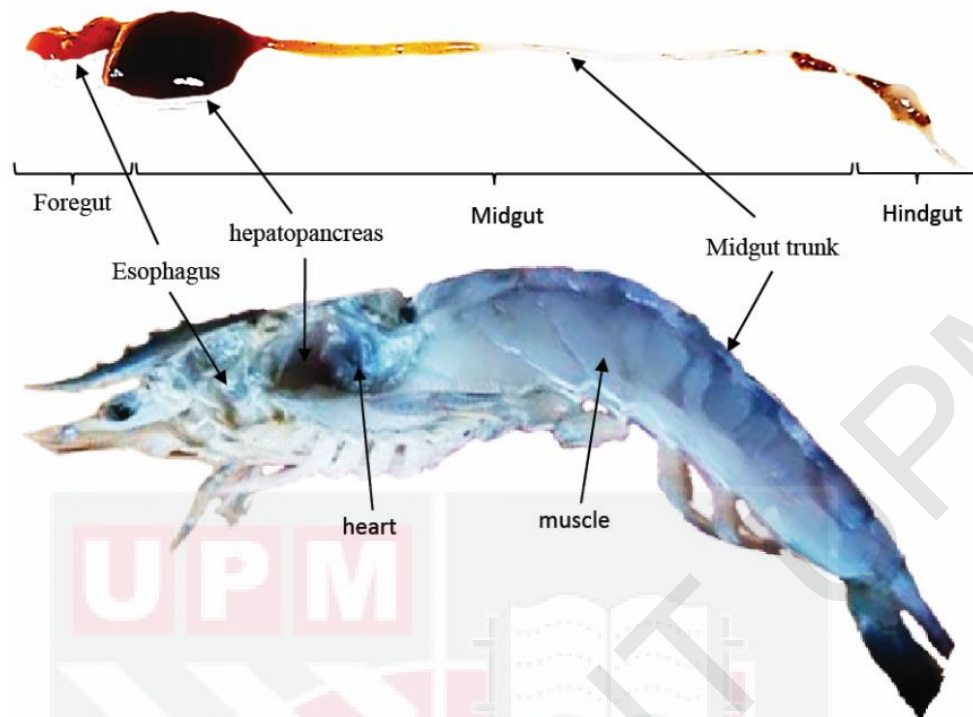


Figure 2.14: Digestive system morphology in horizontal section of *P. monodon* (Photo by Chin. Y.K.) The main components of gastrointestinal tract including foregut, midgut and hindgut.

2.9.1 Gastrointestinal microbiota in shrimp

The gastrointestinal tract, including hepatopancreas of shrimp was inhabited by multiple microorganisms, known as shrimp gastrointestinal microbiota which crucial for contributing shrimp development and physiological functions (Garibay-Valdez et al., 2021). The gastrointestinal microbiota is important for the shrimp's health, as the functional and stable of shrimp gastrointestinal microbiota can maintain immune response, nutrients absorption, and homeostasis of shrimp (Suo et al., 2017). The common bacterial phyla in cultured shrimp gastrointestinal microbiota consists of Proteobacteria, Actinobacteria, Firmicutes, Planctomycetes, Bacteroidetes, Chlamydiae and Cyanobacteria (Vargas-Albores et al., 2017). Commonly, bacterial phyla of Firmicutes and Bacteroidetes are known as beneficial bacteria that maintain

gastrointestinal immune, homeostasis and improve the shrimp health (Liang et al., 2022). However, different shrimp gastrointestinal microbiota compositions are inhabited in shrimp foregut, midgut and hindgut because differential selection pressures of different structures and functions along the longitudinal axis of the shrimp gastrointestinal tract (Holt et al., 2021). Moreover, the common factors that affected shrimp gastrointestinal microbiota, including water environment and diet composition (Liu et al., 2022). In fact, the changes of shrimp gastrointestinal microbiota are affected by physical and chemical parameters of water environment, such as ammonia nitrogen, water temperature, salinity and pH which favorable for shrimp to adapt its external environment (Zeng et al., 2024). Moreover, different shrimp diet which comprising various of nutrients and/or supplement including probiotic, prebiotic and synbiotic can modulate the shrimp gastrointestinal microbiota composition, which affects growth performance, disease resistance and productivity of shrimp (Ochoa-Romo et al., 2022). Furthermore, administrated probiotics and beneficial indigenous intestinal microbiota secrete various digestive enzymes to degrade several nutritional compounds, including carbohydrate, protein and lipid of ingested food into simple molecules, which can be absorbed easily and modify metabolic pathways of epithelial cells in shrimp gastrointestinal tract (Ringø et al., 2022). Usually, non-digestible and complex structure of prebiotic can be degraded into benefit substances, including nutritional carbohydrate, protein and antimicrobial substances by shrimp gastrointestinal microbiota which then absorbed and utilized by shrimp (Navarro et al. 2019). Conversely, the infectious shrimp diseases and bad water quality can eliminate indigenous beneficial microorganism in shrimp gastrointestinal tract, which lead to the

dynamic imbalance of shrimp gastrointestinal microbiota directly (Gao et al., 2024). The dynamic imbalance of shrimp gastrointestinal microbiota often refers to dysbiosis of shrimp, which can further increase the susceptibility of multiple shrimp diseases, such as acute hepatopancreatic necrosis disease (AHPND), hepatopancreas necrosis syndrome (HPNS), and white feces syndrome (WFS) (Zeng et al., 2024).

2.9.2 Molecular detection of shrimp microbiota composition

Beside traditional microorganisms' culture, isolation, incubation, and chemical examination methods known as gold standard, the molecular biology detection technology, which mostly is conventional polymerase chain reaction (PCR), has been applied recently using specific gene primers and genomic DNA to clone targeted DNA fragments for detecting presence of microorganisms in the shrimp gastrointestinal tract (Li et al., 2022). Moreover, quantitative PCR (qPCR) can be applied by targeting 16S rRNA gene or other specific genes to quantify cloned DNA fragments that hybridized with fluorescent for enumerating the targeted microbial load, abundance or profile accurately in the shrimp gastrointestinal tract (Galazzo et al., 2020). These molecular biology detection methods are more feasible compared to traditional methods. Currently, next-generation sequencing (NGS), known as high-throughput technologies are faster, higher accuracy and cost-effective advanced method without require cloning of DNA fragments to obtain microbial DNA sequence information from shrimp gastrointestinal tract as compared to the conventional PCR and qPCR (Hernández et al., 2020). These high-throughput sequencing technologies enable identification and comparison of entire microbial

communities, known as metagenomics, which typically have two sequencing strategies, such as amplicon sequencing and shotgun sequencing (Rausch et al., 2019). The shotgun sequencing is one of the high-throughput strategies which determine microbiota, including bacterial taxa, non-bacterial taxa, species, strain, and their functional characterization in various environments (Mannion et al., 2023). Amplicon sequencing is high-throughput strategy also which targets a specific region of a partial or full region of the 16S rRNA, 18S rRNA or ITS rRNA gene for determining specific microbiota community, such as bacterial taxa or non-bacterial in different environment (Kinoshita et al., 2021). These high-throughput sequencing techniques were recently applied to investigate shrimp disease pathogenesis and evaluate effectiveness of probiotic, prebiotic and synbiotic for shrimp in aquaculture (Goh et al., 2022).

CHAPTER 3

PROBIOTIC SCREENING AND ASSESSMENT OF *Lactobacillus plantarum* STRAINS IN BLACK TIGER SHRIMP (*Penaeus monodon*): IMPLICATION FOR GROWTH PERFORMANCE, PROBIOTIC-PATHOGEN INTERACTION, AND RESISTANCE AGAINST ACUTE HEPATOPANCREATIC NECROSIS DISEASE

3.1. Introduction

Between 2011 and 2014, the AHPND outbreak led to a significant reduction in local shrimp production in Malaysia, resulting in an estimated economic loss of approximately USD 0.49 billion (Muthukrishnan et al., 2019). Probiotics were known as one of the alternatives to antibiotics, in preventing infection of *Vibrio* spp. positive AHPND in black tiger shrimp by exhibiting antagonistic activities against pathogen in shrimp hepatopancreas and intestine. Most of probiotics used in aquaculture farming were allochthonous strains, strains that were isolated from another animal species instead of the targeted species (Yamashita et al., 2019). These allochthonous strains can beneficially restructure the host's gastrointestinal tract microbiome and against dysbiosis-related diseases (Zhang et al., 2016). *Lactobacillus* spp. and *Bacillus* spp. strains are typically bacterial members of the gut microbiota of some fish and are widely used as probiotics (Yu et al., 2019). In previous evidence, probiotic showed an improvement in the digestion of protein, starch, and lipid in shrimp gastrointestinal tract, which resulted in better shrimp growth (Mirbakhsh et al., 2022). Moreover, *Bacillus* sp. and *Lactobacillus* sp., as probiotic strains, were reported to inhibit the activity of pathogenic AHPND-causing bacteria by producing various antibacterial compounds (Kumar et al., 2021). In addition, *L. plantarum*, *L. fermentum* and *B. velezensis* improved the survival of *P.*

vannamei postlarvae during AHPND-causing *V. parahaemolyticus* and *V. campbellii* infection (Truc et al., 2019; Jeon et al., 2022). Despite that, no study has described the local strains of *Lactobacillus* sp. and *Bacillus* sp. as effective probiotic for better growth performance and disease resistance in black tiger shrimp (*P. monodon*) against AHPND-causing *V. parahaemolyticus*, that are prevalent in Malaysia currently. Therefore, the bacteria isolation was conducted using different aquatic animals because the diverse fish and crustacean species could be sources of potential probiotic candidates, including lactic acid bacteria (LAB) and other bacteria that possessed antimicrobial properties (Ringø et al., 2018). Besides the bacterial isolates, the probiotic strains, *L. plantarum* strain L8 and L20 were also used for subsequent *in vitro* and *in vivo* experiments of this study because these probiotic strains possessed multiple pathogen antagonistic activity, high salinity tolerance, bile salt tolerance and acid tolerance without hemolytic activity which were previously isolated, screened and identified by Ida-Muryany et al., (2017).

Some strains of probiotic with transient properties do not colonize aquatic animal intestine and consequently not contribute to the health of the aquatic animal (Ibrahim 2015). Hence, careful selection of probiotics for shrimp is important to optimize their beneficial effects and improve disease resistance of shrimp (Wei et al. 2022). Determining the effectiveness of probiotics for host health benefits in aquaculture requires careful consideration of both the cell viability and the amount of probiotic administered (Vinderola et al., 2022). Reverse-transcriptase quantitative PCR (RT-qPCR) assay is suitable to be used as probing method to detect and enumerate active, viable and live

bacterial cell because mRNA can rapidly degrade in dead cells but remain stable in live cells (Molaei et al., 2015).

Besides, the conducted experimental flow charts of Chapter 3 are shown in Figure 3.1. The study aims of Chapter 3 are shown below.

1. To screen the potential probiotic of *Lactobacillus* spp. strains through isolation, characterization and screening on isolated bacteria from fresh seafoods in local wet market.
2. To analyze the effectiveness of different *Lactobacillus* spp. strains supplemented feeds on the growth performance and AHPND-causing *V. parahaemolyticus* S2-4 resistance of *P. monodon*.
3. To elucidate the modulatory effects of different *Lactobacillus* spp. strains supplemented feeds on digestive tissue structure, probiotic and pathogen viability in *P. monodon*.

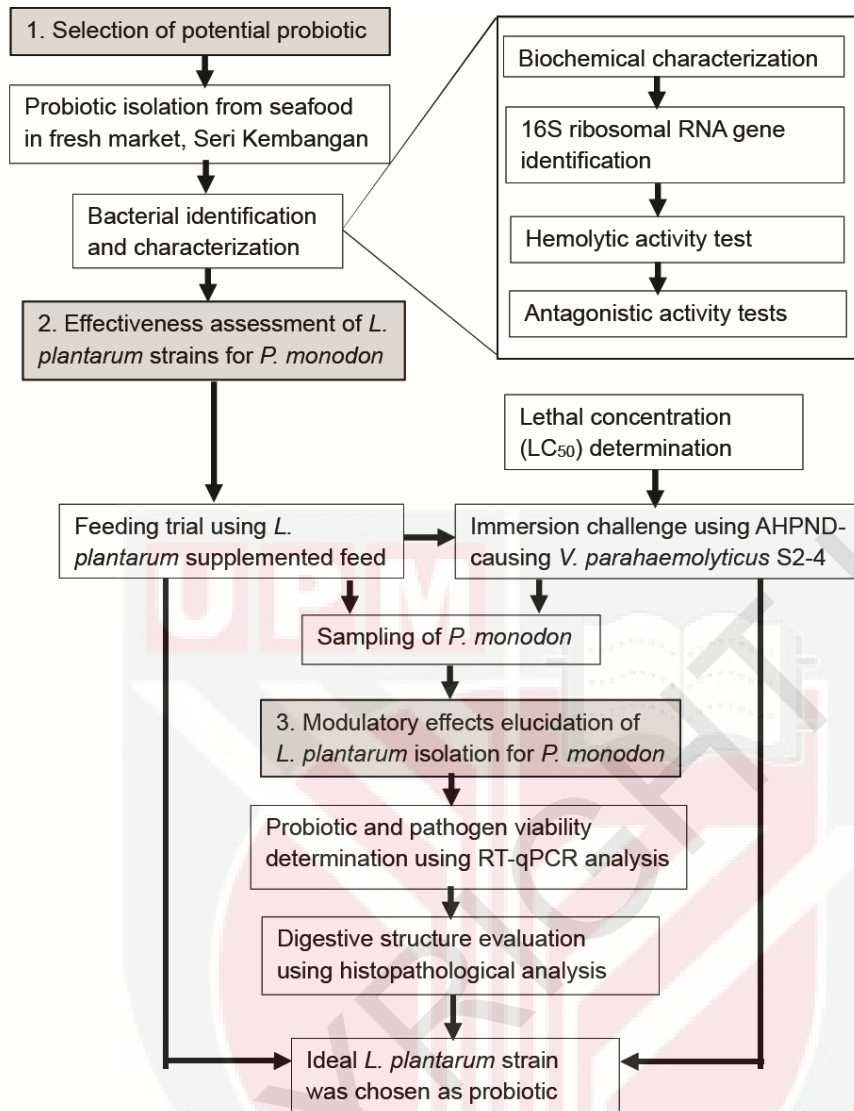


Figure 3.1: Flow charts of conducted experiments in Chapter 3.

3.2. Material and methods

3.2.1. Selection of potential probiotic

3.2.1.1. Sample collection

Diverse fish species and other aquatic organisms were potential sources of bacteriocinogenic lactic acid bacteria as potential probiotic candidate which can produce organic acid and bacteriocin that possessed antimicrobial

properties against aquatic pathogens (Ringø et al., 2018). The sampling period of this study was conducted in August 2020 to September 2020 but non-essential economic activities, including sampling activity in aquaculture farm was not allowed for long-distance travel (more than 10 KM radius from own residential place) because within Recovery Movement Control Order (RMCO) period during COVID-19 pandemic in Malaysia (Zamri et al., 2021). Therefore, multiple species of fresh wild-caught fish and shrimp from nearby wet market were chosen as potential probiotic candidates sampling sources (Table 3.1). In this study, different species of fresh samples including 20 samples of captured fresh marine fish and 4 samples of cultured fresh crustacean (killed and stored in ice within 2 days from fishing port before sell) were purchased from the nearby wet market, Pasar Borong Selangor at Sri Kembangan, Selangor, Malaysia in year 2021. The fish samples were collected 3 whole pieces per species, including Quoy's garfish (*Hyporhamphus quoyi*), blackspot Snapper (*Lutjanus ehrenbergii*), streaked spinefoot (*Siganus javus*), largescale tonguesole (*Cynoglossus arel*), half-smooth golden Pufferfish (*Lagocephalus spadiceus*), fourfinger threadfin (*Eleutheronema tetradactylum*), moonfish (*Mene maculate*), Threadfin bream (*Nemipterus japonicus*), silver pomfret (*Pampus argenteus*), Malabar trevally (*Carangoides malabaricus*), common ponyfish (*Leiognathus equulus*), yellowtail scad (*Atule mate*), bigeye scad (*Selar crumenophthalmus*), herring scad (*Alepes vari*), Belanger's croaker (*Johnius belangerii*), Yellowtail fusilier (*Caesio cuning*), milkfish (*Chanos chanos*), orange-spotted Grouper (*Epinephelus coioides*), red Snapper (*Lutjanus erythropterus*), Asian seabass (*Lates calcarifer*) and crustacean samples were Pacific white shrimp (*P. vannamei*), black tiger

shrimp (*P. monodon*), freshwater giant prawn (*Macrobrachium rosebergii*), Banana shrimp (*Penaeus merguensis*) (Figure 3.2). After the samples were bought in the retail markets, they were placed in sterile plastic bags and immediately transferred to the Laboratory of Aquatic Animal Health and Therapeutic (AquaHealth), Institute of Bioscience, Universiti Putra Malaysia.

Table 3.1: The list of sampled species and their details.

No	Sample	Species description	Sample weight (g)
1		Common name: Quoy's garfish Scientific name: <i>Hyporhamphus quoyi</i> Morphology: Prolonged an beak-like lower jaw; blunt short upper jaw; forked caudal fin (Ambak et al., 2012).	95 ± 4.08
2.		Common name: Blackspot Snapper Scientific name: <i>Lutjanus ehrenbergii</i> Morphology: A black blotch on lateral line below soft dorsal fin; yellow longitudinal line (Ambak et al., 2012).	226.67 ± 20.55
3.		Common name: Streaked spinefoot Scientific name: <i>Sejanus javus</i> Morphology: White or whitish blue spots on upper head and body; wavy horizontal on lower body (Ambak et al., 2012).	304.67 ± 6.18
4.		Common name: Largescale tonguesole Scientific name: <i>Cynoglossus arel</i> Morphology: Yellow brown to greyish body; absent of pectoral fin (Ambak et al., 2012).	132.67 ± 9.53

5.		Common name: Half-smooth golden pufferfish Scientific name: <i>Lagocephalus spadiceus</i> Morphology: Round body; three broad brown band across back; silverly yellow on side body; yellow dorsal and pectoral fins (Ambak et al., 2012).	113.67 ± 14.52
6.		Common name: Fourfinger threadfin Scientific name: <i>Eleutheronema tetradactylum</i> Morphology: Silver body; four free pectoral rays; yellow pectoral and anal fins (Ambak et al., 2012).	198 ± 9.02
7.		Common name: Moonfish Scientific name: <i>Mene maculate</i> Morphology: Compressed body; long anal fin; fused and prolonged pelvic fins (Ambak et al., 2012).	86 ± 8.6
8.		Common name: Threadfin bream Scientific name: <i>Nemipterus japonicus</i> Morphology: Yellow stripes present on body, dorsal and anal fins; Red blotch on posterior lateral line (Ambak et al., 2012).	167 ± 17.96
9.		Common name: Silver pomfret Scientific name: <i>Pampus argenteus</i> Morphology: Anterior part of dorsal and anal fins elongated to appear as sick shaped body (Ambak et al., 2012).	77.33 ± 7.59
10.		Common name: Malabar trevally Scientific name: <i>Carangoides malabaricus</i> Morphology: Silver body; blackspot on opercle; the straight portion of lateral line has 20 scutes (Ambak et al., 2012).	70.67 ± 4.11

11.		Common name: Common ponyfish Scientific name: <i>Leiognathus equulus</i> Morphology: Silver body; strong arched back; protracted mouth; yellow anal fin, transparent dorsal fin (Ambak et al., 2012).	74 ± 4.55
12.		Common name: Yellowtail scad Scientific name: <i>Atule mate</i> Morphology: Yellowish vertical band on body; yellow dorsal and caudal fin (Ambak et al., 2012).	150.33 ± 18.8 g
13.		Common name: Bigeye scad Scientific name: <i>Selar crumenophthalmus</i> Morphology: Big eye; lateral line scutes on short straight portion (Ambak et al., 2012).	124.67 ± 12.92 g
14.		Common name: Herring scad Scientific name: <i>Alepes vari</i> Morphology: Spinous dorsal hyaline; black spot on opercle (Ambak et al., 2012).	152.33 ± 7.93 g
15.		Common name: Belanger's croaker Scientific name: <i>Johnius belangerii</i> Morphology: Snout and lower jaw with conspicuous pores; long dorsal fin with deep notch between spinous and soft ray; dark caudal fin (Ambak et al., 2012).	140.67 ± 14.08 g
16.		Common name: Yellowtail fusilier Scientific name: <i>Caesio cuning</i> Morphology: Yellow dorsal fin and upper body; grayish-blue anterior body; yellow caudal fin; pinkish belly, pectoral, pelvic and anal fins (Ambak et al., 2012).	173.33 ± 11.32 g

17.		Common name: Milkfish Scientific name: <i>Chanos chanos</i> Morphology: Elongated body; protruded lower jaw; long dorsal and anal fin bases (Ambak et al., 2012).	923.67 ± 86.40 g
18.		Common name: Orange-spotted Grouper Scientific name: <i>Epinephelus coioides</i> Morphology: Brownish round spots on all fins; yellowish brown large round spot on body (Ambak et al., 2012).	828 ± 46.88 g
19.		Common name: Red Snapper Scientific name: <i>Lutjanus erythropterus</i> Morphology: Pink body and all fins; sloped head; scale rows on back rising obliquely above lateral line (Ambak et al., 2012).	804.67 ± 65.85 g
20.		Common name: Asian seabass Scientific name: <i>Lates calcarifer</i> Morphology: Elongated body; upper jaw extending behind eye; low edge serrated preopercle (Ambak et al., 2012).	833.67 ± 52.80 g
21.		Common name: Pacific white shrimp Scientific name: <i>Penaeus vannamei</i> Morphology: Translucent white body; moderate rostrum with dorsal and ventral teeth; red tail (Holthuis 1980).	15.33 ± 3.68 g
22.		Common name: Black tiger shrimp Scientific name: <i>Penaeus monodon</i> Morphology: Long rostrum with dorsal and ventral teeth; dark colour carapace; transversely banded with black and white on abdomen (Holthuis 1980).	28.33 ± 4.11 g

23.		Common name: Giant freshwater prawn Scientific name: <i>Macrobrachium rosenbergii</i> Morphology: Long rostrum with dorsal and ventral teeth; adult male has very long elongated blue second chelipeds with blunt spines (Holthuis 1980).	27.67 ± 4.03 g
24.		Common name: Banana shrimp Scientific name: <i>Penaeus merguensis</i> Morphology: Short rostrum with dorsal and ventral teeth; Cream, brownish yellow body; pink tinged yellowish pleopds (Holthuis 1980).	21.33 ± 2.87 g

3.2.1.2. Isolation of potential probiotic bacteria

For isolation, the streak plate method was applied for potential probiotic from the sampled seafood. De Man, Rogosa, Sharpe (MRS) broth and agar supplemented with 1% - 9% NaCl can be used for isolation of lactic acid bacteria (e.g., *Lactobacillus* spp.) and *Bacillus* spp. with halotolerant properties (Nwagu et al., 2020), which suitable for marine environment. Once arrived at the laboratory, 100 µg of intestine were extracted from the fish and decapod samples (n = 3), and homogenized in MRS broth (Himedia, Mumbai, India) supplemented with 2.5% NaCl. The homogenized samples were incubated at 30 °C for 48 h. The two glycerol stored probiotic *L. plantarum* strains L8 and L20 which previously isolated from Belanger's croaker, *Johnius Belangerii* and screened by Ida-Muryany et al. (2017) in laboratory collection were used also for the potential probiotic isolation. Then, one mL of the MRS broth containing the homogenized organ from sampled species was pipetted onto MRS agar

(Himedia, Mumbai, India), supplemented with 2.5% NaCl, and incubated at 30 °C for 48 h. One mL of the glycerol-stored MRS broth containing the isolates from laboratory collection was pipetted onto MRS agar (Himedia, Mumbai, India), supplemented with 2.5% NaCl respectively, and incubated at 30 °C for 48 h. Repeated subculturing was performed using MRS agar supplemented with 2.5% NaCl until single colonies were observed. Convex circular shape, smooth surface, and opaque creamy white color (10 isolates/individual fish) were selected and cultured on MRS agar supplemented with 2.5% NaCl for morphological and biochemical characterization.

3.2.1.3. Biochemical characterization of presumptive lactic acid bacteria isolates

Briefly, selected bacterial isolates were grown on tryptic soy agar (TSA) broth (Oxoid, Hampshire, UK) supplemented with 1.5% NaCl, incubated at 30 °C for 24 h. The bacterial isolates were further characterized phenotypically by a series of biochemical tests, including Gram-staining, oxidase, catalase, sulfide, indole, and motility tests. The isolates with the presence of Gram-positive, rod, coccus or coccobacillus shape, negative oxidase, negative catalase, negative sulfide, negative indole, and negative motility were considered as presumptive lactic acid bacteria (LAB) and chosen for subsequent molecular identification (Karami et al. 2017). The L8 and L20 strains were re-characterized by repeated the procedure above.

3.2.1.4. 16S ribosomal RNA gene identification

For DNA isolation, selected presumptive LAB isolates were grown separately on MRS broth supplemented with 2.5% NaCl at a temperature of 30 °C for 48 h with constant stirring at 150 rpm. Then, the genomic DNA were first extracted by using the enzymatic lysis buffer (20 mM Tris Cl, pH 8.0, 2 mM NaEDTA, 1.2% Triton X-100, 20mg/ml lysozyme) (Chemiz, Shah Alam, Malaysia). Then, DNA extraction and purification was performed on the samples of the pellet using Qiagen Blood and Tissue kits (Qiagen, Hilden, Germany) with manufacturer's procedures for Gram-positive bacteria. Extracted DNA was subjected to amplification using PCR. Amplification of the 16S rRNA gene was performed using universal primers, forward (5'-AGAGTTTGATCC TGGCTCAG-3') and reverse (5'-ACGGCTACCTTGTTACGACTT-3') (Ida-Muryany et al., 2017). The PCR mixture consisted of 100 ng of 2.5 uL template DNA, 6X of 12.5 µL PCR Master Mix (Apical Scientific Laboratories, Sri Kembangan, Malaysia), 1 µL of 20 pmol of each primer. The total volume was brought up to 25 µL with sterile DEPC-treated water. Pre-denaturation at 95 °C for 5 min, followed by 40 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min then were terminated by final extension at 72 °C for 5 min in in an Eppendorf Mastercycler Nexus Thermal Cycler (Eppendorf, Hamburg, Germany). Then, the PCR products were subjected to electrophoresis using a 1.0% agarose gel in 1×Tris-acetate EDTA (TAE) buffer (Apical Scientific Laboratories, Sri Kembangan, Malaysia). A 1 kB DNA ladder (Apical Scientific Laboratories, Sri Kembangan, Malaysia) was used as a molecular marker and later the bands produced were visualized under UV using a gel documentation system (Acinterlab, Miami,

USA). The PCR products were sent for sequencing (First Base Laboratories, Sri Kembangan, Malaysia). The L8 and L20 strains were re-identified by repeated the procedure above.

The 16S rRNA nucleotide sequences of the samples were compared with references sequences available in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank>) using the nBlast program. Phylogenetic analysis was conducted using molecular evolutionary genetics analysis software (MEGA version 7.0). After the addition of 40 reference sequences from the GenBank, the database of 16S rRNA gene sequence was aligned using the ClustalW tool (Appendix 2). The phylogenetic construction of 16S rRNA genes of the identified strains and reference sequences was computed using the maximum likelihood method and Kimura 2-parameter model with 1000 bootstrap replicates (Kumar et al. 2016b). The identified strains of *Lactobacillus* spp. and *Bacillus* spp. were chosen for the subsequent experiment as they shared same genera with most of widely applied probiotic species in Aquaculture (Ringø et al., 2020).

3.2.1.5. Hemolytic activity of *Lactobacillus* and *Bacillus* strains

Selected strains of *Lactobacillus* sp. (KD2, T31, DJ1, L8, L20) and *Bacillus* sp. (T4, T51, L21, KF2, SS1) were streaked on blood agar (5% of horse blood) (Oxoid, Hampshire, United Kingdom) and incubated at 30 °C for 24 h. Strains that produced complete hemolysis (β -hemolysis) were rejected, whereas strains of partial hemolysis (α -hemolysis) and no hemolysis (γ -hemolysis) were selected for subsequent antagonistic activity assay.

3.2.1.6. Antagonistic activity of *Lactobacillus* strains

The antagonistic activities of *Lactobacillus* spp. strains against multiple shrimp pathogenic *Vibrio* spp. were carried out using colony top assay, well diffusion assay, and co-culture assay (Adetoye et al. 2018). Three AHPND-causing *Vibrio* spp. (*V. parahaemolyticus* S2-4, *Vibrio campbellii* S10-4, *Vibrio rotiferianus* S24-4) and a non-AHPND-causing *Vibrio* sp. (*V. parahaemolyticus* J21Y) that were previously isolated by Haifa-Haryani et al. (2022) were chosen for the antagonistic activity assay.

3.2.1.6.1. Colony top assay

For the colony top assay, five μL of 10^8 CFU/mL MRS broth culture of each *Lactobacillus* sp. strain was punctured on MRS agar plate supplemented with 1.5% NaCl and incubated at 30 °C for 24 h. Non-cultured MRS broth supplemented with 1.5% NaCl was punctured on MRS agar plate as control. After incubation, the MRS agar plates were overlaid with 10^5 CFU/mL of an overnight broth culture of the four *Vibrio* spp. pathogens inoculated in 10 mL of Mueller Hinton (MH) (Oxoid, Hampshire, United Kingdom) soft agar (0.7% agar-agar) supplemented with 1.5% NaCl. The overlay was allowed to set and incubated at 30 °C under aerobic condition for 24 h and the zones of inhibition were measured.

3.2.1.6.2. Well diffusion assay

For the well diffusion assay, the cultured broth, cell free supernatant (CFS) and neutralized cell free supernatant of *Lactobacillus* spp. were further tested for antibacterial activities. The *Lactobacillus* spp. strains were grown overnight in MRS broth supplemented with 1.5% NaCl with 30°C and centrifuged at 10000 rpm for 10 min. The pellets were discarded, and the pH of the CFS was adjusted to 6.2 using 1.0 M NaOH (Chemiz, Shah Alam, Malaysia). One hundred µl of CFS and neutralized CFS (adjusted pH of CFS) of the *Lactobacillus* spp. strains were placed in wells (6 mm) bored into MH agar supplemented with 1.5% NaCl pre-seeded with 10^5 CFU/ml of the test *Vibrio* spp. The non-cultured MRS broth supplemented with 1.5% NaCl was placed in wells bored into the MH agar supplemented with 1.5% NaCl pre-seeded with 10^5 CFU/ml of the test *Vibrio* spp. as control. The broth and supernatant were allowed to diffuse for one hour before incubation at 37 °C for 24 h. The plates were examined, and clear zones of inhibition were measured. For the subsequent coculture assay, *Lactobacillus* spp. strains will be selected only if the clear zones of inhibition are at least 15 mm.

3.2.1.6.3. Co-culture assay

For the co-culture assay, the double strength tryptic soy broth (TSB) broth (Oxoid, Hampshire, United Kingdom) supplemented with 1.5% NaCl, was inoculated with 10^8 CFU/ml of *Lactobacillus* spp. strains and 10^8 CFU/ml of *V. parahaemolyticus* S2-4. Monoculture of each strain was used as control. All the cultures were incubated in 30 °C in shaking conditions with 150 rpm. Serial

dilution was carried out immediately after inoculation and appropriate dilutions of the co culture mixture were plated onto thiosulfate citrate bile salts sucrose (TCBS) (Oxoid, Hampshire, United Kingdom) agar. The cultures were serially diluted and plated out at an interval of 0 h, 12 h, 24 h and 48 h and the viable cell count (CFU/ml) at each time were compared with the control grown in monoculture. The rate of inhibition of growth of the *V. parahaemolyticus* S2-4 by the *Lactobacillus* sp. strain was determined. Bactericidal activity was defined as a reduction of at least 99.9% ($\geq 3 \log_{10}$) of the total count of CFU/mL in the original inoculum (Pankey and Sabath 2004; Zadrazilova et al. 2015). Consequently, *Lactobacillus* spp. strains exhibiting bactericidal activity against *V. parahaemolyticus* S2-4 will be chosen as a probiotic in the subsequent diet formulation after the previous screening activities.

3.2.2. Assessment of *L. plantarum* effectiveness for *P. monodon*

3.2.2.1. Preparation of *L. plantarum* strains supplemented feed

Four groups of experimental feeds were selected for feeding trial experiment (3 strains of *Lactobacillus* spp. + control). After the serial of previous screening activities, the three selected *Lactobacillus* strains were *Lactobacillus* sp. strain T31, strain KD2, and strain L20 from Quoy's garfish, Blackspot snapper and lab collection (Appendix 1). Therefore, an overnight TSB culture of *Lactobacillus* sp. strain was centrifuged to get the pellet. After supernatant was discharged, the bacterial pellet was resuspended with sterile phosphate-buffered saline (PBS) respectively. Before diet preparation was made, the basal feed was ground to pass through a 60-mesh screen. The three probiotic

diets were mixed with *Lactobacillus* spp. strain suspensions (10^8 CFU/g), while the control diet was mixed with blank PBS at a ratio of 1:1. The mixtures were put into a stirring machine and water was added slowly until a stiff dough was formed. The resulting stiff dough was then passed through a mincer to form spaghetti-like strings and was cut into pellets approximately 2 mm in diameter and length. Pellets were dried at a temperature of 20 °C. The pellets were then grounded into powdered form. The experimental diets were individually preserved in transparent sterile plastic bags and kept at a temperature of 4 °C before used. The viability of probiotic in the experimental diets were assessed using plate count enumeration. *Lactobacillus* spp. counts were determined using ten-fold serial dilution of a pour plate method on MRS agar and incubated at 30 °C for 48 h. The *Lactobacillus* spp. count enumeration were recorded every 24 h for 7 days. The proximate analysis for moisture, crude ash, crude protein, crude lipid and crude fiber of the experimental diets were analyzed by laboratory services of Unipeq Sdn. Bhd, Bangi, Malaysia according standard AOAC methods (AOAC, 2016).

3.2.2.2. Acclimatization of *P. monodon*

Black tiger shrimp, *P. monodon* postlarvae (PL15; n = 960) were brought from hatchery of Teluk Senangin, Perak, Malaysia, to the hatchery of Institute of Biosciences, Universiti Putra Malaysia, Serdang, Malaysia. During acclimatization, shrimp were assigned to a fiberglass tank (500 L) with 30 ppt of seawater, constant aeration, covered with black plastic cloth for preventing light, and a heater to maintain the temperature at about 27 °C. They were fed daily with the commercial feed (Star Feedmill, Beranang, Malaysia; crude

protein 40%, crude fat 5%, crude fiber 3%, moisture 12%) at 3% of their body weight for 7 days and 30% of water was exchanged every three days. After acclimatization, the 400 shrimps were used for determining lethal concentration 50 (LC₅₀), while 600 shrimps were used for feeding trial. LC₅₀ is mathematical model to qualify and quantify pathogenicity, and virulence of certain pathogen by determining concentration that cause mortality in 50% of tested animals during experiment (Saganuwan 2020).

3.2.2.3. Feeding management for *P. monodon*

The three treatment groups (n = 150 per group) were administered with three different *Lactobacillus* sp. supplemented feeds, while a control tank was administered with basal feed as shown in Table 3.2. Shrimp were fed three times daily for 35 days with the experimental diet, which was 4% of their body weight. In the growth trial, weight was measured on the 0th day (beginning of the feeding trial), the 7th day, and the 35th day (end of feeding trial) respectively. On the end of the feeding trial (35th day), shrimp juveniles were taken for gene expression study (3 shrimp/group) and histopathology study (3 shrimp/group). Figure 3.2. shows the timeline of the feeding trial regime and immersion challenge.

The parameters of growth performance were calculated as follows:

1. Survival (%) = (final shrimp number/initial shrimp number) × 100 %.
2. Weight gain (%) = (final weight – initial weight)/initial weight × 100 %
3. Specific growth rate = (ln final weight – ln initial weight)/days × 100 %
4. Feed conversion ratio = Total dry feed intake (g)/Total wet weight gain (g)

5. Protein efficiency ratio = Total fresh flesh gain (g)/Total protein intake (g)

Table 3.2: Experimental design of feeding trial and immersion challenge

Group	Supplemented feed	Feeding trial		Immersion challenge	
		Number of shrimp per tank	Replicate tank	Number of shrimp per tank	Replicate tank
T31	10 ⁸ CFU/g of <i>L. plantarum</i> T31 with PBS water	50	3	20	4
KD2	10 ⁸ CFU/g of <i>L. plantarum</i> KD2 with PBS water	50	3	20	4
L20	10 ⁸ CFU/g of <i>L. plantarum</i> L20 with PBS water	50	3	20	4
Control	PBS water only	50	3	20	4

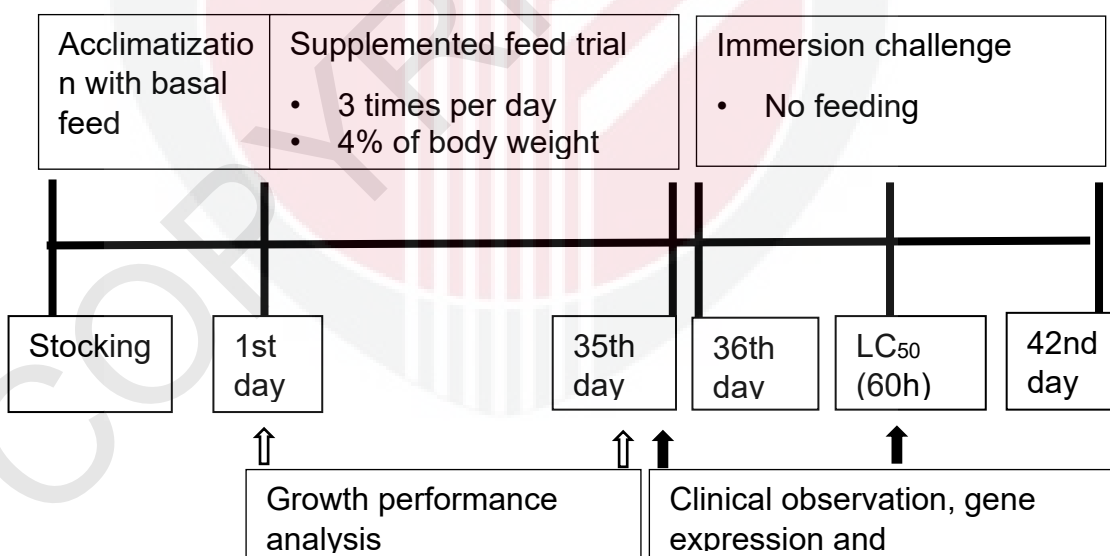


Figure 3.2: Experimental timeline of feeding trial regime and immersion challenge. Growth performance was recorded along the experiment. Clinical observation, gene expression and histopathology assessments were performed at end of feeding trial (35th day) and a lethal concentration time point between challenge periods.

3.2.2.4. Immersion challenge of AHPND-causing *V. parahaemolyticus*

For the determination of median lethal concentration (LC_{50}), 360 shrimp were divided into eighteen 10 L aquariums. Each tank was immersed with different AHPND-causing *V. parahaemolyticus* S2-4 concentrations whereas control tank (autoclaved seawater) as shown in Table 3.3. Mortality was observed daily for 7 days and LC_{50} was determined by using the probit analysis (Juárez-Morales et al., 2019).

Table 3.3: Experimental design of lethal concentration (LC_{50}) determination

Group	Concentration of AHPND-causing <i>V. parahaemolyticus</i> S2-4	Number of shrimps/replicate	Number of replicate tank
Group 1	1×10^6 CFU/mL	20	3
Group 2	8×10^5 CFU/mL	20	3
Group 3	6×10^5 CFU/mL	20	3
Group 4	4×10^5 CFU/mL	20	3
Group 5	2×10^5 CFU/mL	20	3
Control	Autoclaved seawater	20	3

The shrimps from each group ($n = 80$ per group) at the end of feeding trial (35th day) were collected randomly and transferred into six of 8 L of UV treated seawater filled aquariums (30 cm $\times$ 20 cm $\times$ 20 cm). After a day of acclimatization (36th day), shrimp of four aquariums per group were challenged with LC_{50} concentration (6×10^5 CFU/mL) of *V. parahaemolyticus* S2-4 by mixing the calculated volume of bacterial suspension with UV treated seawater in the aquarium as shown Table 3.2. The immersion challenge was conducted without feeding and water exchanged. Mortality and clinical signs were observed for 7 days. At 3rd day (60 h of post-challenge), nine shrimps

were taken for gene expression study while three shrimps were taken for histopathology study.

3.2.3. Elucidation of bacterial profile in shrimp body

3.2.3.1. RNA extraction and cDNA synthesis

For shrimp RNA extraction, nine shrimps from each group (35th day of feeding trial and 60 h of post-challenge) were kept in RNAlater (Invitrogen, USA) after anaesthetized with MS-222 (Western Chemical Inc., Ferndale, WA, USA) at a concentration of 300 ppm. After being dried from RNAlater, the shrimp shell were removed, the shrimps were cut into the cephalothorax and abdomen portion (Figure 3.3). The cephalothorax portion contained the shrimp hepatopancreas and stomach, while the abdomen portion consisted of the shrimp intestinal tract. The collected shrimp parts were homogenized, and its RNA was extracted using the same method specified above. The integrity of the RNA was validated by using 1.0 % agarose of gel electrophoresis.

The RNA samples were treated with the RapidOut DNA Removal Kit (Thermo Fischer Scientific, Waltham, USA) according to the manufacturer's manual. The concentration and quality of the total RNA was checked based on its optical density at 260 and 280 nm measured using spectrophotometry. The concentration of total RNA was adjusted into 1 µg/mL by using nuclease-free water. The RNA was added with 4 mL of reverse transcription buffer (OneScript®, Canada) and 1 mL of reverse transcription enzyme (OneScript®, Canada), 1 uL of dNTP, 1uL of random primer, 1 uL of oligo (Dt) and nuclease-

free water up to 20 mL. The final mixture was incubated at 25 °C for 10 min, and then 60 °C for 30 min, followed by the transcription enzyme inactivation for 5 min at 85 °C. The first strand complementary DNA (cDNA) of bacteria and shrimp were subjected for indicator gene confirmation and gene expression analysis respectively.

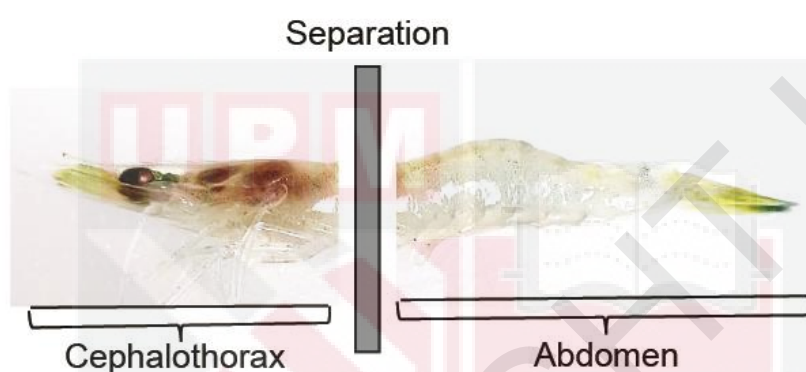


Figure 3.3: Cephalothorax and abdomen separation of *P. monodon*

3.2.3.2. Gene expression by real time-quantitative PCR

To conduct real-time PCR analysis on bacteria specific protein genes (GHKL domain containing protein and *pirA*), the primer sets that are specific to the obtained full or partial cDNA sequences were used (Table 3.4). The quantitative PCR was performed by using SYBR Green real-time PCR mix (QuantiNova, Germany) following the manufacturer's instructions. A total of 1 mL of cDNA sample was added with 10 mL of 2X SYBR Green PCR Master mix, 1 mL of each forward and reverse primer, and 7 mL of nuclease-free water into a PCR tube. The samples were run by using Rotor Gene Q Real-time PCR machine (Qiagen, Germany). The result of cycle threshold value (CT value) was analysed and recorded using Rotor Gene Q software (Qiagen, Germany). The standard cycling included an initial denaturation at 95 °C for 2 min,

followed by 40 cycles of denaturation at 95 °C for 3 seconds and annealing/elongation at 60 °C for 30 s. To evaluate amplification specificity, melting curve analysis was performed at the end of each PCR run. A melting curve profile was obtained by heating the mixture to 95 °C, cooling to 60 °C for each 10 s.

3.2.3.3. Investigation of bacterial profile of *P. monodon*

For quantifying *L. plantarum* strain KD2, T31 and L20 by detecting their cDNA concentration, specific conserved protein-encoding gene, GHKL domain containing protein gene was used as indicator because they can distinguish closely related species and strains of *Lactobacillus* spp. (Huang et al., 2018; Kim et al., 2020). For quantifying *V. parahaemolyticus* strain S2-4 by detecting their cDNA concentration, Photorhabdus insect related (*pir*) toxins (*pirA*) genes were used as indicator because crucial toxin gene in virulent AHPND plasmid of AHPND strain of *V. parahaemolyticus* which can cause lethal effect for penaeid shrimp (Feng et al. 2017; Han et al., 2020). To quantify unknown concentrations of each *Lactobacillus* sp. cells and *V. parahaemolyticus* S2-4, standard curves were generated by the amplification of a 10-fold dilution series of the targeted protein genes in the presence of the bacterial cDNA. The primer sequence of the specific bacterial genes were shown in Table 3.4. The coefficient of correlation (R^2) between the threshold value (CT) and targeted cDNA concentration was between 0.990 – 0.997, while the PCR efficiency was between 90 and 110%. Absolute quantification was applied for detecting and quantifying bacterial protein genes in *in vitro* gastrointestinal digestion system of shrimp (Zhang et al. 2020).

Table 3.4: The primer sequence of specific genes that used for reverse transcriptase quantitative PCR (RT-qPCR)

Bacterial protein gene	Primers	Sequences (5' to 3')	Reference
GHKL domain containing protein	Forward	GCGGTATCGATTGCGATTGGT	Kim et al. (2020)
	Reverse	TGATGTCAATCGCCTCTTGG	
<i>pirA</i> toxin	Forward	CAAACGGAGGGCGTCACAGA	Mai et al. (2021)
	Reverse	GACCGACTTCCGGGATGAT	

3.2.3.4. Histopathological assessment of digestive tract

Shrimp from the successful treatment group were chosen for histopathological analysis. The whole shrimp were fixed in 10% neutral buffered formalin for 24 h and then transferred to 70% ethanol. Then, the samples were dehydrated using series of increasing concentration of ethanol (50% - 100%) embedded in paraffin wax, sectioned at 4 μ m, and stained with haematoxylin and eosin. The samples were observed under microscope and microphotographed with magnification 100 $\times$ and 400 $\times$ under the Olympus BX51 digital light microscope (Olympus, Japan). The hepatopancreas and gut of the shrimps were observed histologically.

3.2.4. Data analyses

Differences of all data were analyzed by one-way analysis of variance (ANOVA) following post-hoc Duncan test. All statistical analysis were performed via software IBM SPSS Statistics® version 22 (SPSS, Chicago, USA). The results were considered significant at $p < 0.05$.

Institutional Review Board Statement

The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM) under approval number UPM/IACUC/AUP-R061/2022 (Appendix 5).

3.3. Results

3.3.1. Screened *L. plantarum* strains as potential probiotic

3.3.1.1. Identification of presumptive lactic acid bacteria for *Lactobacillus* and *Bacillus* isolates screening

Out of 242 presumptive LAB isolates (Appendix 1), a total of 49 LAB isolates were identified by using 16S ribosomal RNA gene sequencing where PCR-positive isolates showing the production of a 1500 bp band as Figure 3.4. The 49 LAB isolates were from Quoy's garfish (10.20 %), blackspot snapper (4.08 %), streaked spinefoot (6.12 %), largescale tonguesole (4.08 %), half-smooth golden pufferfish (2.04 %), moonfish (6.12 %), Threadfin bream (0 %), Malabar trevally (6.12 %), orange-spotted grouper (4.08%), red snapper (2.04 %), fourfinger threadfin (4.08%), yellowtail scad (2.04 %), bigeye scad (4.08%), herring scad (2.04 %), Belanger's croaker (2.04 %), Yellowtail fusilier (0 %), common ponyfish (2.04 %), silver pomfret (4.08 %), milkfish (6.12 %), Asian seabass (4.08 %), banana shrimp (4.08 %), freshwater giant prawn (4.08 %), Pacific white shrimp (4.08 %), black tiger shrimp (8.16 %) and strains (4.08%) of Ida-Muryany et al. (2017). Based on the unrooted maximum likelihood tree of the partial 16S ribosomal RNA (1500 bp) of 49 LAB isolates and 40 reference strains in Figure 3.5, the species identified were *L. plantarum*

(8.16 %), *L. sakei* (2.04 %), *L. curvatus* (2.04 %), *B. cereus* (4.08 %), *B. subtilis* (2.04 %), *B. thuringiensis* (2.04 %), *B. wiedmannii* (4.08 %), *Lactococcus lactis* (12.24 %), *L. taiwanensis* (2.04 %), *L. garvieae* (16.33 %), *Streptococcus parauberis* (2.04 %), *Macrococcus caseolyticus* (2.04 %), *Staphylococcus epidermidis* (2.04 %), *S. edaphicus* (2.04), *S. saprophyticus* (2.04 %), *Enterococcus faecium* (2.04 %), *E. faecalis* (2.04 %), *Vagococcus lutrae* (2.04 %), *Leuconostoc mesenteroides* (2.04 %), *L. pseudomesenteroides* (2.04 %), *L. citreum* (4.08 %), *Weissella paramesenteroides* (2.04 %), *W. ceti* (6.12 %) and *W. cibaria* (6.12%). Out of 49 of LAB strains, the *Lactobacillus* sp. strains (KD2, T31, DJ1, LT1, L8, L20) and *Bacillus* sp. strains (T4, T51, L21, KF2, SS1) were selected. Then, the strains were proceeded with hemolytic test.

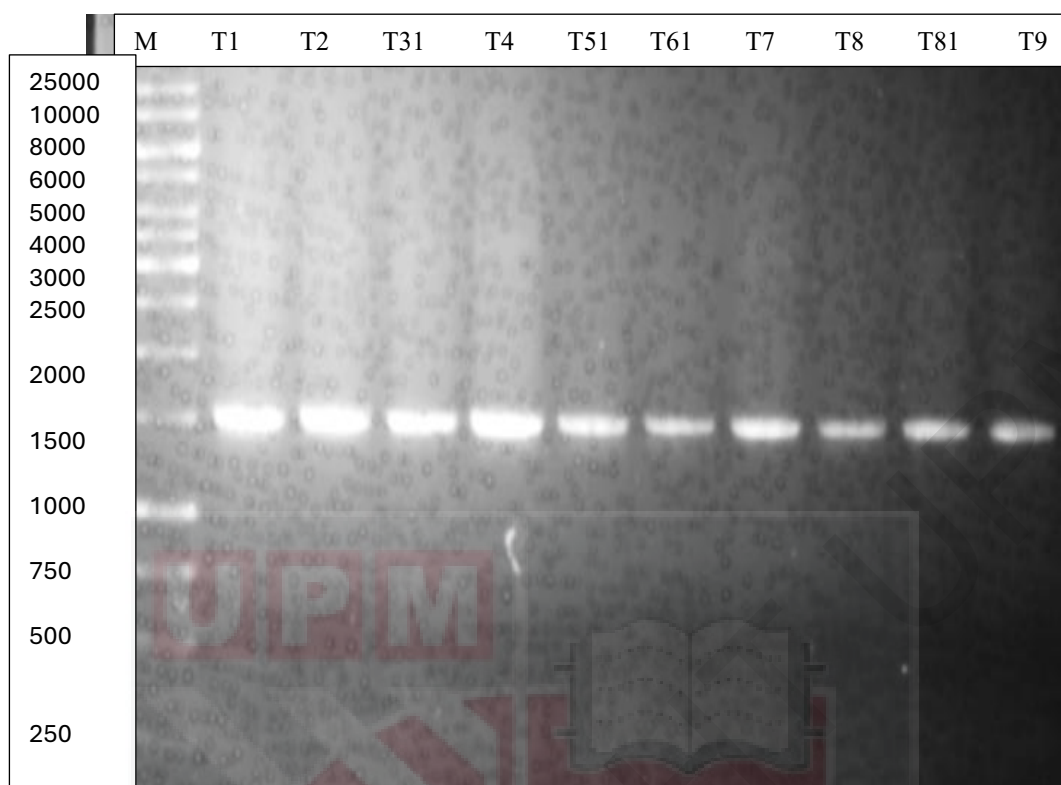


Figure 3.4: Gel electrophoresis of 16S ribosomal RNA gene sequencing where PCR-positive isolates showing the production of a 1500 bp band. M indicate 1000 bp DNAladder (Thermo Fisher scientific, Waltham, United states). T1, T2, T31, T4, T51, T61, T7, T8, T81 and T9 are 10 presumptive LAB isolates from Quoy's garfish.

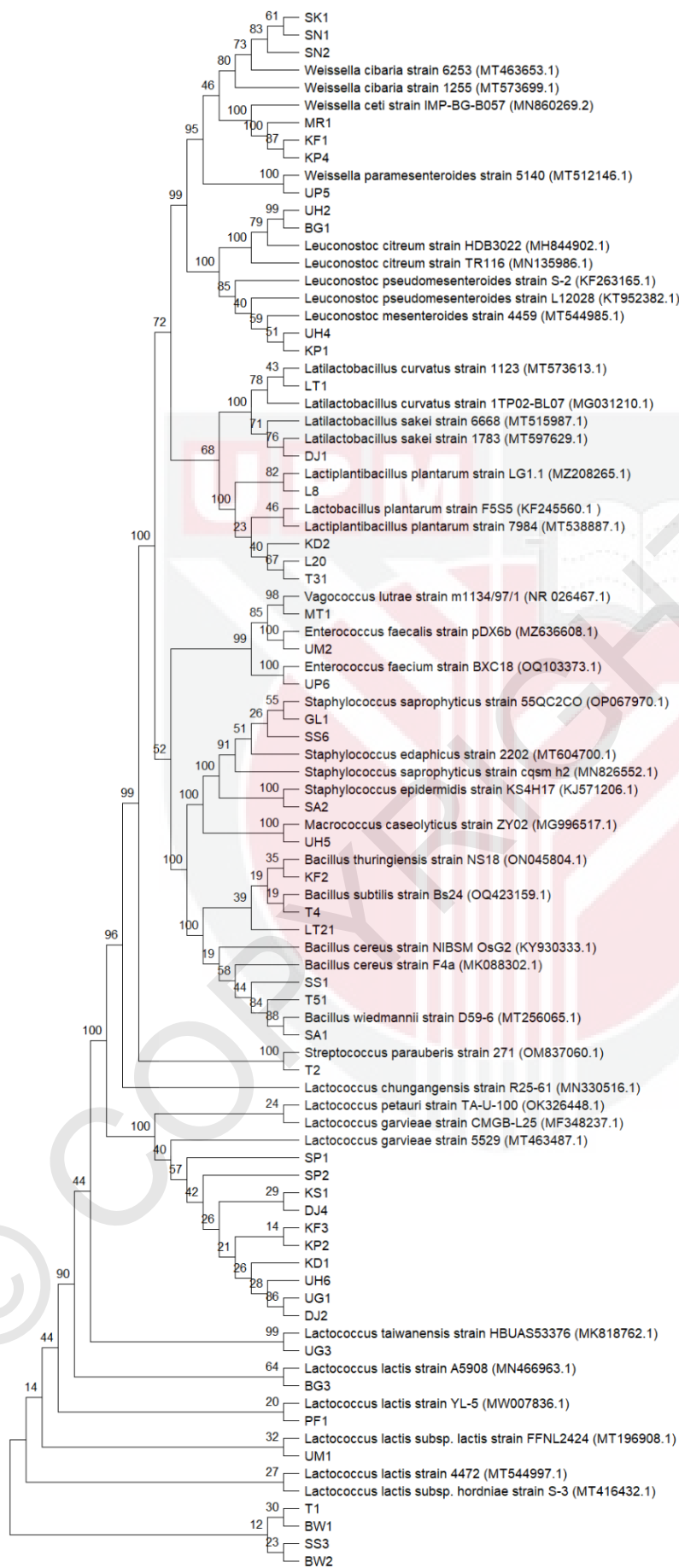


Figure 3.5: Maximum likelihood of environmental and reference lactic acid bacteria isolates based on the partial 16S ribosomal RNA (1500 bp). Numbers shown next to each node indicate bootstrap values (percentage of 1000 replicates).

3.3.1.2. Hemolytic test of lactic acid bacteria

As the result, the *Lactobacillus* sp. strains (KD2, T31, DJ1, LT1, L8, L20) did not exhibit any hemolytic effect on the blood agar. However, *Bacillus* sp. strains (T4, T51, L21, KF2, SS1) exhibited hemolytic effect on the blood agar. Therefore, the *Lactobacillus* sp. were chosen for antagonistic experiments.

3.3.1.3. Antagonistic activities of *Lactobacillus* sp. strains in colony top assay and well diffusion assay.

Table 3.5 showed all *Lactobacillus* spp. strains were able to inhibit all tested *Vibrio* spp. ($p < 0.05$). However, no inhibition was observed for control. In subsequent well diffusion assay, cultured broth of *L. plantarum* T31, KD2, L8, and L20 have significantly ($p < 0.05$) higher inhibition than *L. sakei* DJ1 and *L. curvatus* L1. On the other hand, CFS of *Lactobacillus* sp. T31, KD2, L8, L20, and DJ1 has significant ($p > 0.05$) inhibition for all tested *Vibrio* spp., except for *L. curvatus* L1 where no significant inhibition ($p > 0.05$) for *V. parahaemolyticus* S2-4 and *V. campbellii* S10-4 was observed. Furthermore, CFS of *L. plantarum* T31, KD2, L8, and L20 has significantly ($p < 0.05$) higher inhibition than *L. sakei* DJ1 and *L. curvatus* L1 in well diffusion assay as well. Therefore, *L. plantarum* T31, KD2, L8, and L20 are chosen for the co-culture test with *V. parahaemolyticus* S2-4. Table 3.5 shows the colony top assay and well diffusion assay.

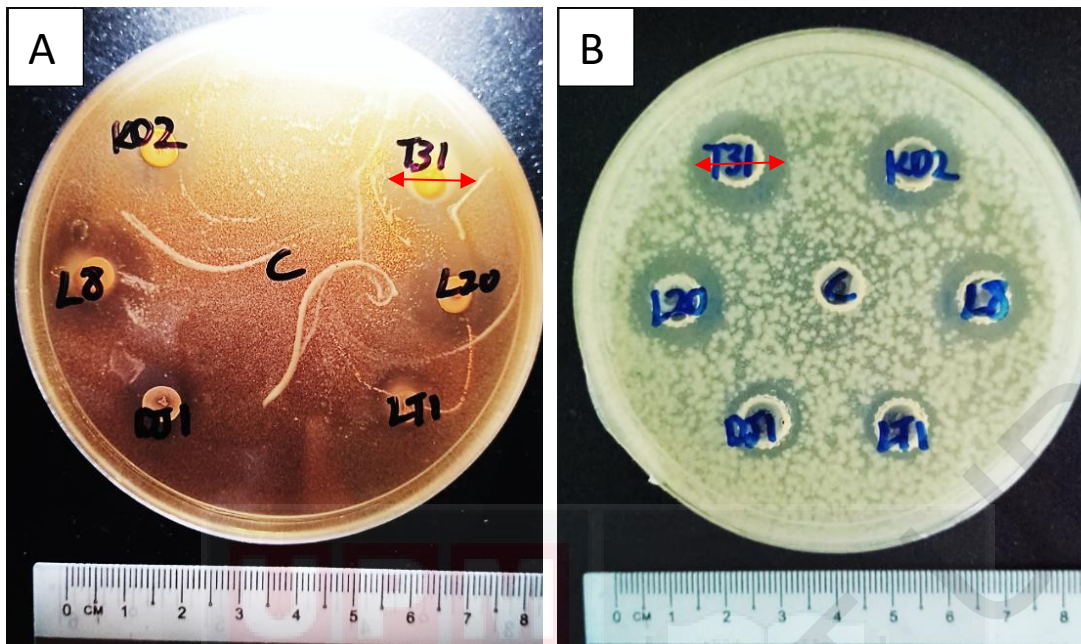


Figure 3.6: Colony top assay and well diffusion assay of *V. parahaemolyticus* S2-4 against *Lactobacillus* sp. strains. Figure 3.5A shows red arrow line indicates the diameter of inhibition zone (15 mm) were measured for colony top assay; Figure 3.5B shows red arrow line indicates the diameter of inhibition zone (16 mm) were measured for well diffusion assay. T31, KD2, L20, L8, DJ1 and LT1 indicates *L. plantarum* T31, *L. plantarum* KD2, *L. plantarum* L20, *L. plantarum* L8, *L. sakei* DJ1 and *L. curvatus* LT1. The clear area surrounding the wells indicates inhibition of *L. plantarum* strains on the *Vibrio* spp.

Table 3.5: Antagonistic activity of selected *Lactobacillus* spp. against other *Vibrio* spp. in colony top assay, well diffusion assay with cultured MRS broth and well diffusion assay with cell free supernatant (CFS)

Zone of inhibition (mm)													
Isolate (10 ⁸ CFU/mL)	<i>Vibrio parahaemolyticus</i> S2-4 (10 ⁵ CFU/mL)				<i>Vibrio campbellii</i> S10-4 (10 ⁵ CFU/mL)				<i>Vibrio rotiferanus</i> S24-4 (10 ⁵ CFU/mL)				<i>Vibrio</i> J21Y (10 ⁵ CFU/mL)
	Colony top assay	Well diffusion assay (Broth)	Well diffusion assay (CFS)	Well diffusion assay (CFS)	Colony top assay	Well diffusion assay (Broth)	Well diffusion assay (CFS)	Well diffusion assay (CFS)	Colony top assay	Well diffusion assay (Broth)	Well diffusion assay (Broth)	Well diffusion assay (Broth)	Colony top assay
T31	15.67 ± 3 ^{bc}	17.67 ± 0.47 ^c	14.67 ± 0.47 ^c	14.67 ± 0.47 ^c	32 ± 0.82 ^d	14.67 ± 0.47 ^c	14.67 ± 0.47 ^c	14.67 ± 0.47 ^c	26.33 ± 1.25 ^e	15.33 ± 0.47 ^{bc}	14.67 ± 0.47 ^b	14.67 ± 0.47 ^b	12.33 ± 0.47 ^a
KD2	11.67 ± 0.93 ^{ab}	17.67 ± 0.47 ^c	14 ± 0.82 ^{bc}	15.33 ± 0.47 ^c	18.33 ± 2.05 ^b	12.67 ± 0.47 ^b	15.33 ± 0.47 ^c	14.67 ± 0.47 ^c	14 ± 0.82 ^b	13.67 ± 0.47 ^b	14.67 ± 0.47 ^c	13.33 ± 0.47 ^b	17.33 ± 0.94 ^c
L20	17.67 ± 0.47 ^c	18 ^c	14.67 ± 0.47 ^c	15 ± 0.82 ^c	20.67 ± 0.47 ^b	15.33 ± 0.94 ^c	15 ± 0.82 ^c	14.33 ± 0.47 ^c	16.67 ± 0.94 ^c	15.67 ± 0.47 ^c	14.33 ± 0.47 ^b	14.33 ± 0.47 ^c	19.67 ± 1.25 ^d
L8	14 ± 1.41 ^{bc}	17.67 ± 0.47 ^c	14.67 ± 0.47 ^c	14.67 ± 0.47 ^c	27.67 ± 2.05 ^c	14.67 ± 0.47 ^c	14.67 ± 0.47 ^c	14.67 ± 0.47 ^c	20.67 ± 0.47 ^d	15.33 ± 0.47 ^{bc}	14.67 ± 0.47 ^c	14.67 ± 0.47 ^b	12.67 ± 0.47 ^a
DJ1	8.67 ± 1.7 ^a	12 ± 2.45 ^a	12 ± 0.82 ^a	11 ± 0.82 ^b	14.33 ± 0.47 ^a	11.33 ± 1.25 ^{ab}	11 ± 0.82 ^b	11 ± 0.82 ^a	12 ± 0.82 ^a	10.33 ± 1.25 ^a	11.33 ± 1.25 ^a	11.33 ± 1.25 ^a	14.67 ± 0.47 ^b
LT1	12 ± 2.45 ^{ab}	8.67 ± 1.7 ^b	8 ± 0.82 ^b	8.67 ± 0.47 ^a	11.67 ± 0.47 ^a	10.33 ± 0.47 ^a	8.67 ± 0.47 ^a	9.67 ± 1.25 ^a	12.67 ± 0.47 ^{ab}	11.33 ± 1.25 ^a	9.67 ± 1.25 ^a	10 ± 0.82 ^a	16.33 ± 0.47 ^c

Different superscript letter indicates significant differences ($p < 0.05$) of the same column.

3.3.1.4. Antagonistic activities of *Lactobacillus* spp. in co-culture assay

Figure 3.7 shows the difference in the concentration of *V. parahaemolyticus* S2-4 co-cultured with different strains of *L. plantarum*. It was observed that there was no significant difference ($p>0.05$) in *V. parahaemolyticus* S2-4 concentration in co-cultures of *L. plantarum* T31, KD2, L20, and L8, compared to monoculture of *V. parahaemolyticus* S2-4 at 0 h and 12 h. However, at 24 h and 48 h, the concentration of *V. parahaemolyticus* S2-4 in co-cultures with *L. plantarum* T31, KD2, L20, and L8, was significantly lower ($p<0.05$) compared to the single culture of *V. parahaemolyticus* S2-4. Among the co-cultures at 48 h, co-culture L20 + S2-4 exhibited the lowest concentration ($3.933 \pm 0.065 \log_{10}$ CFU/mL) of *V. parahaemolyticus* S2-4, followed by co-culture KD2 + S2-4, co-culture T31 + S2-4, and L8 + S2-4 that exhibited the concentrations ($5.423 \pm 0.116 \log_{10}$ CFU/mL), ($6.105 \pm 0.182 \log_{10}$ CFU/mL), and ($7.464 \pm 0.140 \log_{10}$ CFU/mL) of *V. parahaemolyticus* S2-4, respectively, compared with the single culture of *V. parahaemolyticus* S2-4 ($9.234 \pm 0.065 \log_{10}$ CFU/mL). Notably, no concentration of *V. parahaemolyticus* was detected in single culture of *L. plantarum*. Overall, *L. plantarum* L20, KD2, and T31 exhibited a reduction of at least 99.9% ($\geq 3 \log_{10}$) CFU/mL of *V. parahaemolyticus* S2-4 at 48 h. Therefore, *L. plantarum* L20, KD2, and T31 were chosen for feed formulation (Figure 3.7).

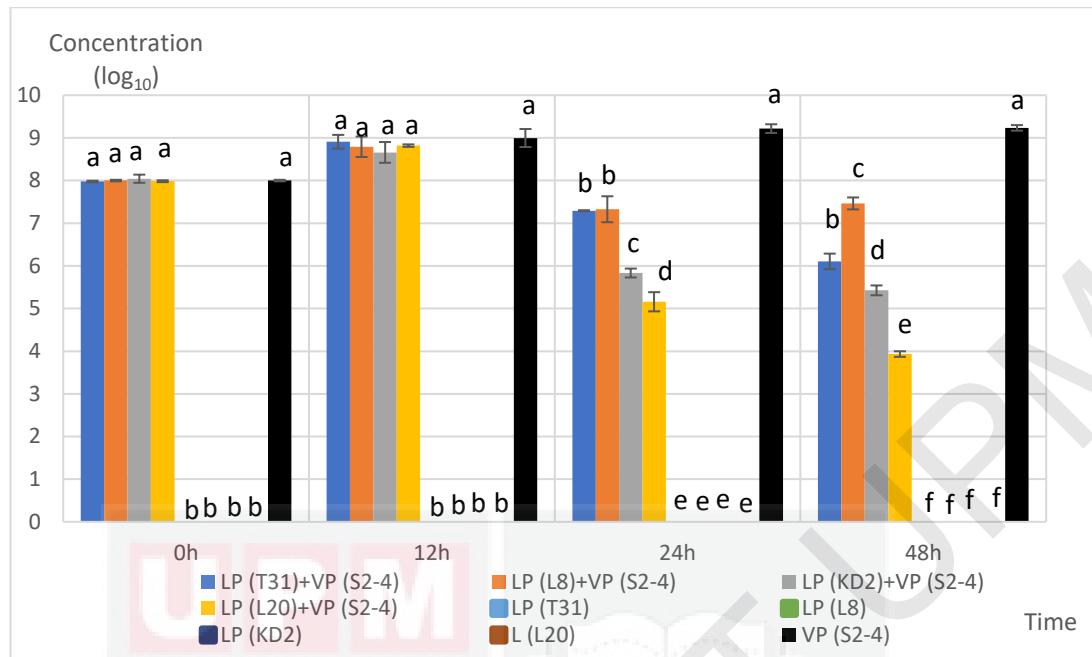


Figure 3.7: The concentration of *V. parahaemolyticus* S2-4 in the coculture experiment. Different superscript letter above bar indicates significant differences ($p<0.05$). LP (T31) indicates *L. plantarum* T31; LP (L20) indicates *L. plantarum* L20; LP (KD2) indicates *L. plantarum* KD2; VP (S2-4) indicates *V. parahaemolyticus* S2-4.

3.3.2. Effectiveness of *L. plantarum* strain for *P. monodon*

3.3.2.1. Proximate analysis and viability test

The concentration of *L. plantarum* T31, KD2 and L20 remained relatively consistent over time, with no significant changes ($p>0.05$) observed with concentration of 10^8 CFU/mL in T31, KD2 and L20 feed respectively, whereas control feed was no presence of *Lactobacillus* spp. for 15 days at 4 °C. All parameters of proximate analysis show not significantly different for all *L. plantarum* strains supplemented diets with control diets (Table 3.6).

Table 3.6: Proximate analysis of supplemented diets

	10 ⁸ CFU/g of <i>L. plantarum</i> T31	10 ⁸ CFU/g of <i>L. plantarum</i> KD2	10 ⁸ CFU/g of <i>L. plantarum</i> L20	PBS water
Ash	9.51 ± 0.03 ^a	9.54 ± 0.09 ^a	9.69 ± 0.10 ^a	9.53 ± 0.12 ^a
Moisture	17.59 ± 0.15 ^a	17.89 ± 0.27 ^a	17.36 ± 0.19 ^a	17.65 ± 0.24 ^a
Crude fiber	1.07 ± 0.12 ^a	1.07 ± 0.05 ^a	0.97 ± 0.09 ^a	1.00 ± 0.08 ^a
Protein	39.13 ± 0.41 ^a	38.40 ± 0.57 ^a	39.00 ± 0.82 ^a	38.53 ± 0.45 ^a
Fat	3.70 ± 0.08 ^a	3.73 ± 0.12 ^a	3.7 ± 0.08 ^a	3.7 ± 0.14 ^a
Carbohydrate	28.99 ± 0.69 ^a	29.38 ± 0.76 ^a	29.38 ± 0.76 ^a	29.28 ± 0.78 ^a

Different superscript letter indicates significant differences ($p < 0.05$) of the same row.

3.3.2.2. Growth performance in feeding trial

For final weight and specific growth rate (SGR), Group L20, KD2 and T31 were significant ($p < 0.05$) higher compared with control group (Table 3.7). For weight gain, Group T31 and KD2 were significant ($p < 0.05$) higher than other groups (Table 3.4). For feed conversion ratio (FCR), Group KD2 and L20 were significant ($p < 0.05$) lower than other groups (Table 3.7). For protein conversion ratio, Group L20 was significant ($p < 0.05$) high than other groups. In overall, *L. plantarum* L20 effectively improved growth performances of *P. monodon* (Table 3.7). No mortality was observed during the feeding trials.

Table 3.7: Parameter of growth performance for *L. plantarum* T31, KD2 and L20 supplemented feed administrated *P. monodon* for 35 days

	10 ⁸ CFU/g of <i>L. plantarum</i> T31	10 ⁸ CFU/g of <i>L. plantarum</i> KD2	10 ⁸ CFU/g of <i>L. plantarum</i> L20	PBS water	
Initial weight (g)	0.01 ± 0.00 ^a	0.01 ± 0.00 ^a	0.01 ± 0.00 ^a	0.01 ± 0.00 ^a	±
Final weight (g)	0.05 ± 0.00 ^b	0.06 ± 0.00 ^b	0.06 ± 0.01 ^b	0.04 ± 0.00 ^a	±
Weight gain (%)	502.09 ± 71.25 ^{ab}	521.42 ± 26.15 ^b	547.71 ± 65.14 ^b	341.23 ± 29.16 ^a	±
Specific growth rate (%)	5.11 ± 0.34 ^b	5.22 ± 0.12 ^b	5.32 ± 0.29 ^b	4.23 ± 0.19 ^a	±
Feed conversion ratio	2.17 ± 0.23 ^{ab}	2.06 ± 0.14 ^b	1.98 ± 0.01 ^b	2.58 ± 0.13 ^a	±
Protein conversion ratio	0.03 ± 0.00 ^{ab}	0.03 ± 0.00 ^{ab}	0.03 ± 0.00 ^b	0.03 ± 0.00 ^a	±

Different superscript letter indicates significant differences ($p < 0.05$) of the same row.

3.3.2.3. Lethal concentration (LC₅₀) of *V. parahaemolyticus* S2-4

The control group and group 5 (2×10^5 CFU/mL) did not show any shrimp mortality (Figure 3.8). However, group 1 (1×10^6 CFU/mL), group 2 (8×10^5 CFU/mL), and group 3 (6×10^5 CFU/mL) started to exhibit mortality at 24 hours post-infection (hpi), while group 4 exposed to 4×10^5 CFU/mL of *V. parahaemolyticus* S2-4 started to show mortality at 48 hpi (Figure 3.7). All shrimps from group 1 died by 60 h. Therefore, based on the Probit analysis, the lethal concentration 50% (LC_{50-60 h}) was determined to be 6×10^5 CFU/mL.

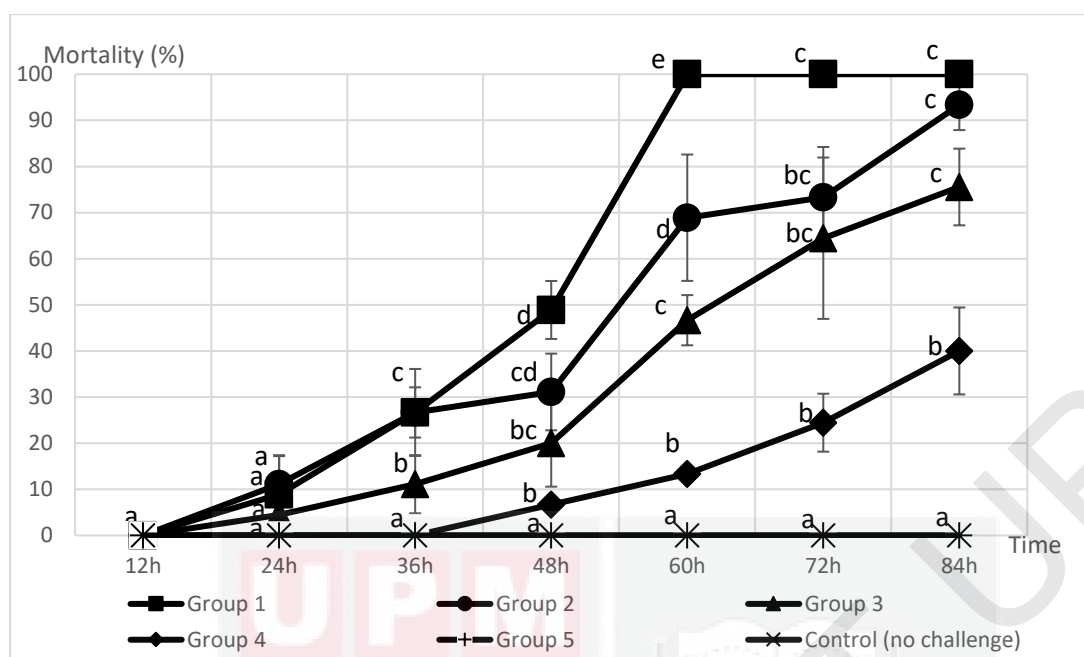


Figure 3.8: Mortality of *P. monodon* after different concentrations of *V. parahaemolyticus* S2-4 immersion challenge. Different superscript letter above bar indicates significant differences ($p < 0.05$). Group 1 indicates 1×10^6 CFU/mL immersion challenge, Group 2 indicates 8×10^5 CFU/mL immersion challenge, Group 3 indicates 6×10^5 CFU/mL immersion challenge, Group 4 indicates 4×10^5 CFU/mL immersion challenge, Group 5 indicates 2×10^5 CFU/mL immersion challenge whereas control tank (immersed in UV treated seawater).

3.3.2.4. Survival percentage in immersion challenge

At the median lethal concentration ($LC_{50-60\text{ h}}$) of *V. parahaemolyticus* S2-4 for *P. monodon*, the shrimp fed *L. plantarum* T31 supplemented feed (Group T31) achieved about 60% survival which slightly significantly ($p < 0.05$) higher than control group (Figure 3.9). The shrimp fed *L. plantarum* KD2 supplemented feed (Group KD2) and *L. plantarum* L20 supplemented feed (Group L20) achieved about 70% and 80% survival respectively, which significantly ($p < 0.05$) higher than control group (Figure 3.9). Group KD2 was slightly significantly ($p < 0.05$) lower survival than Group L20 (Figure 3.9). In comparison, *L. plantarum* L20 effectively improved survival of *P. monodon* during challenge test.

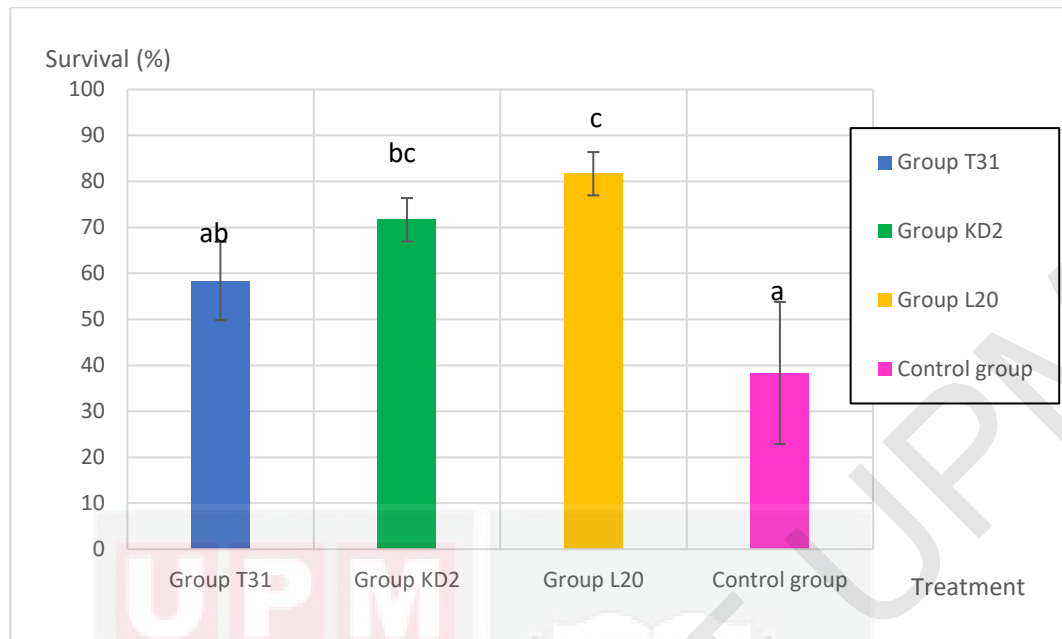


Figure 3.9: Survival percentage of 1×10^8 CFU/mL different strains *L. plantarum* supplemented feed administered *P. monodon* after immersion challenge of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 at 60 h as LC₅₀₋₆₀ h. Different superscript letter above bar indicates significant differences ($p < 0.05$).

3.3.2.5. Clinical symptom observation

No clinical sign was observed in all treatment groups during the feeding trial (Figure 3.10). On the other hand, pale hepatopancreas of moribund shrimp was observed at 60 h post-challenge in control group, whereas normal hepatopancreas were found in Group T31, Group KD2 and Group L20 (Figure 3.10).

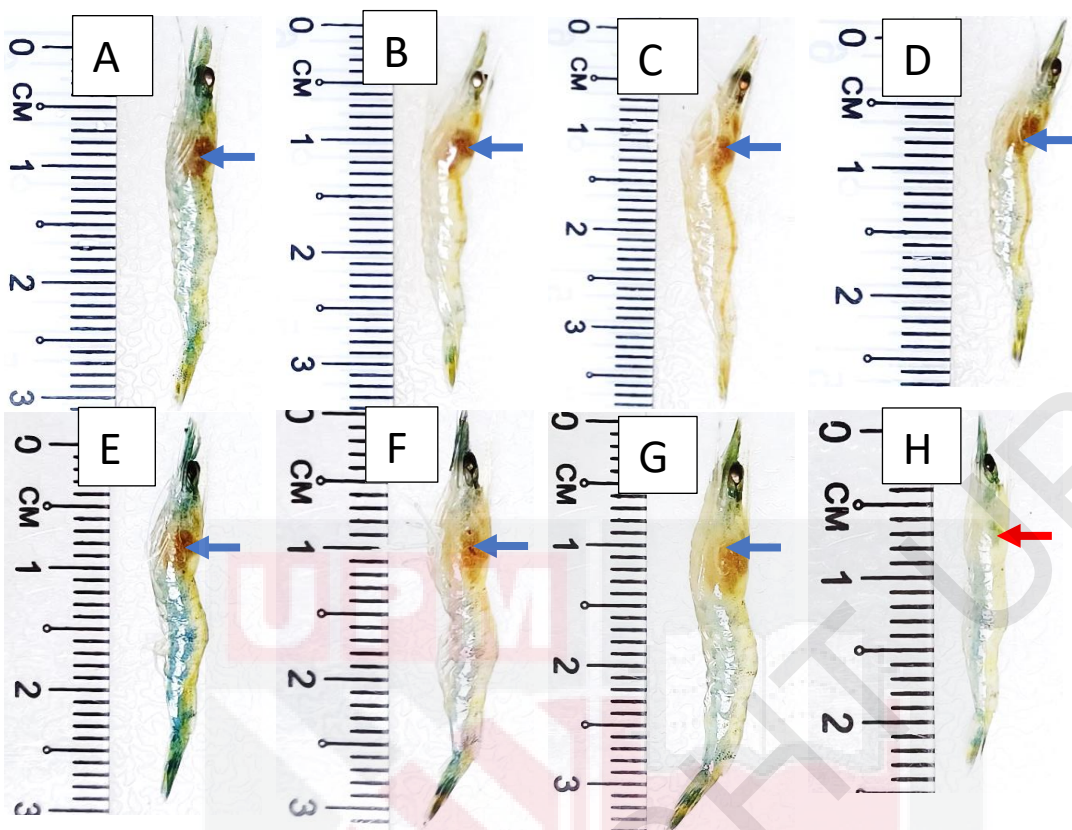


Figure 3.10: Clinical sign of different strains *L. plantarum* administered *P. monodon*. (A – D) showed shrimp at end of feeding trial (35th day), whereas (E – H) showed shrimp at 60 h of post-immersion challenge with 6×10^5 CFU/mL *V. parahaemolyticus* S2-4. (A) and (E) indicate Group T31; (B) and (F) indicate Group KD2; (C) and (G) indicate Group L20; (D) and (H) indicate control group. (A – G) show Normal hepatopancreas (→) and (H) show pale white hepatopancreas (→).

3.3.3. Bacterial gene expression

3.3.3.1. Quantification of *L. plantarum* strains in shrimp body

The cycle threshold value (CT value) of GHKL domain containing protein gene as *L. plantarum* strains' specific gene indicated viable concentration of *L. plantarum* strains by referring to the standard curve in shrimp (Appendix 3A).

In end of feeding trial (35th day), the concentration of *L. plantarum* strains was significantly the lowest ($p < 0.05$) in the cephalothorax of shrimp from Group KD2 compared other groups (Figure 3.11A). In contrast, concentration of *L.*

plantarum strains was significantly ($p < 0.05$) showed highest (5.436 ± 0.161 $\log_{10}$ CFU/mL) in the abdomen of shrimp from Group L20 compared to other groups (Figure 3.11A). No detected CT value in cephalothorax and abdomen of control group (Figure 3.11A). In the 60 h post-immersion challenge (LC_{50-60} h), the concentration of *L. plantarum* strain was significantly ($p < 0.05$) showed highest (2.947 ± 0.420 $\log_{10}$ CFU/mL) in the cephalothorax of shrimp from Group T31 and highest (2.712 ± 0.174 $\log_{10}$ CFU/mL) in the abdomen of shrimp from Group L20 compared to other groups (Figure 3.11B). No detected CT value in cephalothorax and abdomen of control group (Figure 3.11B).

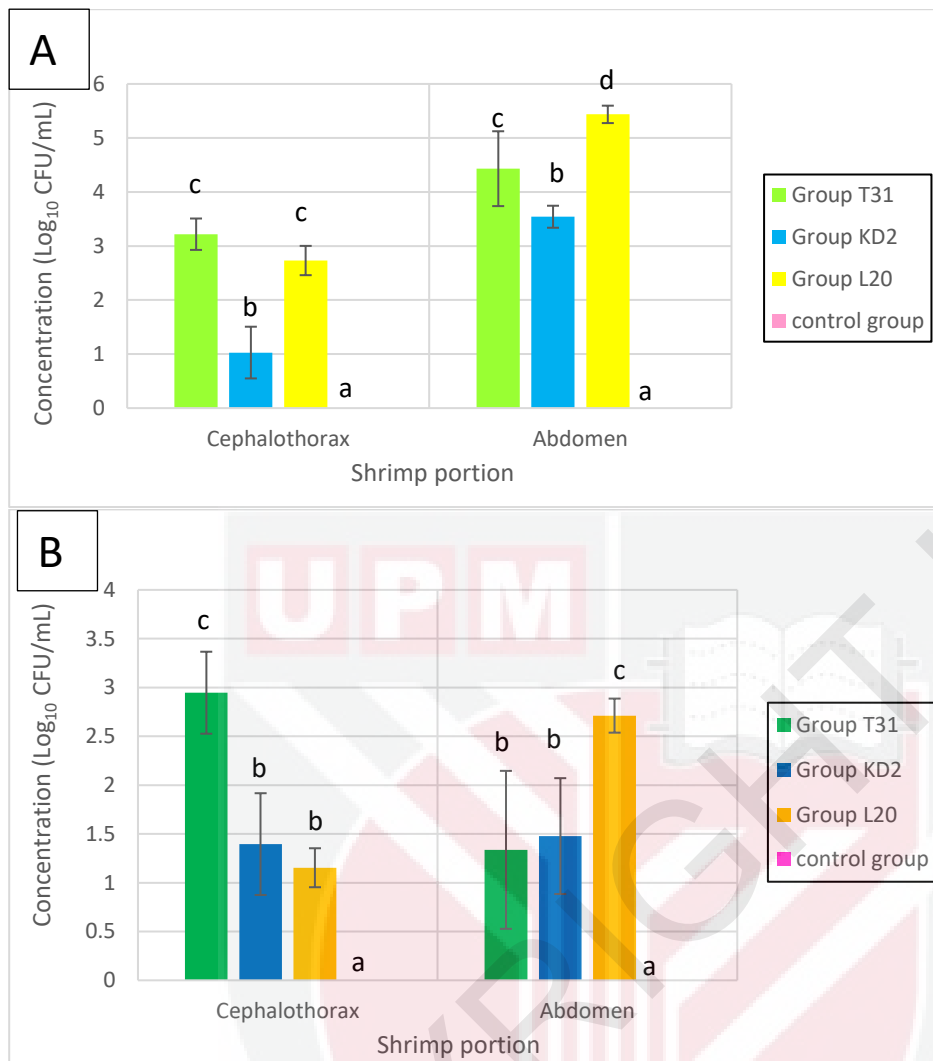


Figure 3.11: Comparative concentration of *L. plantarum* strains by GHKL domain containing protein gene quantification in cephalothorax and abdomen of *P. monodon* fed with 1×10^8 CFU/g different *L. plantarum* strains supplemented feed in feeding trial and challenge test. (A) is 35th day of feeding period while (B) is 60 h of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 post-immersion challenge. Different superscript letter above bar indicates significant differences ($p < 0.05$).

3.3.3.2. Quantification of *V. parahaemolyticus* S2-4 in shrimp body

The CT value of *pirA* toxin gene as AHPND-causing *V. parahaemolyticus* 's specific gene indicated viable concentration of *V. parahaemolyticus* S2-4 by referring the standard curve (Appendix 3B) in shrimp. No detected CT value in cephalothorax and abdomen of all groups in 35th day of feeding trial. In 60 h of post-immersion challenge, Group L20 shows the concentration of the *V.*

parahaemolyticus S2-4 significantly ($p < 0.05$) showed lowest (2.771 ± 0.598 $\log_{10}$ CFU/mL) in the shrimp cephalothorax and lowest (2.603 ± 0.336 $\log_{10}$ CFU/mL) in abdomen (Figure 3.12). Control group shows the concentration of the *V. parahaemolyticus* S2-4 significantly ($p < 0.05$) showed highest (5.191 ± 0.170 $\log_{10}$ CFU/mL) in the shrimp cephalothorax and highest (4.116 ± 0.307 $\log_{10}$ CFU/mL) in abdomen (Figure 3.12).

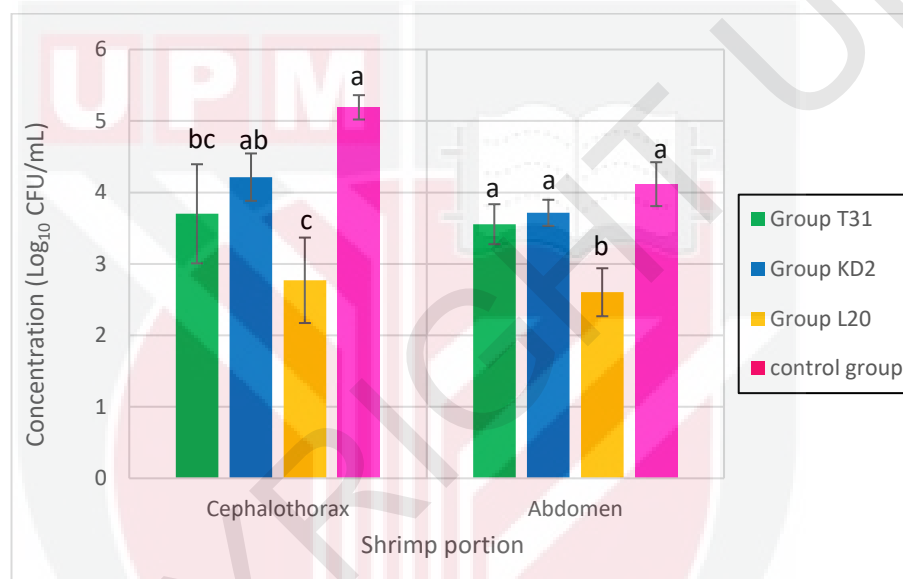


Figure 3.12: Comparative concentration of *V. parahaemolyticus* S2-4 by *pirA* toxin gene quantification in cephalothorax and abdomen of *P. monodon* were fed 1×10^8 CFU/g different *L. plantarum* strains supplemented feed in 60 h of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 post-immersion challenge. Different superscript letter above bar indicates significant differences ($p < 0.05$).

3.3.4. Histological observation

3.3.4.1. Histopathological evaluation of hepatopancreas in *P. monodon*

Normal structures of hepatopancreas including resorptive cell (R-cell), blister-like cell (B-cell), fibrillar cell (F-cell), hepatic lumen was shown in representative shrimp from Group T31 (Figure 3.13A), Group KD2 (Figure 3.13B), Group L20

(Figure 3.13C) and control group (Figure 3.13D) at the 35th day of feeding trial. Group T31 (Figure 3.13E), Group KD2 (Figure 3.13F) and Group L20 (Figure 3.13G) at 60 h of post-immersion challenge showed mild infection signs of hepatocytes mild-vacuolated R-cell, mild-vacuolated F-cell and presence of hemocytes. However, Group T31 (Figure 3.13E) and Group KD2 (Figure 3.13F) show ruptured B-cell but Group L20 (Figure 3.13G) shows normal B-cell. Severe infection signs included ruptured karyomegaly R-cell, ruptured karyomegaly F-cell, ruptured B-cell, sloughed hepatic lumen and hemocyte infiltration in control group (Figure 3.13H) at 60 h of post-immersion challenge.

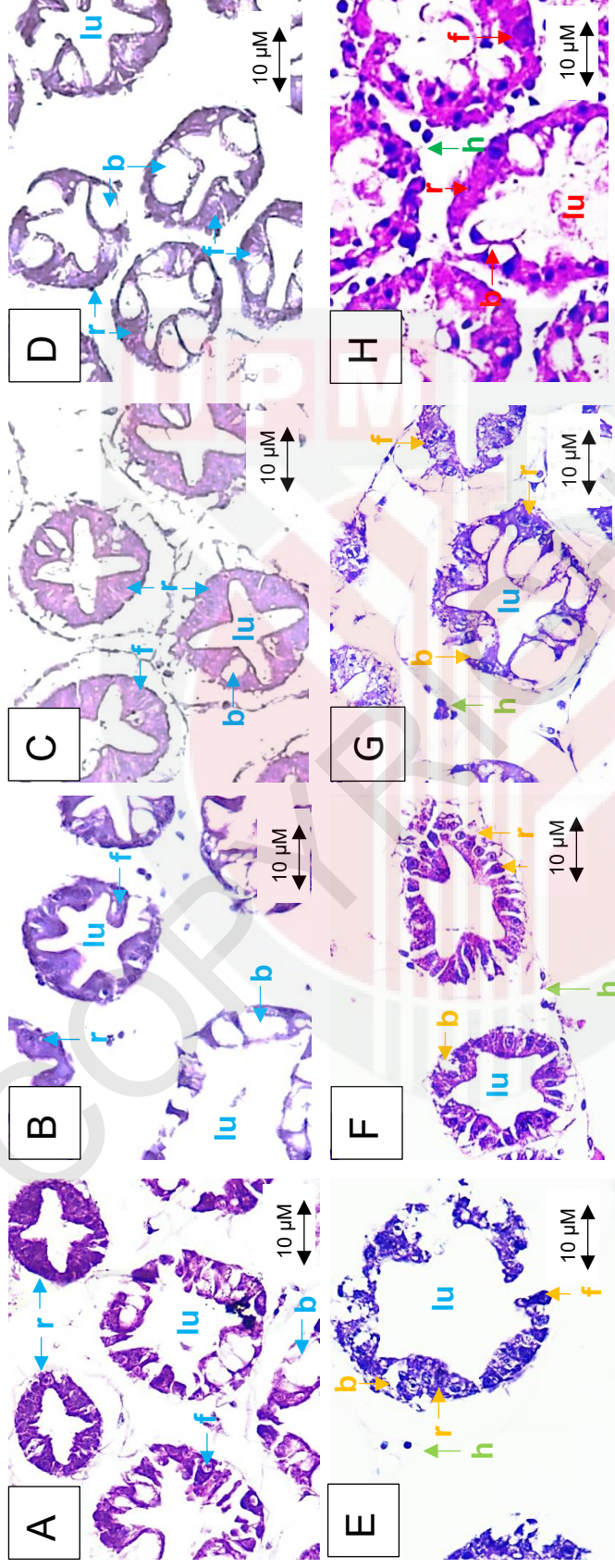


Figure 3.13: Histopathology for hepatopancreas in cephalothorax of *P. monodon*. (A – D) showed shrimp at 35th day of feeding trial, whereas (E – H) showed shrimp at 60 h of post-immersion challenge with 6×10^5 CFU/mL *V. parahaemolyticus* S2-4. (A) and (E) indicates Group T31; (B) and (F) indicates Group KD2; (C) and (G) indicates Group L20; (D) and (H) indicates control group. (A – D) shows resorptive cell (R-cell: **r**), blister-like cell (B-cell: **b**), fibrillar cell (F-cell: **f**), hepatic lumen (**lu**) ($\times 400$). (E – G) shows hepatic lumen (**lu**), mild-vacuolated R-cell (**r**), mild-vacuolated F-cell (**f**), mild-ruptured B-cell (**b**) and presence of hemocyte (**h**) ($\times 400$). (H) shows ruptured karyomegaly R-cell (**r**), ruptured B-cell (**b**), sloughed hepatic lumen (**lu**) and hemocyte infiltration (**h**) ($\times 400$).

Histopathological evaluation of intestine in *P. monodon*

Normal structure of intestinal epithelium included brush border, intestinal epithelium, intestinal lumen and hemocytes were shown in representative shrimp from Group T31 (Figure 3.14A), Group KD2 (Figure 3.14B), Group L20 (Figure 3.14C) and control group (Figure 3.14D) at 35th day of feeding trial. Representative shrimp from Group KD2 (Figure 3.14F) and Group L20 (Figure 3.14G) at 60 h of post-immersion challenge show brush border, mild-vacuolated intestinal epithelium, intestinal lumen and presence of submucosa hemocytes. However, severe infection signs included severe degeneration of brush border, disintegration and severe vacuolization of mucosa in Group T31 (Figure 3.14E) and control group (Figure 3.14H) at 60 h of post-immersion challenge.

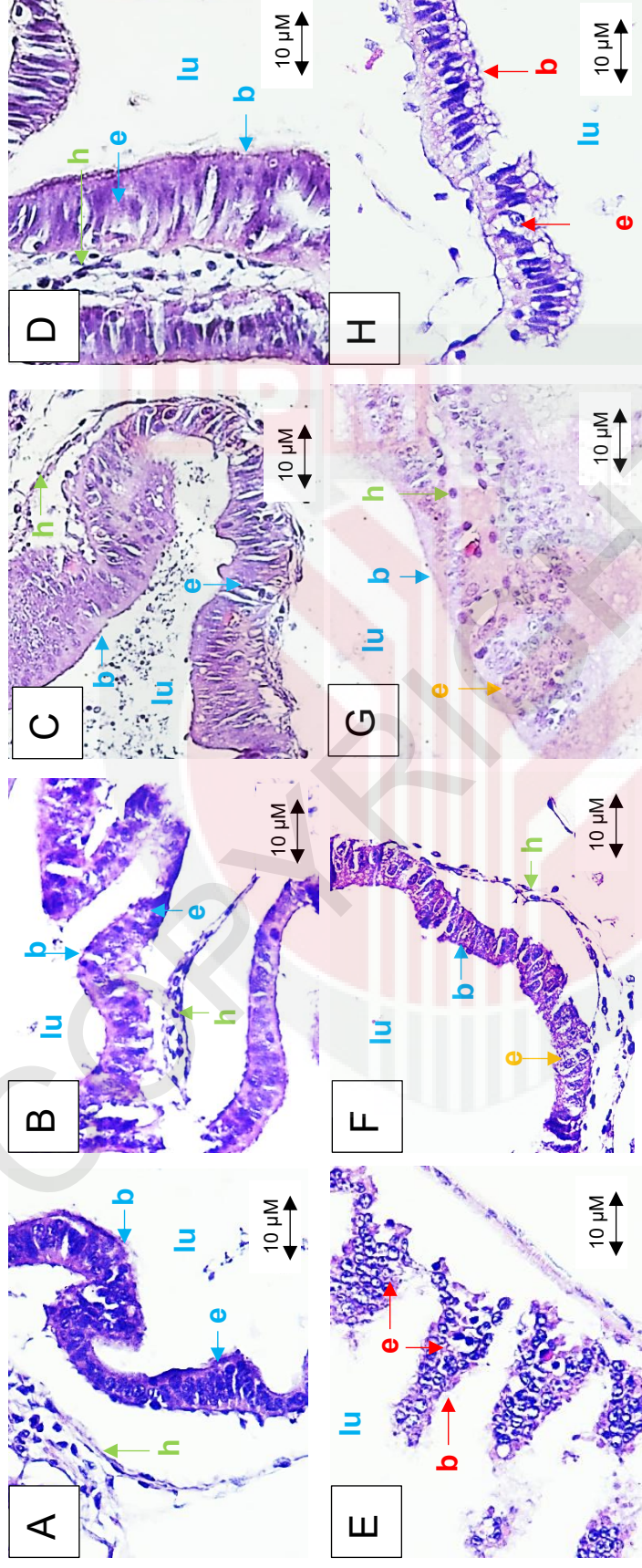


Figure 3.14: Histopathology for intestine in abdomen of *P. monodon* fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. (A – D) showed shrimp at 35th day of feeding trial, whereas (E – H) showed shrimp at 60 h of post-immersion challenge with 6×10^5 CFU/mL *V. parahaemolyticus* S2-4. (A) and (E) indicates Group T31; (B) and (F) indicates Group KD2; (C) and (G) indicates Group L20; (D) and (H) indicates control group. (A) – (D) show brush border (**b**), intestinal epithelium (**e**), intestinal lumen (**lu**) and submucosa hemocytes (**h**) ($\times 400$). (E) and (G) show brush border (**b**), mild-vacuolated intestinal epithelium (**e**), intestinal lumen (**lu**) and submucosa hemocytes (**h**) ($\times 400$). (E) and (H) shows degenerated brush border (**b**), vacuolated intestinal epithelium (**e**), and intestinal lumen (**lu**) ($\times 400$).

3.4. Discussion

Probiotics either need to adapt or to be protected in dynamic environments before delivering their health benefits for targeted hosts (Terpou et al., 2019). The lactic acid bacteria are one of indigenous composition of fish gut microbiota plays an important role in fish gastrointestinal tract development, digestive function, mucosal tolerance, host immune response stimulation, and protection against pathogenic infections (Ringø et al., 2018). Since probiotic supplemented feed will be immersed into shrimp pond water that usually between 20-30 ppt by using feeding trays and feed tables for shrimp feeding (Gao et al. 2016; Taily et al. 2021). Therefore, probiotics with salinity tolerance are needed for survive in high salinity water environment before enter shrimp gastrointestinal tract. Therefore, lactic acid bacteria isolated from marine-related environment were selected, since it was hypothesized that these strains would have high salt tolerance and adaptation to the marine environment, attributed to their origin from high-salt concentration environment (Zhang et al., 2017). Among lactic acid bacteria, *Lactobacillus* spp. is the most effective and widely use probiotic as a feed supplement for aquatic animals' health by controlling microbial diseases in aquaculture (Zheng et al., 2020). From previous study, the *L. plantarum* L20 was proved to have gastric acidity resistance, bile acid resistance, mucus and/or human epithelial cells adhesion and bile salts hydrolysis, which suitable as human probiotic and animal feed additive (Ida-Muryany et al., 2017).

Generally, 16S rRNA phylogeny of the isolated bacteria included *Bacillus* spp., *Enterococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Staphylococcus* spp.,

Streptococcus spp., *Vagococcus* spp., and *Leuconostoc* spp. implied the bacteria belongs to gram-positive bacteria cluster with low G+C of genomic nucleotide content (Kurokawa et al. 2012). *Vagococcus* spp., *Enterococcus* spp. and *Lactococcus* spp. are closely related phylogenetically and phenotypically (Jimenez et al. 2022). In other study, closely related evolutionary relationship between *Weissella* spp., *Leuconostoc* spp., *Enterococcus* spp., and *Lactobacillus* spp. was revealed upon nucleotide sequences alignment of multidrug transporter involved in fosfomycin resistance encoding gene (Abriouel et al., 2015). *Lactobacillus sakei* DJ1 was found similar with the *L. curvatus* LT1 in this study because *L. curvatus* belongs to the *L. sakei* clade (Terán et al., 2018).

Different strains of bacteria species in the phylogeny demonstrated genetic divergence in their ancestral lineages which could related with distinct environmental adaptation (Teng et al. 2022). Unlike others of *Lactobacillus* spp. with environment and/or host specific properties, *L. plantarum* strains are nomadic bacterial species comprised variable and flexible gene which developed versatile functional capacities independently of its habitat, to thrive in many different environments (Martino et al., 2016). In vitro antagonism tests were considered for pre-selecting potential probiotics by testing inhibitory substances or antimicrobial metabolites of potential probiotic against targeted pathogens in the same medium (Medina et al., 2020). For antagonistic activity, other studies demonstrated *L. plantarum* strain inhibit *V. parahaemolyticus* strain robustly in well diffusion assay and coculture assay of their experiment (Le and Yang 2018; Truc et al., 2019; Han et al. 2022), which similar with *L.*

plantarum T31, L20, KD2 exhibited greater antibacterial activity against all *Vibrio* spp. Therefore, we inferred that different strains and species of *Lactobacillus* likely have unique antimicrobial mechanisms. For instance, *L. plantarum* have been reported to produce a range of antimicrobial compounds, including organic acids such as lactic and acetic acid, hydrogen peroxide, and antimicrobial peptides (Hu et al., 2019). Additionally, *L. plantarum* was also reported to be among the best producers of lactic acid (Brinques et al., 2009). Organic acids, particularly lactic acid, effectively inhibit the growth of various *Vibrio* spp., including *V. parahaemolyticus*, by creating an unfavorable low pH environment and damaging the cell wall of the bacteria (Kaur et al., 2018).

In comparison, the result of the feeding trial was aligned by previous research related with farmed crustacean species wherein other commercial probiotic *L. plantarum* strains, such as Xinhailisheng, MTCC1407, NatureWay and KC426951 are safe and improved growth performances on Pacific white shrimp (*P. vannamei*), giant freshwater prawn (*Macrobrachium rosenbergii*), smooth marron (*Cherax cainii*), and narrow clawed crayfish (*Astacus leptodactylus*) respectively (Dash et al., 2015; Zheng et al., 2017; Valipour et al., 2019; Foyosal et al., 2020; Wei et al., 2022). Moreover, the result of the challenge test was aligned also with previous research, which probiotic *L. plantarum* strains SGLAB01 and L1 improved survival of Pacific white shrimp during challenge experiment with AHPND-causing *V. parahaemolyticus* (Chomwong et al., 2018; Truc et al. 2019;).

Crucial criteria of probiotics included ability to adhere, colonize and survive in their host's gastrointestinal tract (Bagon et al., 2018). Commonly, autochthonous strain microbes are known to have longer duration of gastrointestinal mucosa-adhering properties while allochthonous strain microbes have shorter period of the properties (Rimoldi et al., 2020). *L. plantarum* T31, KD2 and L20 demonstrated different scenario of gastrointestinal colonization. This may be due to the complex spatial organization of microbial communities and the various cooperative and competitive mechanisms involved (Xiong et al., 2018). During feeding trials, concentration of the *L. plantarum* strains in the abdomen of Group L20 demonstrated some allochthonous *Lactobacillus* spp. have been found to exhibit ecological functions like autochthonous strains, even when they are not present in their evolutionary habitats (Duar et al., 2017). In the challenge test, the highest concentration of the *L. plantarum* strain in the abdomen of L20 group indicated a high population of *L. plantarum* L20 that had colonized the shrimp gastrointestinal tract, which could inhibit the invasion and growth of pathogen, *V. parahaemolyticus* S2-4 (Du et al., 2022). This mechanism could be related to the high aggregation activities of *L. plantarum* against *V. parahaemolyticus* S2-4 (Le & Yang, 2018). Therefore, even as an allochthonous strain, *L. plantarum* L20 can exhibit ecological functions like autochthonous beneficial bacteria in the shrimp gastrointestinal tract, making it a potential probiotic candidate.

Vibrio parahaemolyticus S2-4 have been found to change the community composition and function of the microbiota in the shrimp gastrointestinal tract

(Zhang et al., 2021). During the challenge test, *V. parahaemolyticus* S2-4 positive AHPND colonized shrimp stomach initially, then entered and colonized shrimp hepatopancreas, released that *pirA* and *pirB* toxins that specifically recognize only glycoproteins on the epithelium of the hepatopancreas, cause AHPND lesions in cephalothorax of control group (Phiwsaiya et al., 2017; Soto-Rodriguez et al., 2022). Similar with abdomen of control group, *V. parahaemolyticus* S2-4 positive AHPND and other opportunistic *Vibrio* spp. dominated shrimp intestine in result intestinal dysbiosis (Kumar et al., 2020; Petrova et al., 2022). Moreover, we postulated *L. plantarum* L20 have colonizable space competition, bacteriocins and lactic acid secretion (Gao et al., 2018), which could inhibit activity of AHPND-causing *V. parahaemolyticus* S2-4 in stomach and hepatopancreas of L20 group.

In the challenge test, histological deformation of hepatopancreas of the shrimp samples from control group could related with AHPND mechanism, *pirA* toxin plays a crucial role in target-specific recognition by binding to receptors on the cell membrane, which then allows *pirB* toxin to bind to another region of the same receptor. *pirB* toxin then causes cell death through the formation of pores, resulting in deformation of the hepatopancreas and ultimately leading to shrimp mortality (Lee et al. 2015; Kumar et al. 2019). Histological vacuolation with tissue necrosis of intestinal epithelium of the shrimp samples from control group inferred the lipopolysaccharide of *V. parahaemolyticus* can damage the intestine mucosal barrier by decreasing the viability of epithelial cells, disrupting tight junctions, and disordering the stability of mucosal mucins of shrimp gastrointestinal tract (Duan et al. 2018). Moreover, the *L. plantarum*

L20 might have adhesive ability to colonize host intestinal mucosa and potential immunomodulatory effect for host (Santarmaki et al. 2017). Therefore, it is possible that *L. plantarum* L20 could prevent *V. parahaemolyticus* infection by blocking the pathogen's entry from the shrimp gastrointestinal tract, as *Vibrio* spp. colonization usually begins in the shrimp's gastrointestinal tract (Valente and Wan, 2021).

3.5. Conclusion

Overall, *L. plantarum* L20 effectively inhibited AHPND-causing *V. parahaemolyticus* *in vitro*, improved growth performance, and demonstrated enhanced survival of *P. monodon* postlarvae against infection by the pathogen *in vivo*. Therefore, this study proposed the use of *L. plantarum* L20 as probiotic candidate in shrimp aquaculture industry to prevent further losses due to AHPND.

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CHAPTER 4

ELUCIDATION OF THE SYNBIOTIC FEED ENRICHED WITH (*Lactobacillus plantarum* STRAIN L20 AND *Sargassum polycystum*) SUPPLEMENTED DIET FOR GROWTH, IMMUNE RESPONSE, BACTERIAL PROFILE AND DISEASE RESISTANCE OF BLACK TIGER SHRIMP (*Penaeus monodon*) AGAINST ACUTE HEPATOPANCREATIC NECROSIS DISEASE

4.1. Introduction

Acute hepatopancreatic necrosis disease (AHPND) is a disease that can cause sudden mass mortalities of up to 100% within the initial 30–35 days after stocking *P. monodon* in their postlarvae to early-stage juvenile. As they progress to the late-stage juveniles, mortalities can still reach 40-60% within 56-94 stocking days (Peña et al., 2015). For the pathogenesis in shrimp, AHPND-positive *V. parahaemolyticus* and other *Vibrio* sp. first colonise the digestive tract including the stomach of penaeid shrimp species, where upon reaching the hepatopancreas, the pathogen would induce the sloughing of tubule epithelial cells via the production of *pirAB* toxin, ultimately leading to shrimp mortality (Kumar et al. 2020). The Chapter 3 revealed that *L. plantarum* L20 had a high inhibition activity against *Vibrio* spp. and higher survival rate of *P. monodon* postlarvae compared to other *L. plantarum* strains during *V. parahaemolyticus* immersion challenge. For better improvement, the synbiotic was proposed which combination of the probiotic with a prebiotic to achieve an integrated or greater effect as nutritional approach for targeting the host gastrointestinal microbiota (Sergeev et al. 2020). Thus, prebiotic was required for the establishment of synbiotic.

Generally, International Scientific Association of Probiotics and Prebiotics (ISAPP) defined dietary prebiotics as “a selectively fermented ingredient that cause specific changes in the composition and/or activity of the host’s gastrointestinal microbiota to contribute advantage(s) for host health” in November of 2008 (Gibson et al. 2010). In latest, the definition of prebiotics was updated by ISAPP as “diverse categories of substrates, including non-carbohydrate based, which are selectively consumed by the inhabiting microbiota to contribute benefit(s) for host’s health” in December of year 2016 (Gibson et al. 2017). In previous findings, powder form of *Macrocystis* spp. and *Lessonia* spp as prebiotic candidates supplemented shrimp diet demonstrated high growth performance and digestibility of *P. vannamei* (Cárdenas et al., (2015). The marine macrophytes, seaweeds show prebiotic effects, which can be utilized as prebiotic or feed additive for farmed aquatic animals because consist multiple various type of complex carbohydrates with prebiotic properties including alginates, fucoidans, mannitol and laminarin (Makkar et al., 2016). Moreover, Brown seaweed, *Sargassum* spp. can be applied as prebiotic directly because consists of fermentable dietary polysaccharides (alginates, laminarins, fucoidans) for human and animal consumption (Wan et al. 2018). In fact, the dry powder form of *S. polycystum* (Patent No: PI2013702168) consists of several carbohydrate components, including alginate, fucoidan, fucose, with minor quantities of other monosaccharides, especially xylose, galactose, mannose and rhamnose, which have prebiotic properties (Nazarudin et al., 2021). Additionally, our research demonstrated that incorporating prebiotic *S. polycystum* powder (Patent No: PI2013702168) into feeds improved the growth performance of Asian seabass, *Lates calcarifer*

(Nazarudin et al. 2020). Thus, in this study, we developed prebiotic and probiotic is anticipated to yield more promising results.

Ideally, prebiotic substrate of synbiotic mixture can improve the constituted probiotic and host intestinal microbiota to achieve improved effects for targeted host, including additional supplemented nutrients, digestibility enhancement and immune responses improvement (Swanson et al. 2020). Studies by Huynh et al. (2018) and Huang et al. (2023) reported the beneficial effects of synbiotic diets (*L. plantarum* 7-40 with galactooligosaccharide and *Leuconostoc mesenteroide* B4 with dextran) on shrimp growth performance and disease resistance against vibriosis for *P. vannamei*. Consequently, in this study, synbiotic was formulated by combining probiotic *L. plantarum* L20 with nutrient rich-prebiotic *S. polycystum* powder, integrated into shrimp feeds. This formulation is hypothesized to yield synergistic effects, enhancing growth performance, immune response, and disease resistance for penaeid shrimp farming. However, no study has described the synergistic potential of *L. plantarum* L20 and *S. polycystum* as an effective synbiotic for improving *P. monodon*'s growth performance, bacterial profiles, immune responses of digestive system and disease resistance against Malaysia-prevalent *V. parahaemolyticus* isolate, associated with AHPND infection. In addition, no study has also described *L. plantarum* L20 enumeration in shrimp by using RT-qPCR technique for quantitative bacteria profiling. Besides, the conducted experimental flow charts of Chapter 4 are shown in Figure 4.1. The study aims of Chapter 4 are shown below.

1. To assess the efficacy of probiotic (*L. plantarum* L20), prebiotic (*S. polycystum*) and synbiotics (*L. plantarum* L20 and *S. polycystum*) supplemented feed on the growth performance and AHPND-causing *V. parahaemolyticus* S2-4 resistance of *P. monodon*.
2. To determine the immune response stimulation and modulatory effects of probiotic (*L. plantarum* L20), prebiotic (*S. polycystum*) and synbiotics (*L. plantarum* L20 and *S. polycystum*) supplemented feed for *P. monodon*.

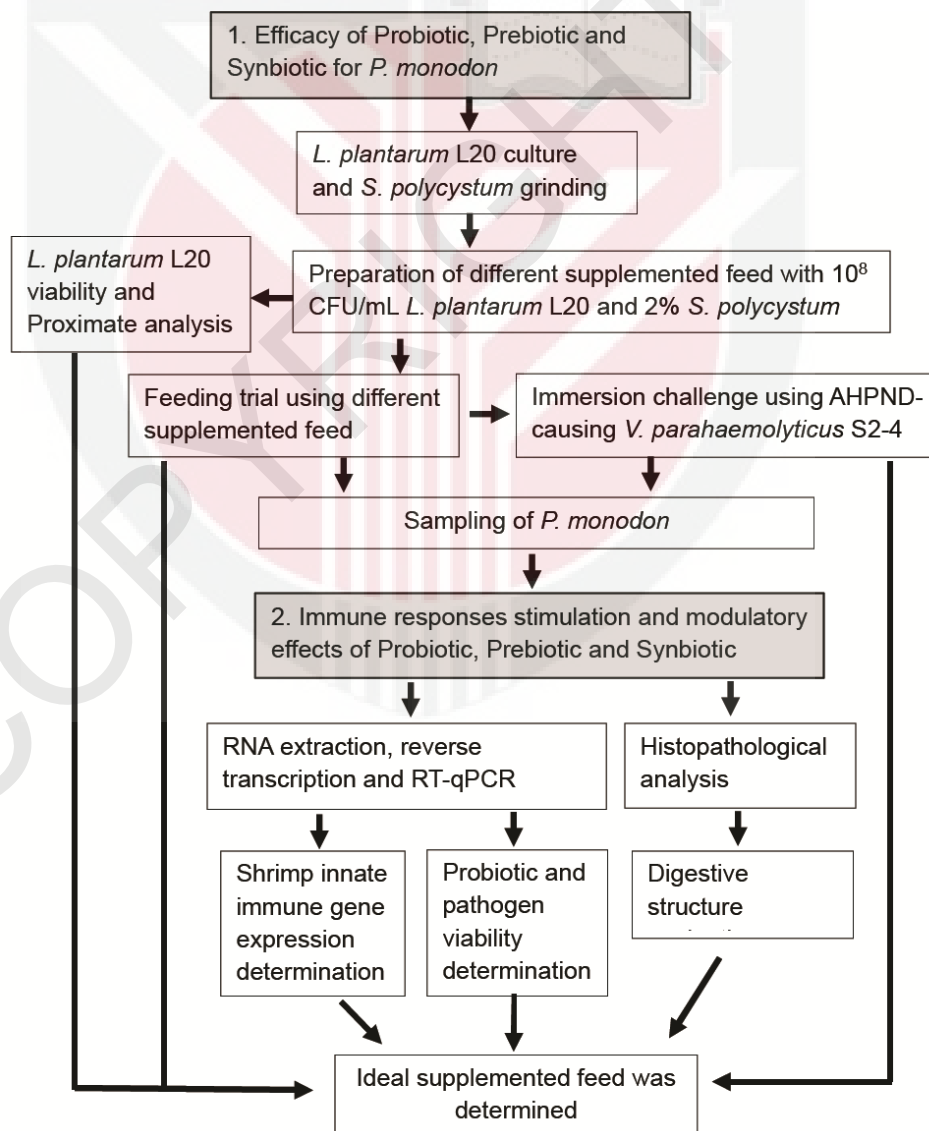


Figure 4.1: Flow charts of conducted experiments in Chapter 4

4.2. Materials and Methods

4.2.1. Effectiveness assessment of Probiotic, Prebiotic and Synbiotic

4.2.1.1. Bacterial culture

Lactobacillus plantarum L20 was inoculated on MRS agar (Himedia, Mumbai, India) supplemented with 1.5% NaCl and incubated at 30 °C for 48 h. Repeated subculturing was performed until single colony was found. Each single colony was then inoculated into the MRS broth (Himedia, Mumbai, India) supplemented with 1.5% NaCl and incubated at 30 °C for 48 h. AHPND-causing *V. parahaemolyticus* S2-4 that was previously isolated from diseased shrimp by Wan Haifa-Haryani et al. (2022), was selected for the challenge test. The pathogen was grown on tryptic soy broth (TSB; Oxoid, Hampshire, England) supplemented with 1.5% NaCl for 24 h at 27 °C and harvested by centrifugation for 10 min, at 1000 rpm and 4 °C. The pellet was washed and re-dissolved in sterile autoclaved filtrated seawater and adjusted to 10⁸ CFU/mL as stock by using total plate count technique.

4.2.1.2. Preparation of *L. plantarum* L20 and *S. polycystum* powder supplemented feed

Four experimental diets including a control diet without probiotic and prebiotic supplement, and three experimental diets consisting of probiotic diet (PRO) containing 10⁸ CFU/g *L. plantarum* L20, prebiotic diet (PRE) containing 2% (w/w) *S. polycystum* powder (Patent No: PI2013702168; Nazarudin et al. 2020), and synbiotic diet (SYN) containing 10⁸ CFU/g *L. plantarum* L20 and 2% *S.*

polycystum powder were prepared. The cultured broth of *L. plantarum* L20 was centrifuged to obtain pellets. After the supernatant was discarded, the bacterial pellet was resuspended with cool autoclaved PBS saline water. Before diet preparation, the basal feed (Star Feedmill, Beranang, Malaysia; crude protein 40%, crude fat 5%, crude fiber 3%, moisture 12%) was ground to pass through a 60-mesh screen. The diet mixed with blank phosphate buffered saline (PBS) water (Sigma-Aldrich, Missouri, USA) only was assigned as control diet; the diet mixed with *L. plantarum* L20 suspension as a probiotic diet; the diet mixed with 2% *S. polycystum* powder and PBS water (Sigma-Aldrich, Missouri, USA) as a prebiotic diet; and the diet mixed with *L. plantarum* L20 suspension and 2% *S. polycystum* powder as a synbiotic diet. The ratio of feed powder with bacterial suspension or PBS water (Sigma-Aldrich, Missouri, USA) was 1:1. The four mixtures were placed into a stirring machine and slowly added with water until a stiff dough was formed. The dough was then passed through a mincer to form spaghetti-like strings and was cut into pellets of approximately 2 mm in diameter and length. The pellets were dried by air-conditioner at 20 °C. The pellets were ground into powder form. The experimental diets were individually preserved in transparent sterile plastic bags and kept at 4 °C before use. The viabilities of *L. plantarum* in the experimental diets were assessed by total plate count technique with MRS agar (Himedia, Mumbai, India) supplemented with 1.5% NaCl and incubated at 30 °C for 48 h, before the feeding trial experiment. The proximate analysis for moisture, crude ash, crude protein, crude lipid and crude fibre of the experimental diets was conducted by Unipeq Sdn. Bhd. Bangi, Malaysia according standard AOAC methods (AOAC 2016).

4.2.1.3. Feeding management for *P. monodon*

Healthy black tiger shrimp (*P. monodon*) postlarvae approximately 10 mg (n = 600) were selected randomly from the hatchery of Teluk Senangin, Perak, Malaysia. Six hundred shrimps were brought to the hatchery of Institute of Biosciences, Universiti Putra Malaysia, Serdang, Malaysia. During acclimation, 200 shrimps were randomly assigned to three fiberglass tanks (500 L) each tank. They were fed three times daily with the control diet and 4% of feed per times according to their body weight and cultured for 7 days with 30 ppt of UV treated and filtered natural seawater. The tanks were equipped with air stones for aeration, covered with black plastic cloth for preventing light and a heater for controlling the temperature at approximately 27 °C. All tanks were set within the same space and area of the hatchery to maintain the same environmental condition. Water quality was monitored by using YSI professional plus multiparameter meter (YSI, Ohio, United states) and maintained by water exchange with 1/3 of total tank water volume once per 2-3 days.

After acclimation, shrimps were distributed into 4 treatment groups (n = 150 per group) of fiberglass tanks (90 cm× 60 cm × 30 cm, containing 100 L of seawater) as shown Table 4.1. All tanks were set within the same space and area of the hatchery to maintain the same environmental condition. Shrimps were fed three times daily for 35 days with the experimental diets, corresponding to 4 % of their body weight. In the growth trial, weight was measured at the beginning of the trial, 7 days interval and after 35 day (Figure 4.2). The tanks were equipped with air stones for aeration, artificial shrimp shelters, covered with black plastic cloth for preventing light and a heater for

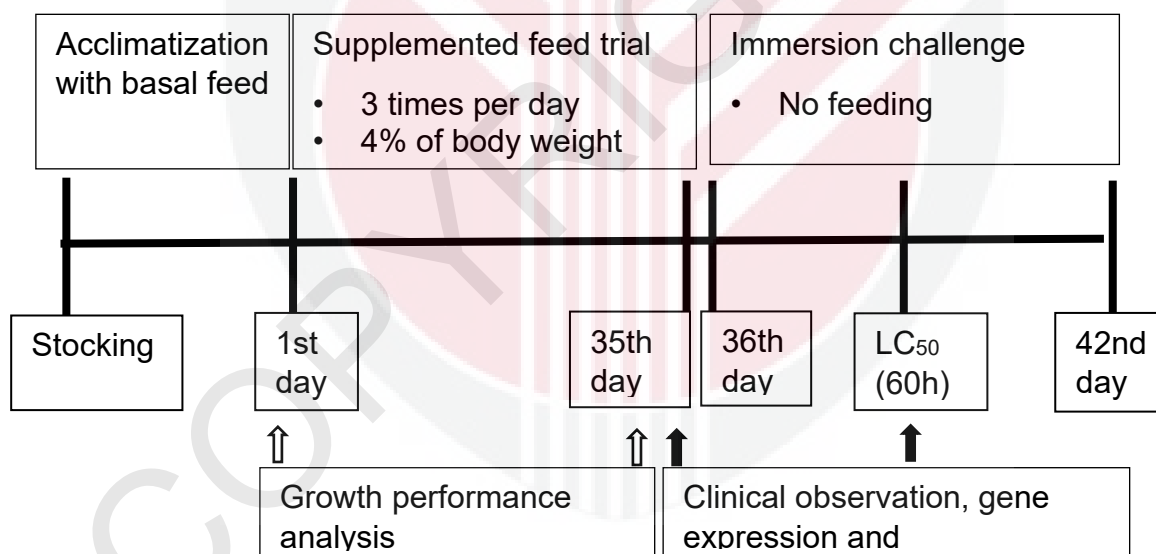
controlling the temperature at approximately 27 °C. All tanks were set within the same space and area of the hatchery to maintain the same environmental condition. Water quality was monitored by using YSI professional plus multiparameter meter (YSI, Ohio, United states) and maintained by water exchange with 1/3 of total tank water volume once per 2-3 days. Uneaten feed was collected daily, dried in an oven, and weighed to determine the food conversion ratio (FCR). Prior to morning feeding, feces were removed daily. Shrimp survival was monitored throughout the trial period. At 35th day, measurements including final length and weight of the shrimp, as well as the dry weight of uneaten feed, were recorded to calculate growth parameters. Nine shrimps were sampled from each group for qPCR study upon RNA extraction and cDNA synthesis as described in section 4.2.7 to 4.2.10 while three shrimp were sampled from each group for histopathology study as described in section 4.2.8.

The parameters of growth performance were calculated as follows:

1. Survival = (final shrimp number/initial shrimp number) × 100 %;
2. Weight gain = (final weight – initial weight)/initial weight × 100 %;
3. Specific growth rate = (ln final weight – ln initial weight)/days × 100 %;
4. Feed conversion ratio = Total dry feed intake/Total wet weight gain
5. Protein efficiency ratio = Total fresh flesh gain/Total protein intake

Table 4.1: Experimental design of feeding trial and immersion challenge

Group	Supplemented feed	Feeding trial		Immersion challenge	
		Number of shrimps per tanks	Replicate tanks	Number of shrimps per tanks	Replicate tanks
Probiotic	10 ⁸ CFU/mL of <i>L. plantarum</i> L20 with PBS water	50	3	20	4
Prebiotic	2% (w/w) <i>S. polycystum</i> powder with PBS water	50	3	20	4
Synbiotic	10 ⁸ CFU/mL of <i>L. plantarum</i> L20 & 2% (w/w) <i>S. polycystum</i> powder with PBS water	50	3	20	4
Control	PBS water	50	3	20	4

**Figure 4.2: Experimental timeline of feeding trial regime and immersion challenge.** Growth performance was recorded during the experiment. Survival, clinical observation, gene expression and histopathology assessments were performed at end of feeding trial (35th day) and immersion challenge test with LC₅₀ of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 (3rd day, 60h).

4.2.1.4. Immersion challenge of AHPND-causing *V. parahaemolyticus* S2-4

For the immersion challenge experiment, shrimps (n = 80 per group) at 35th day feeding trial were chosen and challenged with LC₅₀ concentration of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 by mixing the calculated volume of bacterial suspension with UV treated seawater in the aquarium (Chapter 3) shown in Table 4.1. During the immersion challenge period, all aquariums were maintained with 30 ppt of the seawater, equipped with air stones for aeration, artificial shrimp shelters, covered with black plastic cloth for preventing light and a heater for controlling the temperature at 27 °C. No water exchange occurred during the immersion challenge test. Four replicates of each group were used for survival observation, qPCR and histopathological study. Mortality and clinical signs were observed for 7 days. On the 3rd day (60 h of post-challenge), nine shrimps were sampled from each group for qPCR study while three shrimp was sampled from each group for histopathological study.

4.2.2. Shrimp immune stimulation and modulatory effect of Probiotic, Prebiotic and Synbiotic

4.2.2.1. RNA extraction and cDNA synthesis

Nine shrimps were sampled at end of the feeding trial, while another nine shrimps were sampled at 60 h post challenge from each group. All shrimps from each treatment group (pre- and post-challenge) were kept in RNAlater (Invitrogen, Massachusetts, USA) after anaesthetised with 300 ppm pH buffered tricaine methanesulphonate (MS-222; Sigma-Aldrich, Missouri, USA). Then, shrimp shells were removed, and shrimp were cut into cephalothorax

portion and abdomen portion separately (Figure 4.3) after being dried from RNAlater (Invitrogen, Massachusetts, USA). Cephalothorax portion consisted of shrimp hepatopancreas while the abdomen portion consisted of the shrimp intestinal tract. Three cephalothorax portions were assembled as one replicate, while three abdomen portions were assembled as one replicate. Three replicates were obtained from the four treatment groups from the feeding trial and post challenge. The samples of the four treatments were ground using electronic homogeniser. RNA was extracted using Trizol (Invitrogen, Massachusetts, USA) for each homogenised sample according to the manufacturer's manual. The steps were repeated for other treatment groups. The integrity of the RNA was validated by using 1.0 % agarose of gel electrophoresis.

The RNA samples were treated with the RapidOut DNA Removal Kit (Thermo Fischer Scientific, Waltham, USA) according to the manufacturer's manual. The concentration and quality of the total RNA was checked based on its optical density at 260 and 280 nm as measured by GENOVA-NANO Spectrophotometer (Jenway, Staffordshire, UK). The concentration of total RNA was adjusted into 1 µg/µL by using nuclease-free water. The RNA was added with 4 µL of OneScript® reverse-transcription buffer (Applied Biological Materials, Richmond, Canada) and 1 µL of OneScript® reverse-transcription enzyme (Applied Biological Materials, Richmond, Canada), 1 µL of dNTP, 1 µL of random primer, 1 µL of oligo (dT) and up to 20 µL of nuclease-free water. The final mixture was incubated at 25 °C for 10 min and 60 °C for 30 min,

followed by transcription enzyme inactivation for 5 min at 85 °C. The first-strand complementary DNA (cDNA) was subjected for gene expression analysis.

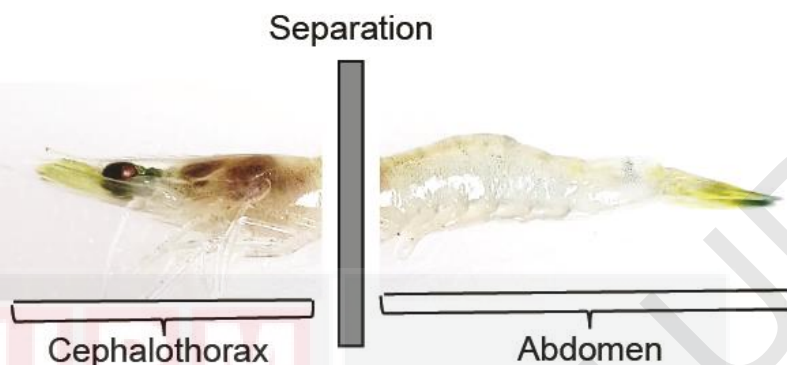


Figure 4.3: Cephalothorax and abdomen separation of *P. monodon*

4.2.2.2. Gene expression by real time-quantitative PCR

Quantitative PCR analysis was performed using QuantiNova® SYBR Green real-time PCR mix (Qiagen, Hilden, Germany) following the manufacturer's instructions. About 1 µL of cDNA sample was added with 10 µL of 2x SYBR Green PCR Master mix, 1 µL of forward primer, 1 µL of reverse primer and 7 µL of nuclease-free water into a PCR tube. Each cDNA sample was performed in triplicate. The samples were run by using Rotor Gene Q Real-time PCR machine (Qiagen, Hilden, Germany). No template control (NTC) and no reverse transcriptase control (NRT) were added also in each run. The CT value was analysed and recorded through Rotor Gene Q software (Qiagen, Hilden, Germany). The primers used in RT-qPCR and their efficiency for bacterial specific genes and shrimp immune genes were listed in Table 4.2. and Table 4.3. respectively. The primer pairs were efficiently determined by performing serial dilutions of reference cDNA and used to quantify cDNA concentration. The standard cycling parameters included an initial denaturation at 95 °C for 2

minutes, followed by 40 cycles of denaturation at 95 °C for 3 seconds and annealing/elongation at 60 °C for 30 seconds. Melting curve analysis was performed at the end of each PCR run. A melting curve profile was obtained by heating the mixture to 95 °C, cooling to 60 °C for each 10 s. Primer specificity was evaluated by determining single peak in all melting curves and single targeted band on 1.0% agarose gel electrophoresis of all RT-PCR amplicons indicating absence of primer-dimer and optimal performance of the primers. The mean CT value was used for gene expression analysis.

Table 4.2: The primer sequence of bacterial specific genes that used for Reverse transcriptase Quantitative PCR (RT-qPCR)

Gene	Primers	Sequences	Efficiency	Reference
GHL domain containing protein	Forward	GCGGTATCGATTGATT GGT	99%	Kim et al. (2020)
	Reverse	TGATGTCAATCGCCTCT TGG		
Photobacterium insect-related toxins A (<i>pirA</i>)	Forward	CAAACGGAGGCGTCAC AGA	100%	Mai et al. (2021)
	Reverse	GACCGACTTCCGGGATGA T		

Table 4.3: The primer sequence of shrimp immune genes that used for Reverse transcriptase Quantitative PCR (RT-qPCR)

Gene	Function	Primers	Sequences	Efficiency	Reference
lipopolysaccharide and β-1,3-glucan-binding protein (LGBP)	Pathogen recognition	Forward	CA TGTCCAAC TTCGCTTT CAGA	100%	Mai et al. (2021)
		Reverse	ATCACC GCGTGGCATCTT		
Peroxinectin	Immune signalling transduction	Forward	ATCCAGCAGCCAGGTATG	98%	Lin et al. (2012)
		Reverse	CAGACTCATCAGATCCATTCC		
Prophenoloxidase	Main component of	Forward	ACCACTGGCACTGGCACCTCGTCTA	100%	Lin et al. (2012)
	Prophenoloxidase cascade	Reverse	TCGCCAGTTCTCGAGCTTCTGCAC		
Toll-like receptor	Main component of	Forward	CTTAGCCTTGGAGACAAC	98%	Deris et al. (2020)
	Toll signalling pathway	Reverse	GATGCTTAACAGCTCCTC		
Crustin	Cationic antimicrobial peptides	Forward	AGCGACTGCAGGTATTGGTG	97%	Mai et al. (2021)
		Reverse	TCGTTGGAACAGGTTGTGG		
Penaeidin	Cationic antimicrobial peptides	Forward	TCGTGGTCTGCCCTGGTCTT	102%	Mai et al. (2021)
		Reverse	CAGGTCTGAACGGTGGTCTTC		
Lysozyme	Cationic antimicrobial peptides	Forward	TGGTGTGGCAGCGATTATG	102%	Deris et al. (2020)
		Reverse	GATCGAGGTCCGATTCTTAC		
Heat shock protein 70 (HSP70)	Stress and immunostimulant protein	Forward	CCTCCTACGTCCCTTCACAGACA	96%	Lin et al. (2012)
		Reverse	GGGGTAGAAGGTCTTCTTGTCTCCC		
Elongation factor 1α (EF1-α)	house-keeping gene	Forward	TCGCCGAACTGCTGACCAAGA	104%	Mai et al. (2021)
		Reverse	CCGGCTTCCAGTTCCCTTACC		

4.2.2.3. Measurement of the expression of immune related genes in *P. monodon*

The immune related genes included lipopolysaccharide and β -1,3-glucan-binding protein (LGBP), peroxinectin, prophenoloxidase, toll-like receptor, crustin, penaeidin, lysozyme, and heat shock protein (HSP70) (Table 4.3). By using three computational programs included geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) for analysing stability of housekeeping genes, the most stable elongation factor 1-alpha (EF1- α) was chosen as control gene among other housekeeping genes (β -actin, GAPDH) for the immune gene expression study of the shrimp cephalothorax and abdomen. Relative quantification was applied for determining expression of immune-related genes in gastrointestinal digestion system of shrimp (Silveira et al. 2018). The relative expression levels of shrimp immune genes were calculated by using the comparative $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

4.2.2.4. Investigation of bacteria in intestinal microbiota of *P. monodon*

For quantifying *L. plantarum*, specific conserved protein-encoding gene, GHKL domain containing protein gene was used as indicator (Table 4.4) because they can distinguish closely related species and strains of *Lactobacillus* sp. strains (Huang et al. 2018; Kim et al. 2020). For quantifying AHPND-causing *V. parahaemolyticus*, Photorhabdus insect related (*pir*) toxin A (*pirA*) gene was used as indicator (Table 4.4) because it is a crucial toxin genes of AHPND-causing *V. parahaemolyticus* (Aranguren Caro et al. 2020; Han et al., 2020).

Absolute quantification was applied for detecting and quantifying bacterial protein genes in gastrointestinal digestion system of shrimp (Zhang et al. 2020). The absolute concentrations of the bacterial protein genes were calculated by using the linear regression of their standard curve (Bustin 2000). Standard curves were generated by the amplification of a 10-fold dilution series of the targeted protein genes in the presence of the bacterial cDNA. The coefficient of correlation (R^2) between the Threshold value (CT) and targeted cDNA concentration was between 0.990–0.997, while the PCR efficiency was between 90 and 110%. Zero concentration of bacteria was determined if CT value was not available or more than 40 (McCall et al. 2014).

4.2.2.5. Histopathological observation

Three shrimp from all treatment groups were chosen for histopathological analysis. The shrimp shells were removed and whole shrimp were fixed in 10% neutral buffered formalin for 24 h after anaesthetised with 300 ppm tricaine methanesulphonate, known as MS-222 (Sigma-Aldrich, Missouri, USA). The samples were transferred to 70% ethanol and dehydrated using series of increasing concentration of ethanol (50% – 100%). The samples were embedded in paraffin wax, cut into 4 μ m thickness and stained with haematoxylin and eosin. The samples were observed under microscope and microphotographed with magnification 100X and 400X under the Olympus BX51 digital light microscope (Olympus, Tokyo, Japan). The hepatopancreas and gut of the shrimps were observed histologically.

4.2.3. Data analyses

Normality of the variance was evaluated using Shapiro–Wilk test. Then, differences in data were analysed by one-way analysis of variance (ANOVA) following post-hoc Duncan test. All statistical analyses were performed using IBM SPSS Statistics® version 22.0. The values were considered significantly different if $p < 0.05$.

Ethical statement

All procedures in this study involving animals were approved by the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee, approval number: UPM/IACUC/AUP-R061/2022 (Appendix 5).

4.3. Results

4.3.1. Effectiveness of Probiotic, Prebiotic and Synbiotic for *P. monodon*

4.3.1.1. Proximate analysis and viability test

The concentration of *L. plantarum* L20 remained relatively consistent over time, with no significant minimal changes ($p>0.05$) observed in the Probiotic feed (*L. plantarum* L20 supplemented feed) and Synbiotic feed (*L. plantarum* L20 and *S. polycystum* supplemented feed), whereas for Prebiotic feed (*S. polycystum* supplemented feed) and control feed (PBS water supplemented feed) was no presence of *Lactobacillus* spp. for 15 days at 4 °C. Prebiotic feed and Synbiotic feed were significantly ($p<0.05$) higher in ash, moisture and carbohydrate than the Probiotic feed and control feed (Table 4.4). Crude fibre, Protein, and fat were not significantly different ($p>0.05$) among all diets (Table 4.4).

Table 4.4: Proximate analysis of supplemented diets

	Probiotic (10 ⁸ CFU/g of <i>L. plantarum</i> L20)	Prebiotic (2% of <i>S. polycystum</i>)	Synbiotic (10 ⁸ CFU/g of <i>L. plantarum</i> L20 with 2% of <i>S. polycystum</i>)	Control (PBS water only)
Ash	9.69 ± 0.10 ^a	10.49 ± 0.04 ^b	10.54 ± 0.01 ^b	9.53 ± 0.12 ^a
Moisture	17.36 ± 0.19 ^a	14.51 ± 0.51 ^b	14.58 ± 0.44 ^b	17.98 ± 0.68 ^a
Crude fiber	0.97 ± 0.09 ^a	1.20 ± 0.08 ^a	1.27 ± 0.09 ^a	1.00 ± 0.08 ^a
Protein	39.00 ± 0.82 ^a	38.83 ± 0.24 ^a	39.47 ± 0.53 ^a	38.53 ± 0.45 ^a
Fat	3.7 ± 0.08 ^a	3.47 ± 0.05 ^a	3.43 ± 0.05 ^a	3.7 ± 0.14 ^a
Carbohydrate	29.28 ± 0.78 ^a	31.50 ± 0.52 ^b	30.72 ± 0.21 ^b	29.26 ± 0.68 ^a

Different superscript letter indicates significant differences ($p<0.05$) of the same row.

4.3.1.2. Growth performance

The final weight and weight gain were significantly higher in the shrimp fed *L. plantarum* L20 supplemented feed (Probiotic group), *S. polycystum* supplemented feed (Prebiotic group) and *L. plantarum* L20 combined *S. polycystum* supplemented feed (Synbiotic group) ($p < 0.05$) than those in the control group but not significantly different ($p > 0.05$) between Probiotic group, Prebiotic group and Synbiotic group (Table 4.5). The specific growth rate and protein conversion ratio were slightly higher ($p < 0.05$) in the Probiotic group and Prebiotic group than those in the control group, but those in the Synbiotic group was significantly highest ($p < 0.05$) among the groups (Table 4.5). The feed conversion ratio was slightly lower in the Probiotic group and Prebiotic group ($p < 0.05$) than those in the control group, but those in Synbiotic group was significantly lowest ($p < 0.05$) among the groups (Table 4.6). No significant ($p > 0.05$) different for survival among all groups (Table 4.5).

Table 4.5: Parameters of growth performance for supplemented diets administrated *P. monodon*

	Probiotic (10 ⁸ CFU/g of <i>L. plantarum</i> L20)	Prebiotic (2% of <i>S.</i> <i>polycystum</i>)	Synbiotic (10 ⁸ CFU/g of <i>L. plantarum</i> L20 with 2% of <i>S.</i> <i>polycystum</i>)	Control (PBS water only)
Initial weight	0.02 ± 0.00 ^a	0.02 ± 0.00 ^a	0.02 ± 0.00 ^a	0.02 ± 0.00 ^a
Final weight	0.07 ± 0.01 ^b	0.06 ± 0.01 ^b	0.07 ± 0.01 ^b	0.04 ± 0.00 ^a
Weight gain (%)	332.03 ± 77.12 ^b	326.95 ± 68.17 ^b	387.55 ± 66.07 ^b	179.15 ± 24.78 ^a
Specific growth rate%	3.61 ± 0.48 ^{ab}	3.60 ± 0.40 ^{ab}	3.94 ± 0.33 ^b	2.56 ± 0.23 ^a
Feed conversion ratio	2.66 ± 0.62 ^{ab}	2.66 ± 0.53 ^{ab}	2.20 ± 0.29 ^b	3.63 ± 0.41 ^a
Protein conversion ratio	0.03 ± 0.01 ^{ab}	0.03 ± 0.01 ^{ab}	0.03 ± 0.00 ^b	0.02 ± 0.00 ^a
Survival (%)	98.00 ± 1.63 ^a	96.67 ± 2.49 ^a	97.33 ± 0.94 ^a	99.33 ± 0.94 ^a

Different superscript letter indicates significant differences ($p < 0.05$) of the same row.

4.3.1.3. Survival of post-challenge

The survival rates of the Prebiotic group was significantly ($p < 0.05$) higher than the control group, Although Probiotic group and Synbiotic group were significantly highest survival rates ($p < 0.05$) among all groups (Figure 4.4). The Synbiotic group were slightly significantly ($p > 0.05$) higher than Probiotic group.

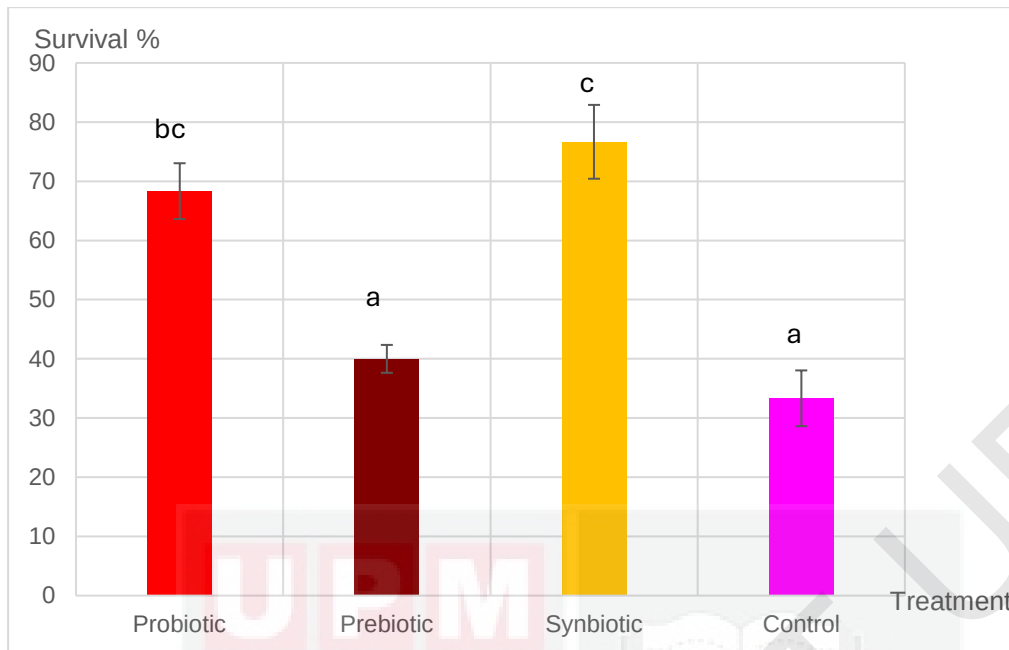


Figure 4.4: Survival percentage of different diet administered *P. monodon* after immersion challenge of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 at 3rd day (60h) post challenge. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; Prebiotic group that fed with 2% *S. polycystum* powder supplemented feed; Synbiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed; Control group that fed with basal feed.

4.3.1.4. Clinical symptom observation

All shrimp shows normal colour of hepatopancreas from all groups (Figure 4.4A-4.4D). Nevertheless, all shrimp shows normal colour of hepatopancreas from Probiotic group, Prebiotic group and Synbiotic group but pale hepatopancreas from control group in the immersion challenge (Figure 4.4E-4.5H).

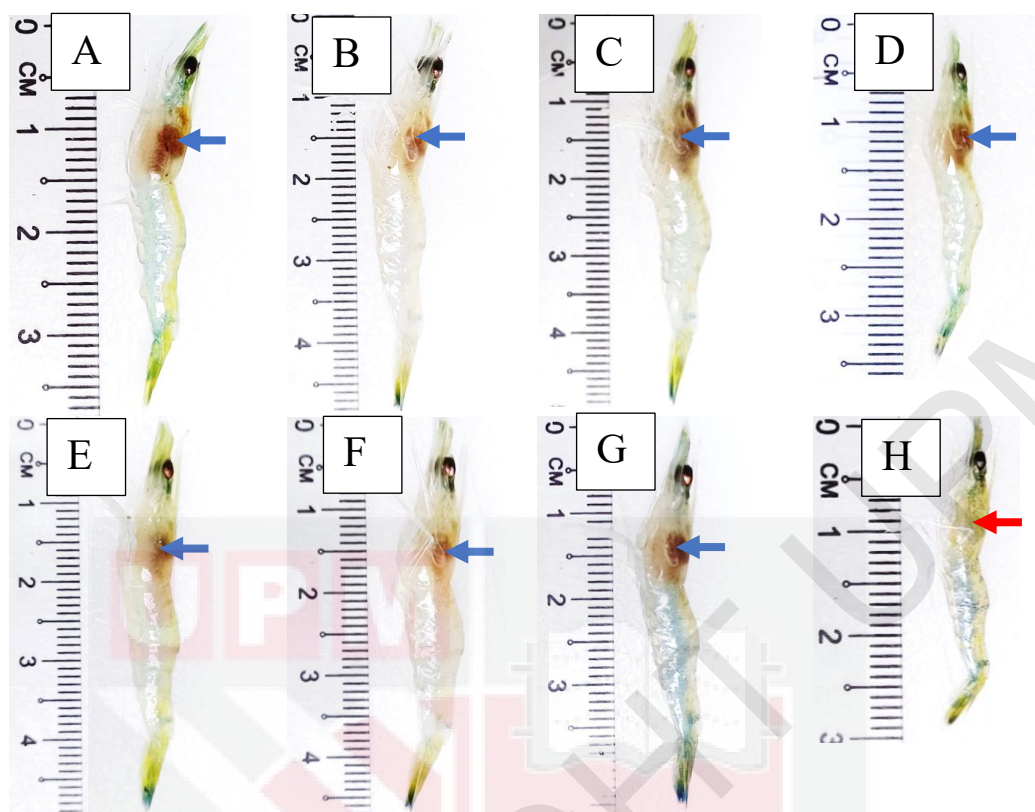


Figure 4.5: Clinical sign observation of different supplemented diets administered *P. monodon*. (A-D) were 35th day of feeding trial. (E-H) were 60 h of post-immersion challenge. (A) and (E) showed Probiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. (B) and (F) show Prebiotic group that fed with 2% *S. polycystum* powder supplemented feed. (C) and (G) show Synbiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed. Normal hepatopancreas (→) (A – G) and pale white hepatopancreas (→) (H) can be observed among the shrimps.

4.3.2. Immune gene expression of *P. monodon*

4.3.2.1. Shrimp immune responses in 35th day of feeding trial

In general, the Probiotic and Synbiotic groups significantly ($p < 0.05$) expressed high cell adhesive activity, subsequently triggered significant ($p < 0.05$) expression of immune deficiency signalling pathway for secreting lysozyme as antimicrobial peptide, and prophenoloxidase system pathway for melanization as compared with control group for shrimp cephalothorax (Figure 4.6B).

Though, the Probiotic, Prebiotic and Synbiotic groups significantly ($p<0.05$) inhibited these immune pathways, but significantly ($p<0.05$) expressed secretion of lysozyme as antimicrobial peptide as compared with control group for shrimp abdomen (Figure 4.7B). The Probiotic, Prebiotic and Synbiotic group significantly ($p<0.05$) inhibited stress responses compared with control group for whole shrimp (Figure 4.6B & Figure 4.7B).

For comprehensive immune gene regulation of shrimp cephalothorax in the feeding trial (Figure 4.6A), lipopolysaccharide and β -1,3-glucan-binding protein (LGBP), peroxinectin, prophenoloxidase and lysozyme were significantly up-regulated ($p<0.05$) but toll-like receptor, crustin, penaeidin and HSP70 were significantly down-regulated ($p<0.05$) from the Probiotic group and Synbiotic group compared with control group. LGBP, peroxinectin, toll-like receptor and HSP70 were significantly down-regulated ($p<0.05$), crustin, penaeidin and lysozyme were not significantly difference ($p>0.05$), prophenoloxidase were significantly up-regulated ($p<0.05$) from the Prebiotic group compared with control group.

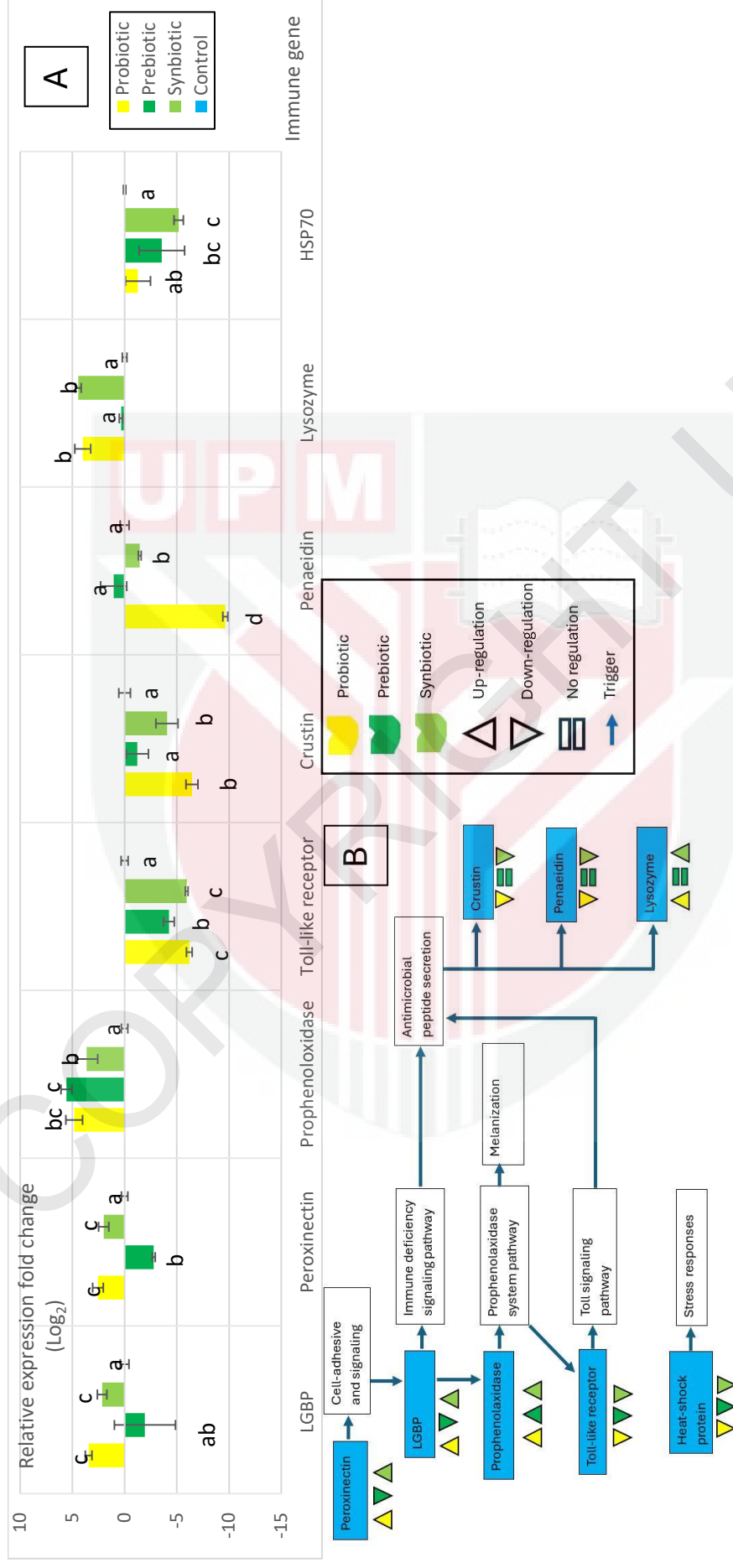


Figure 4.6: Immune gene expression and pathway in cephalothorax of *P. monodon* fed with different supplemented feed during at 35th day of feeding trial. (A) shows immune gene expression while (B) shows immune pathway. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. Prebiotic group that fed with 2% *S. polycystum* powder supplemented feed. Synbiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed.

For comprehensive immune gene regulation of shrimp abdomen in the feeding trial (Figure 4.7A), LGBP, toll-like receptor, HSP70 were significantly down-regulated ($p < 0.05$), but significantly up-regulated ($p < 0.05$) from the Probiotic, Prebiotic and Synbiotic group compared with control group. Crustin and penaeidin were significantly down-regulated ($p < 0.05$) from the Probiotic group and Synbiotic group, but not significantly difference ($p > 0.05$) compared with control group. Peroxinectin was significantly up-regulated ($p < 0.05$) from Probiotic group while prophenoloxidase was significantly down-regulated ($p < 0.05$) from Prebiotic group, but the rest of groups were not significantly different ($p > 0.05$) for the two genes compared with control group.

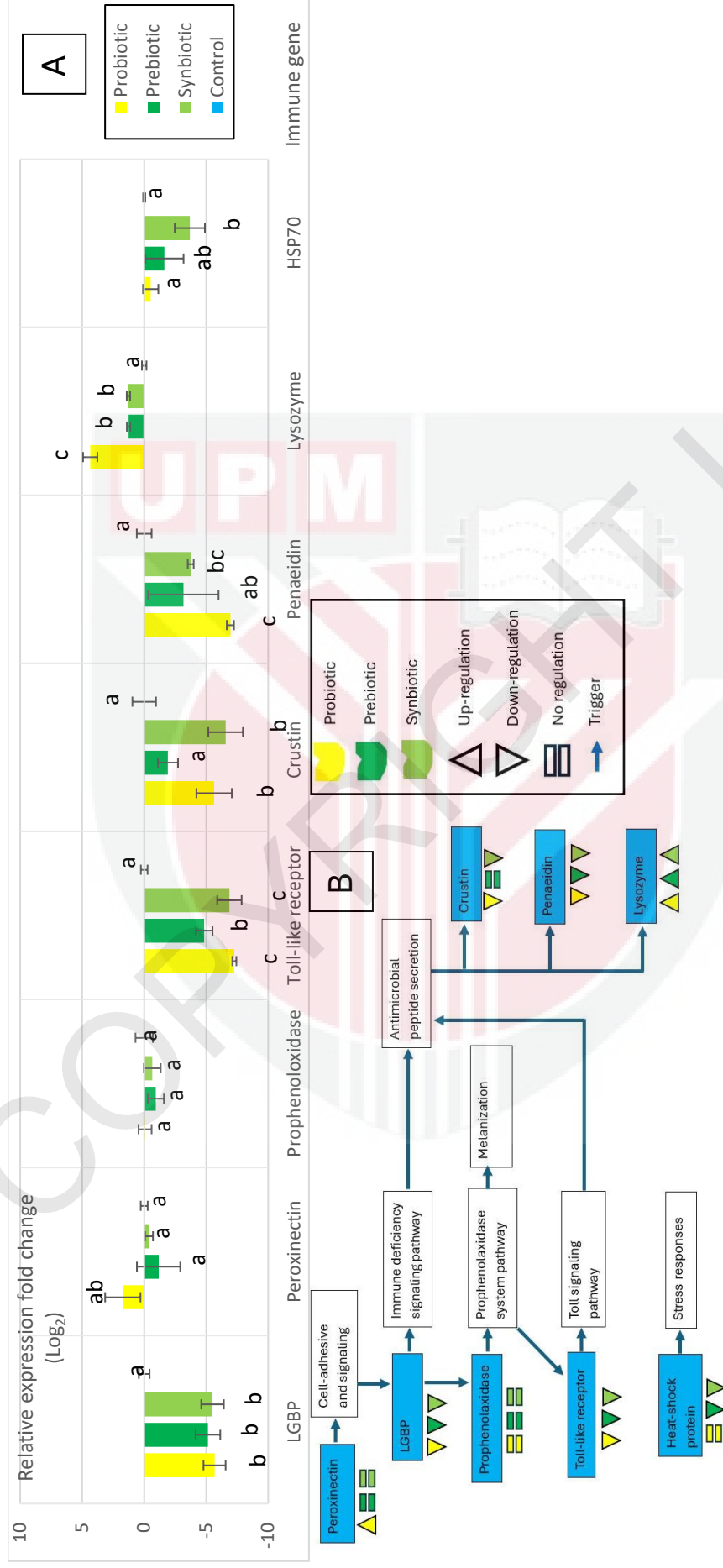


Figure 4.6: Immune gene expression and pathway in abdomen of *P. monodon* fed with different supplemented feed during at 35th day of feeding trial. (A) shows immune gene expression while (B) shows immune pathway. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. Prebiotic group that fed with 2% *S. polycystum* powder supplemented feed. Synbiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed.

4.3.2.2. Shrimp immune responses in 60 h of post-immersion challenge

In general, Probiotic, Prebiotic and Synbiotic groups show significantly ($p < 0.05$) inhibition of low cell-adhesive activity and immune deficiency signaling pathway compared with control group for whole shrimp (Figure 4.8B & Figure 4.9B). However, the Probiotic and Synbiotic groups significantly ($p < 0.05$) expressed prophenoloxidase system pathway for melanization, subsequently significant ($p < 0.05$) triggered expression of toll signalling pathway to secrete penaeidin as antimicrobial peptide in as compared with control group for shrimp cephalothorax (Figure 4.8B). Though, the Probiotic, Prebiotic and Synbiotic groups significantly ($p < 0.05$) expressed prophenoloxidase system pathway for melanization, subsequently significant ($p < 0.05$) triggered expression of toll signaling pathway, but significantly ($p < 0.05$) inhibited any antimicrobial peptide secretion for shrimp abdomen (Figure 4.9B). The Probiotic, Prebiotic and Synbiotic groups significantly ($p < 0.05$) inhibited stress responses compared with control group for whole shrimp (Figure 4.8B & Figure 4.9B).

For comprehensive immune gene regulation of shrimp cephalothorax in the challenge test (Figure 4.8A), LGBP, peroxinectin and HSP70 were significantly down-regulated ($p < 0.05$), crustin was not significant difference ($p > 0.05$) but toll-like receptor and penaeidin were significantly up-regulated ($p < 0.05$) from the Probiotic, Prebiotic and Synbiotic group compared with control group. Prophenoloxidase and lysozyme were significantly up-regulated ($p < 0.05$) from Probiotic group but significantly down-regulated ($p < 0.05$) from Prebiotic group

compared with control group. Synbiotic group exhibited significantly up-regulated prophenoloxidase ($p<0.05$) but significantly down-regulated lysozyme ($p<0.05$) compared with control group.



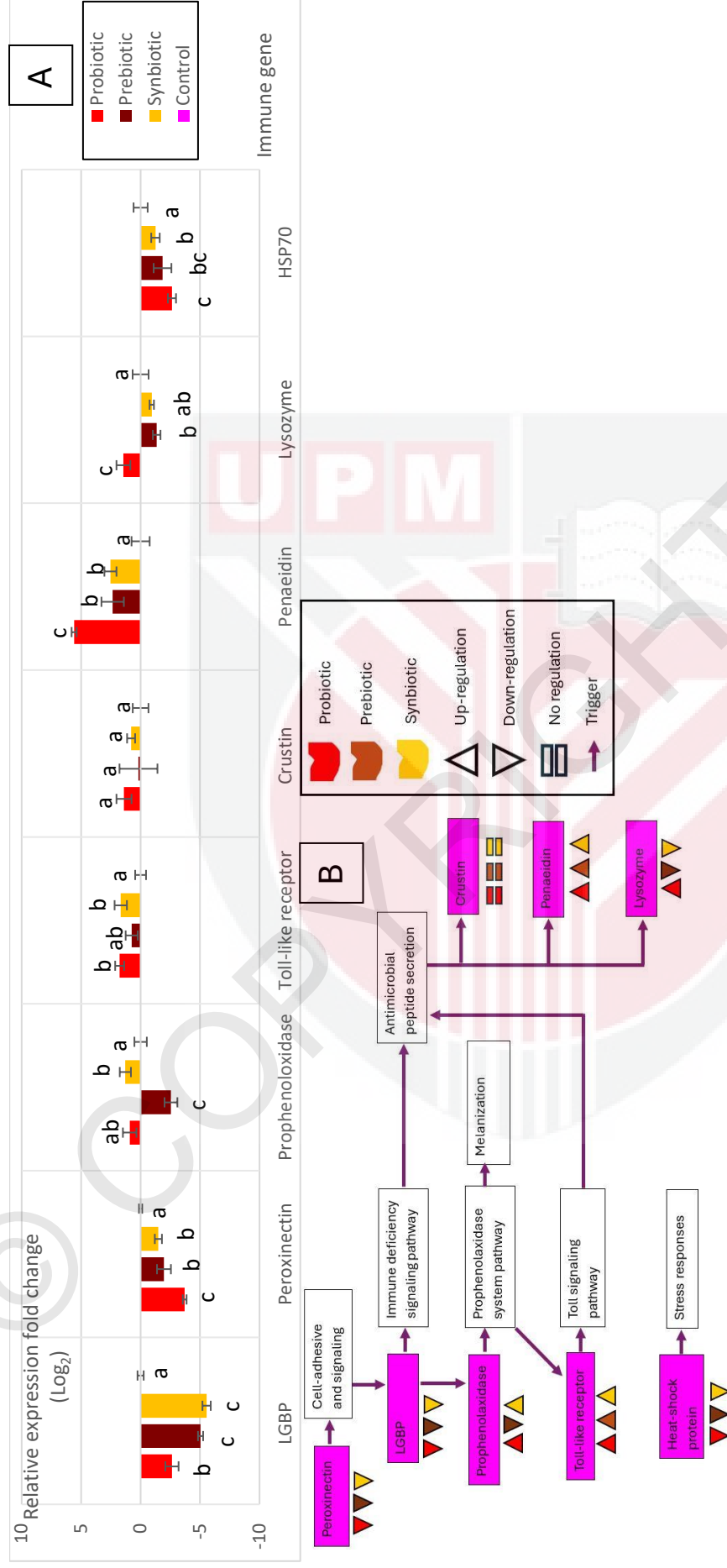


Figure 4.8: Immune gene expression and pathway in cephalothorax of *P. monodon* fed with different supplemented feed during at 60 h of *V. parahaemolyticus* S2-4 immersion challenge. (A) shows immune gene expression while (B) shows immune pathway. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. Prebiotic group that fed with 2% *S. polycystum* powder supplemented feed. showed Synbiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed.

For comprehensive immune gene regulation of shrimp abdomen in the challenge test (Figure 4.9A), LGBP and crustin were not significant difference ($p>0.05$), peroxinectin was significantly down-regulated ($p<0.05$) but toll-like receptor was significantly up-regulated ($p<0.05$) from Probiotic, Prebiotic and Synbiotic group compared with control group. Prophenoloxidase was significantly up-regulated ($p<0.05$) whereas penaeidin was significantly down-regulated ($p<0.05$) from Probiotic group and Synbiotic group but the Prebiotic group were not significantly difference ($p>0.05$) for the prophenoloxidase and significantly up-regulated for penaeidin compared with control group. Lysozyme and HSP70 were not significantly difference ($p>0.05$) from Probiotic group and Prebiotic but significantly down-regulated ($p<0.05$) from Synbiotic group.

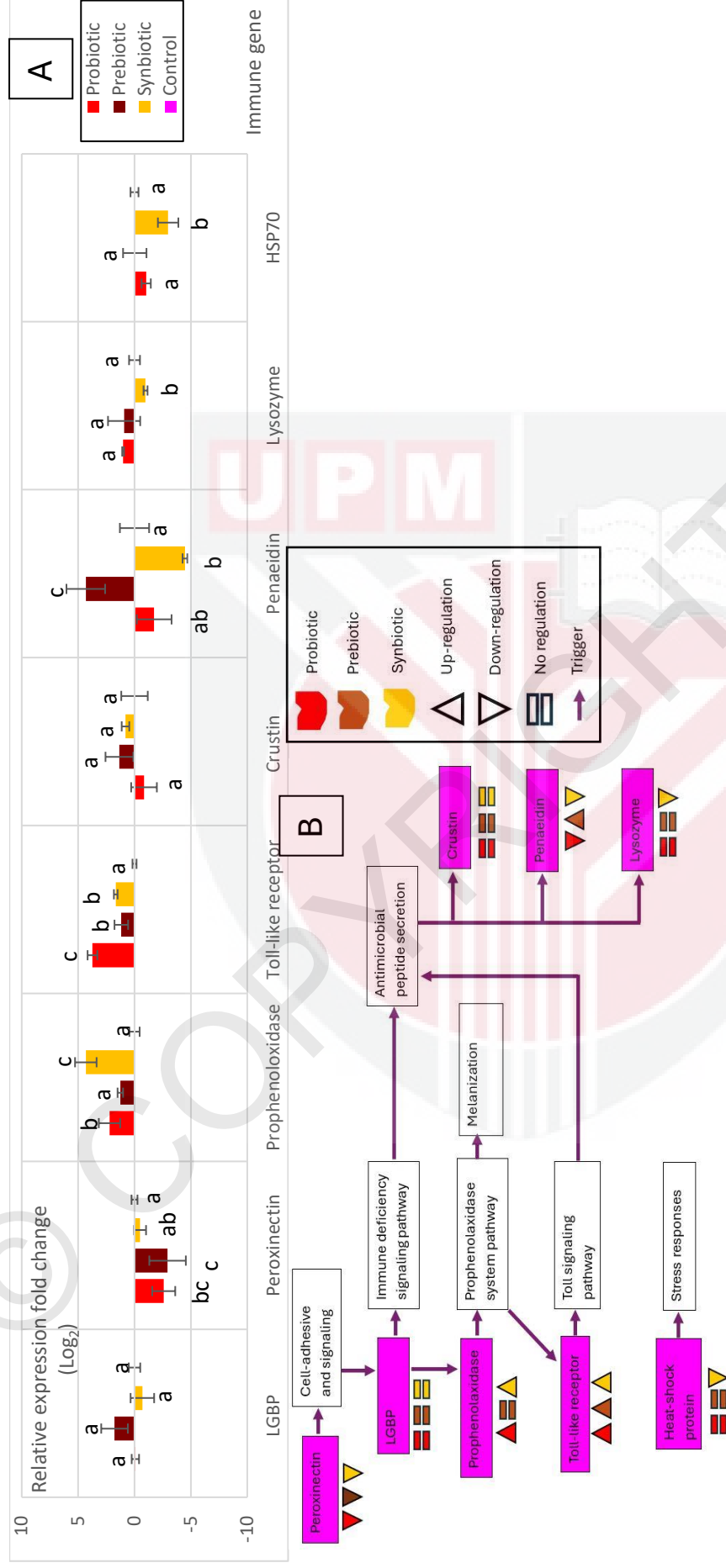


Figure 4.9: Immune gene expression and pathway in abdomen of *P. monodon* fed with different supplemented feed during at 60 h of *V. parahaemolyticus* S2-4 immersion challenge. (A) shows immune gene expression while (B) shows immune pathway. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. Prebiotic group that fed with 2% *S. polycystum* powder supplemented feed. showed Synbiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed.

4.3.3. Bacterial gene expression of probiotic and pathogen in *P. monodon*

4.3.3.1. Concentration of *L. plantarum* determination in *P. monodon*

The cycle threshold value (CT value) of the GHKL domain-containing protein gene indicated the concentration of *L. plantarum* by referencing the standard curve (Appendix 3A), revealed viable *L. plantarum* with specified GHKL domain containing protein gene in shrimps (Figure 4.10). On the 35th day of the feeding trial, shrimp from Probiotic, Prebiotic, and Synbiotic group exhibited the presence of *L. plantarum*, in contrast to the control group where *L. plantarum* was absent. Shrimp from Probiotic group and Synbiotic group exhibited significantly ($p < 0.05$) highest concentration of *L. plantarum* in the shrimp cephalothorax which $2.41 \pm 0.32 \log_{10}$ CFU/mL and $2.63 \pm 0.54 \log_{10}$ CFU/mL respectively (Figure 4.10A), as well as highest concentration of *L. plantarum* which $3.55 \pm 0.34 \log_{10}$ CFU/mL and $3.68 \pm 0.19 \log_{10}$ CFU/mL in the shrimp abdomen of Probiotic group and Synbiotic group respectively compared to other groups (Figure 4.10A).

Similarly, the presence of *L. plantarum* in shrimp from Probiotic, Prebiotic and Synbiotic group, in contrast with control group in 60 h post-immersion challenge with 6×10^5 CFU/mL of *V. parahaemolyticus* S2-4. The concentration of *L. plantarum* was $3.18 \pm 0.27 \log_{10}$ CFU/mL in the shrimp cephalothorax from Prebiotic group which was significantly ($p > 0.05$) highest compared with control group (Figure 4.10B). However, *L. plantarum* concentration were $3.61 \pm 0.29 \log_{10}$ CFU/mL, $3.35 \pm 0.44 \log_{10}$ CFU/mL, $3.82 \pm 0.51 \log_{10}$ CFU/mL in the abdomen of shrimp from Probiotic, Prebiotic

and Synbiotic group respectively which not significantly different ($p>0.05$) with each other (Figure 4.10B).

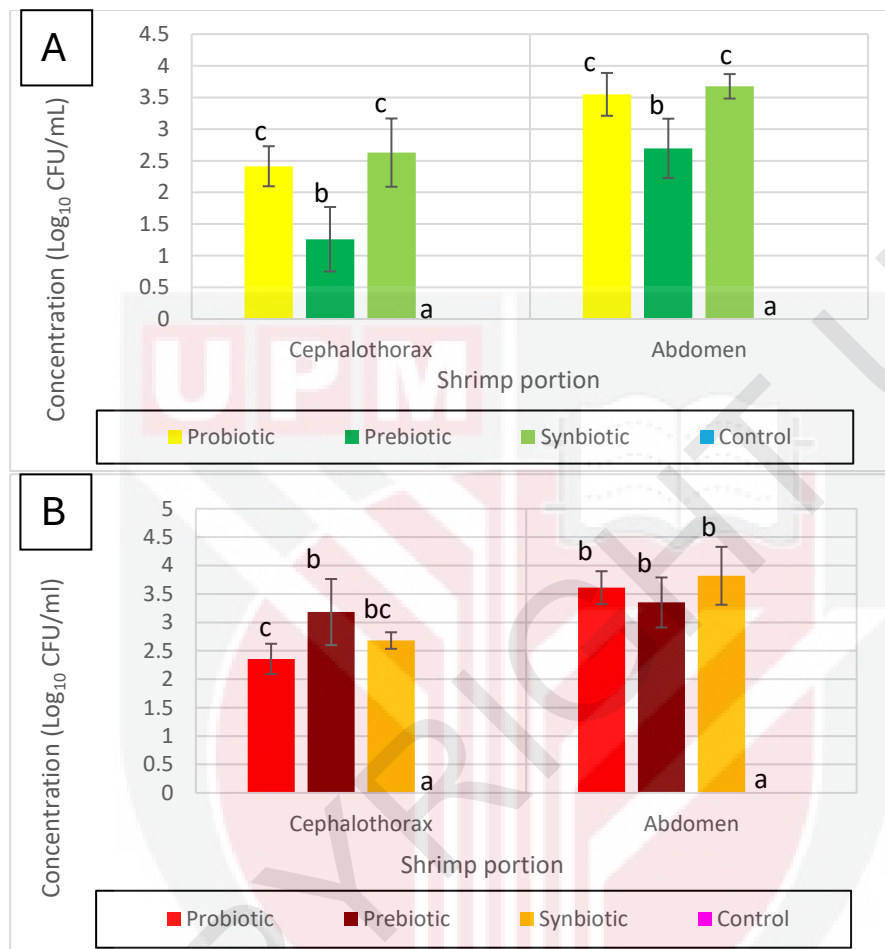


Figure 4.10: Comparative GHKL domain containing protein gene expression in cephalothorax and abdomen of *P. monodon* fed with different supplemented feed. (A) is 35th day of the feeding trial while (B) is challenge test is 60 h of 6×10^5 CFU/mL of *V. parahaemolyticus* S2-4 immersion challenge. No GHKL domain-containing protein gene was detected in cephalothorax and abdomen of control group. Different superscript letter above bar indicates significant differences ($p<0.05$). Probiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. Prebiotic group that fed with 2% *S. polycystum* powder supplemented feed. showed Synbiotic group that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed.

4.3.3.2. Concentration of AHPND-causing *V. parahaemolyticus* in *P. monodon*

The CT value of *pirA* toxin gene indicated concentration of AHPND-causing *V. parahaemolyticus* by referring the standard curve (Appendix 3B), revealed viable AHPND-causing *V. parahaemolyticus* including strain S2-4 in shrimp (Figure 4.11). No concentration of AHPND-causing *V. parahaemolyticus* was determined since no *pirA* gene was detected in cephalothorax and abdomen of shrimp from all groups in 35th day of feeding trial. In the immersion challenge, the concentration of AHPND-causing *V. parahaemolyticus* which 6.41 ± 0.29 Log₁₀ CFU/mL and 4.94 ± 0.10 Log₁₀ CFU/mL were exhibited significantly ($p < 0.05$) highest in cephalothorax and abdomen respectively of shrimp from control groups compared with other groups (Figure 4.11). However, lowest concentration of AHPND-causing *V. parahaemolyticus* was exhibited significantly ($p < 0.05$) by shrimp from Probiotic and Synbiotic group which 4.24 ± 0.41 Log₁₀ CFU/mL and 4.50 ± 0.20 Log₁₀ CFU/mL respectively in cephalothorax, 3.71 ± 0.25 Log₁₀ CFU/mL and 4.13 ± 0.33 Log₁₀ CFU/mL respectively in abdomen (Figure 4.11).

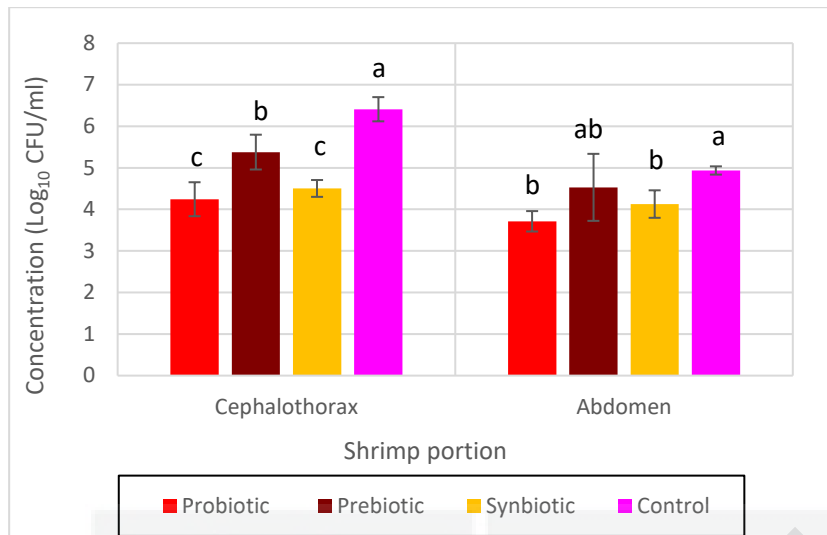


Figure 4.11: Comparative *pirA* toxin expression in cephalothorax and abdomen of *P. monodon* were fed with different supplemented feed in 60h of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 immersion challenge. Different superscript letter above bar indicates significant differences ($p < 0.05$). Probiotic group (PRO) that fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed. Prebiotic group (PRE) that fed with 2% *S. polycystum* powder supplemented feed. showed Synbiotic group (SYN) that fed with 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed.

4.3.4. Histological observation

4.3.4.1. Histopathological evaluation of hepatopancreas in *P. monodon*

The histopathological changes of hepatopancreas in the representative *P. monodon* by referring Sukor et al., (2016). For the feeding trial, the normal structures of different hepatocytes including, resorptive cell (R-cell), blister-like cell (B-cell), fibrillar cell (F-cell), hepatic lumen were shown in representative shrimp from the Probiotic group (Figure 4.12A), Prebiotic group (Figure 4.12B), Synbiotic group (Figure 4.12C) and control group (Figure 4.12D). Moreover, Probiotic group (Figure 4.12A), Prebiotic group (Figure 4.12B) and Synbiotic group (Figure 4.12C) exhibited hemocytes in the shrimp hepatopancreas. For the challenge test, B-cell, hepatic lumen, mild-vacuolated R-cell, mild-

vacuolated F-cell and hemocytes were showed in representative shrimp from the Probiotic group (Figure 4.12E) and Synbiotic group (Figure 4.12G). Hepatic lumen, vacuolated R-cell, vacuolated F-cell, reptured B-cell, and hemocytes were showed in representative shrimp from the Prebiotic group (Figure 4.12F). However, ruptured karyomegaly R-cell, ruptured karyomegaly F-cell, ruptured B-cell, sloughed hepatic lumen and hemocytes inflation were observed in control group (Figure 4.12H).



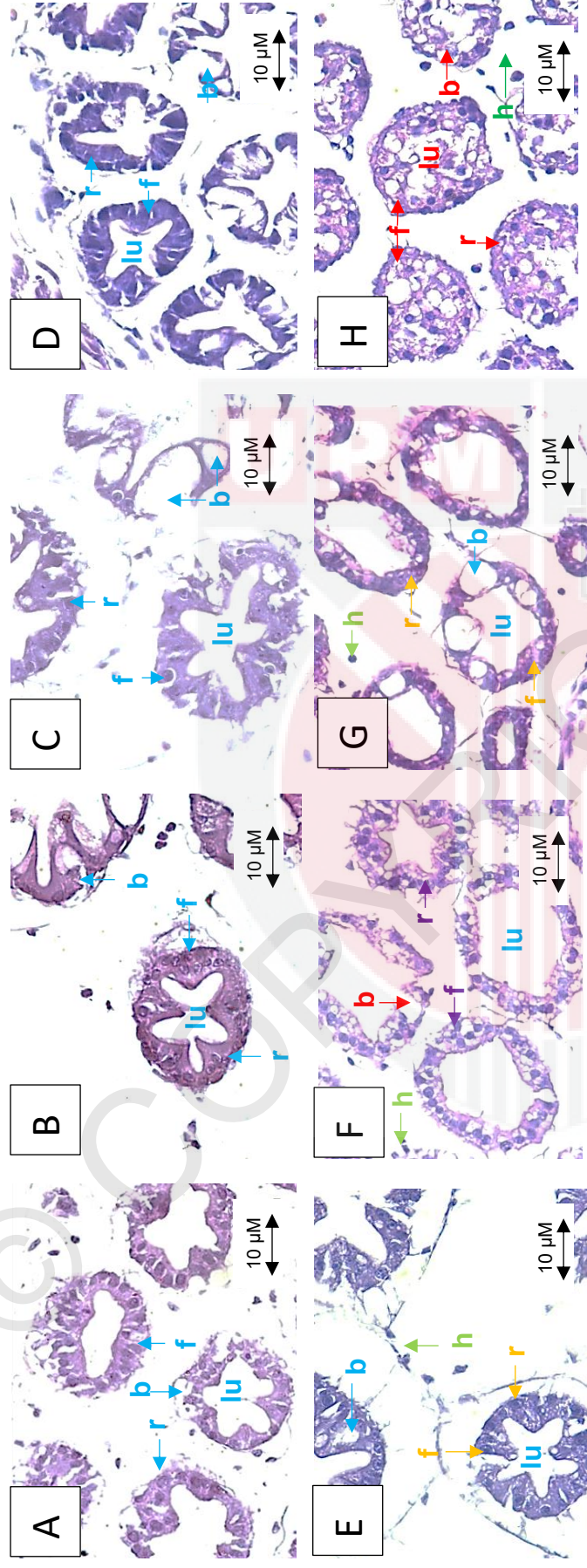


Figure 4.12: Histopathology for hepatopancreas in cephalothorax of *P. monodon* fed with different supplemented feeds. (A-D) are 35th day of feeding trial. (E-H) are 60 h of post-immersion challenge. (A) and ® are probiotic group which fed with 1 × 10⁸ CFU/g *L. plantarum* L20 supplemented feed; (B) and (F) are probiotic group which fed 2% *S. polycystum* powder supplemented feed; (C) and (G) are synbiotic group which fed 1 × 10⁸ CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed; (D) and (H) are control group which fed with control feed. (A-D) show resorptive cell (R-cell: **r**), blister-like cell (B-cell: **b**), fibrillar cell (F-cell: **f**), hepatic lumen (**lu**) (× 400). ® and (G) show blister-like cell (B-cell: **b**), hepatic lumen (**lu**), mild-vacuolated R-cell ®, mild-vacuolated F-cell (f) and hemocyte (**h**) (× 400). (F) shows hepatic lumen (**lu**), vacuolated R-cell ®, vacuolated F-cell (f), reaptured B-cell (**b**) and hemocyte (**h**) (× 400). (H) shows ruptured karyomegaly R-cell ®, ruptured karyomegaly F-cell (f), ruptured B-cell (**b**), sloughed hepatic lumen (**lu**) and hemocyte infiltration (**h**) (× 400).

4.3.4.2. Histopathological evaluation of intestine in *P. monodon*

The histopathological changes of intestine in the representative *P. monodon* by referring Duan et al., (2017). For the feeding trial, the normal structure of brush border, intestinal epithelium, intestinal lumen and hemocytes were shown in representative shrimp from Probiotic group (Figure 4.13A), Prebiotic group (Figure 4.13B), Synbiotic group (Figure 4.13C) and control group (Figure 4.13D). For the challenge test, brush border, mild-vacuolated intestinal epithelium, intestinal lumen and hemocytes infiltration showed in intestine of representative shrimp from Probiotic group (Figure 4.13E), Prebiotic group (Figure 4.13F) and Synbiotic group (Figure 4.13G). However, degenerated brush border, vacuolated intestinal epithelium, disintegrated intestinal epithelium and intestinal lumen were examined in control group (Figure 4.13H).

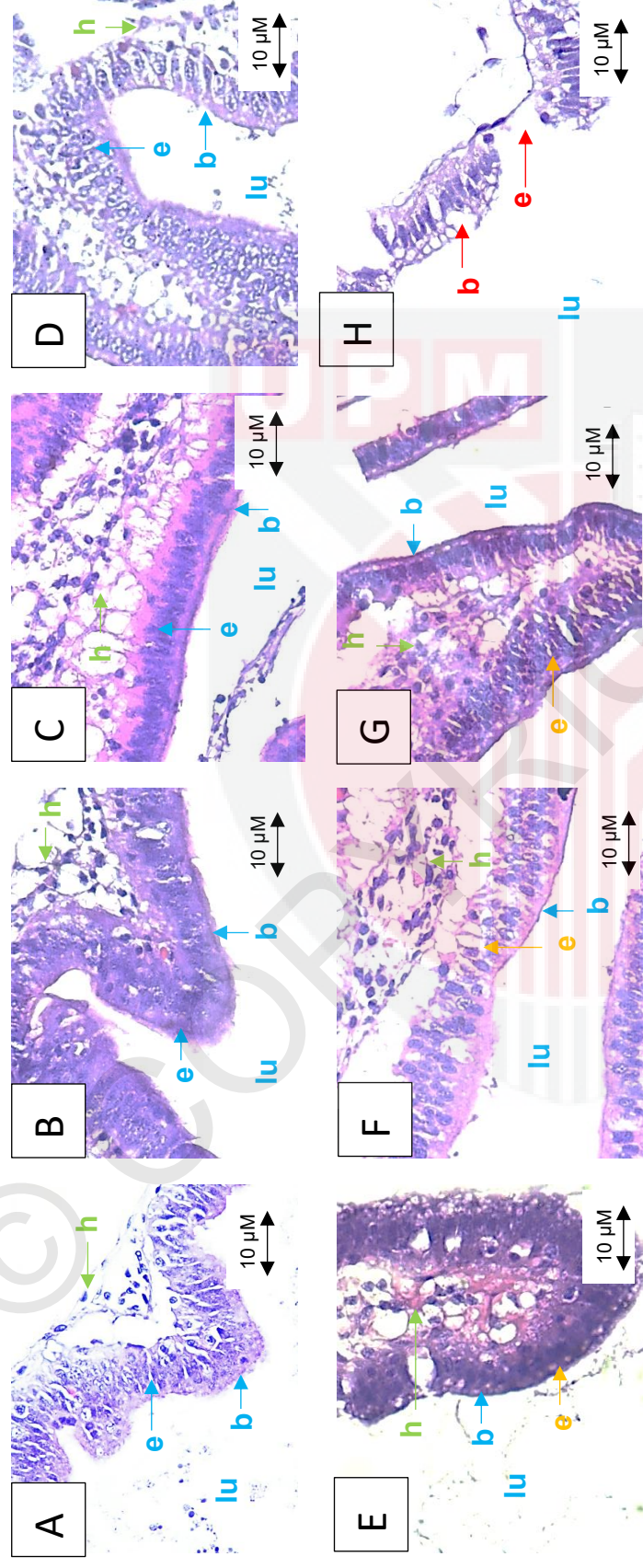


Figure 4.13: Histopathology for intestinal epithelium in abdomen of *P. monodon* fed with different supplemented feed. (A-D) are 35th day of feeding trial while (E-H) are 60 h of 6×10^5 CFU/mL AHPND-causing *V. parahaemolyticus* S2-4 immersion challenge. (A) and (E) are Probiotic group which fed with 1×10^8 CFU/g *L. plantarum* L20 supplemented feed; (B) and (F) are Prebiotic group which fed 2% *S. polycystum* powder supplemented feed; (C) and (G) are Synbiotic group which fed 1×10^8 CFU/g *L. plantarum* L20 and 2% *S. polycystum* powder supplemented feed; (D) and (H) are control group which fed with normal feed. (A-D) show brush border (b), mild-vacuolated intestinal epithelium (e), intestinal lumen (lu) and hemocytes (h) ($\times 400$). (E-G) show brush border (b), mild-vacuolated intestinal epithelium (e), intestinal lumen (lu) and submucosa hemocytes (h) ($\times 400$). (H) shows degenerated brush border (b), disintegrated and vacuolated intestinal epithelium (e) and intestinal lumen (lu) ($\times 400$).

Discussion

Probiotic, Prebiotic and Synbiotic groups demonstrated effectiveness of *L. plantarum* L20 and *S. polycystum* as Probiotic group and Prebiotic group for improving growth performance of shrimp in the feeding trial. Previous studies have reported many varieties of synbiotic feeds by using different types of extracted polysaccharides (galactooligosaccharide, mannan oligosaccharide, chitosan) and gut beneficial bacteria (*Lactobacillus* spp., *Bacillus* spp.) for improving penaeid shrimp's growth performance (Huynh et al. 2018; Chen et al. 2020; Kewcharoen et al. 2022). However, *S. polycystum* were recommended as prebiotic because of its higher nutritional contents (minerals and vitamins) and cheapest market price (USD 0.50 to 1.08 per kg in year 2021) in Malaysia (Shannon and Abu-Ghannam 2019; Asri et al. 2021). Moreover, the dry powder form of *S. polycystum* (Patent No: PI2013702168) consists of several fermentable dietary carbohydrate components, including polysaccharides, including alginate, fucoidan, fucose, with minor quantities of other monosaccharides, especially xylose, galactose, mannose and rhamnose, which have prebiotic properties (Nazarudin et al., 2021). These fermentable carbohydrates of *Sargassum* spp. can potentially modulate innate immunity and improve host gastrointestinal microbiota to confer health for the host (Abdel-Rahim et al. 2021). Interestingly, the highest SGR, PER and best FCR of shrimp from Synbiotic group indicated synergistic effect of combined *L. plantarum* L20 and *S. polycystum*. In the AHPND-causing *V. parahaemolyticus* S2-4 immersion challenge, highest survival of shrimp from Probiotic group and Synbiotic group were aligned with similar study, synergistic combination of *Bacillus* sp. B2 and red seaweed, *Gracilaria* spp. supplemented diet reduced

the mortality of Pacific white shrimp, *P. vannamei* post-challenge with the AHPND-causing *V. parahaemolyticus* strain (Lim et al. 2020).

The immune responses of *P. monodon* were concerned. In the feeding trial, the shrimp cephalothorax of Probiotic group and Synbiotic group expressed consequential cellular innate immune responses and exhibited constant hepatopancreas structure and hemocyte histologically. This is due to peptidoglycan of the Probiotic supplement's bacterial cell wall was possibly recognized by shrimp hemocytes via LGBP (Tseng et al., 2023). LGBP activated peroxinectin and prophenoloxidase system cascade in shrimp hemocyte (Lin et al. 2015). Activated peroxinectin enhanced hemocyte degranulation and facilitated hemocyte phagocytosis (Cai et al., 2020). Activated prophenoloxidase in prophenoloxidase system cascade of hemocytes triggered the production of the melanin and toxic reactive intermediates as immune defends (Amparyup et al., 2013). This is due to the Prebiotic supplement's fucoidan possibly induced also prophenoloxidase system cascade without recognized by LGBP of shrimp from Prebiotic group (Sivagnanavelmurugan et al., 2014). The series of cellular innate immunity were suppressed in shrimp abdomen from Probiotic, Prebiotic and Synbiotic groups. Moreover, humoral innate immune responses were suppressed by hemocyte in the shrimp cephalothorax and abdomen from Probiotic, Prebiotic and Synbiotic groups. We postulated components of Probiotic, Prebiotic and Synbiotic supplements were not activated pro-Spätzle that crucial for toll-like signaling pathways in the shrimp (Liu et al., 2022). However, immune-activated hemocyte associated releasing of lysozyme despite its mechanism behind still

unknown (Morales-Covarrubias et al., 2023). HSP70 induction could indicated oxidative stress response which related with high reactive oxygen species (ROS) level from respiratory burst upon overwhelming of shrimp hemocyte phagocytosis activities (Bautista-Covarrubias et al., 2020; Janewanthanakul et al., 2020). Therefore, absence of oxidative stress was indicated by suppression of HSP70 in shrimp cephalothorax and abdomen from Probiotic, Prebiotic and Synbiotic groups. In the immersion challenge, Suppression of LGBP and peroxinectin in shrimp cephalothorax and abdomen from Probiotic, Prebiotic and Synbiotic groups upon *V. parahaemolyticus* S2-4 immersion challenge. Subsequently, prophenoloxidase and toll-like receptor were highly expressed in shrimp abdomen and cephalothorax from Probiotic group and Synbiotic group except Prebiotic group which expressed toll-like receptor only. This is due to C-type lectins (CTLs) was highly expressed instead of LGBP in shrimp. Evidence shows CTLs recognized *V. parahemolyticus* lipopolysaccharide to evoke subsequential of shrimp innate immune responses and had strong growth inhibition against *V. parahaemolyticus* (Huang et al., 2022). However, the reason of prophenoloxidase suppression in Prebiotic group is yet to investigated. The Probiotic, Prebiotic and Synbiotic supplements boosted expression of penaeidin as effective antimicrobial peptide against *V. parahaemolyticus* S2-4. Evidence shows prophenoloxidase cascade and toll-like pathways were highly expressed in shrimp for melanisation and antimicrobial peptide production respectively during *Vibrio* spp. AHPND infection (Boonchuen et al. 2020). The penaeidins penetrated superficial cell membrane to bind bacterial DNA, thereby eliminate *V. parahaemolyticus* (Xiao et al. 2021). However, suppression of penaeidin was

determined in shrimp abdomen from the Probiotic group and Synbiotic group. We postulated Anti-lipopolysaccharide factors were possible expressed against *V. parahaemolyticus* S2-4 and broad spectrum of opportunistic Gram-negative bacteria to prevent co-infection or secondary infection in shrimp intestine (Supungul et al., 2015).

The interaction of *L. plantarum* in shrimp was concerned undoubtedly during the feeding trial. A notably high abundance of *L. plantarum* was observed in shrimp from Probiotic group and Synbiotic group, indicating effective colonization of administrated *L. plantarum* within the shrimp gastrointestinal tract (Zheng et al. 2018). By referring the result of Probiotic group and Synbiotic group, we inferred *L. plantarum* L20 has been stimulate shrimp hepatopancreas to enhance digestive enzymes secretion (protease, lipase, amylase), thus improved food digestion and nutrient absorption in shrimp intestine (Wang et al., 2020). Similarly, the presence of *L. plantarum* was detected in shrimp from Prebiotic group, inferring that the dietary *S. polyscytum* improved the proliferation of indigenous *Lactobacillus* spp. in the gastrointestinal tract of the host (Nazarudin et al. 2020). The finding supports the idea that the polysaccharides present in the Prebiotic supplement (fructooligosaccharide, galactooligosaccharide, mannanoligosaccharide) positively impacted shrimp gastrointestinal microbiota, promoting the production of short-chain fatty acids utilized by the shrimp intestine for improved nutrient absorption (Nazarudin et al., 2021; Zhou et al., 2023). In synergistic effect of Synbiotic supplement, we inferred that Probiotic component-inducing shrimp protease hydrolysed protein of Prebiotic

component to release additional peptides and free essential amino acids, that could be readily absorbed by the shrimp intestine (Xie et al. 2012; Lee and Lee 2018; Raveschot et al. 2018). The interaction of *L. plantarum* and AHPND-causing *V. parahaemolyticus* were concerned in shrimp during the immersion challenge. High abundance of *L. plantarum* were found in shrimp from Probiotic, Prebiotic and Synbiotic groups compared with control group. We inferred *L. plantarum* L20 which enhanced indigenous *Lactobacillus* spp. to produces various antimicrobial compounds, including organic acids (especially lactic acid and acetic acid), hydrogen peroxide and antimicrobial peptides (Hu et al. 2019). *Sargassum* spp. have antibacterial agents (n-hexane, ethyl acetate, ethanol, alkaloids, flavonoids) inhibited *Vibrio* sp. (Ahmed et al. 2022). Since the deadly *pirAB* toxin was released by *V. parahaemolyticus* via quorum sensing when its population reaches 10^6 CFU/ml (Hong et al. 2015), the decreasing *V. parahaemolyticus* S2-4 in survival shrimp of Probiotic, Prebiotic and Synbiotic groups demonstrated the *V. parahaemolyticus* S2-4 was eliminated by the antimicrobial peptides and phenol oxidase enzymes of the stimulated humoral innate immune system (Kumar et al. 2022). A study described cell wall lipoteichoic acid (LTA) of the *L. plantarum* prevented adhesion and internalization of *Vibrio* spp. on host intestinal epithelial cells (Gao et al. 2016).

Structure changes of shrimp internal organs were crucial for investigation. In the feeding trial, shrimp cephalothorax of Probiotic, Prebiotic and Synbiotic groups exhibited histologically normal structure of hepatic and intestinal epithelial cells. We postulated that Probiotic, Prebiotic and Synbiotic

supplemented feeds improved structural integrity, mucus components and immune molecules of shrimp hepatopancreas and intestine (Duan et al., 2019). It exhibited histologically presence and increasing hemocytes in the shrimp hepatopancreas and intestine. This could be components of the Probiotic, Prebiotic and Synbiotic had immunostimulant properties to increase circulating hemocytes for enhancing shrimp innate immunity (Saptiani et al., 2020). In the immersion challenge, the AHPND-affected shrimp are divided into three phases: initial phase (tissue deterioration), acute phase (tissue necrosis) and terminal phase (tissue sloughing, hemocyte infiltration) (Kumar et al., 2021). Obviously, AHPND initial stage was determined in the Probiotic and Synbiotic group; acute stage was determined in the Prebiotic group; and terminal stage was determined in the control group. Administration of Probiotic, Prebiotic and Synbiotic supplements delayed the progressive infection of AHPND (Pinoargote et al. 2018). Histological vacuolation with tissue necrosis of intestinal epithelium of the shrimp samples from control group inferred the lipopolysaccharide of *V. parahaemolyticus* can damage the intestine mucosal barrier by decreasing the viability of epithelial cells, disrupting tight junctions, and disordering the stability of mucosal mucins of shrimp gastrointestinal tract (Duan et al. 2018). Interestingly, intact histologically structure of intestinal epithelium from Prebiotic group and Synbiotic group demonstrated that *S. polycystum* feed administration strengthens the structure integrity of the intestinal epithelium (Abdel-Rahim et al. 2021).

4.3. Conclusion

Overall, the synbiotic incorporating *L. plantarum* L20 and *S. polycystum* show better effectiveness which improved the growth performance, innate immunity and survival of *P. monodon* compared probiotic and prebiotic. In perspective, this synbiotic supplemented feed can be used potentially against AHPND in aquaculture practices for preventing income loss and generating additional income.

**This chapter was published and entitled as “The synergistic *Lactobacillus plantarum* L20 and *Sargassum polycystum* -added diet for improvement of Black tiger shrimp, *Penaeus monodon* ‘s growth, immune responses, bacterial profiles, and resistance against *Vibrio parahaemolyticus* associated Acute hepatopancreatic necrosis disease (AHPND) infection” in *Aquaculture reports* at year 2024 with DOI: 10.1016/j.aqrep.2023.101903.

CHAPTER 5

MODULATORY EFFECT OF ORAL ADMINISTRATED SYNBIOTIC (*Lactobacillus plantarum* L20 AND *Sargassum polycystum* POWDER) SUPPLEMENTED FEED ON MICROBIOTA AND STRUCTURE OF THE GASTROINTESTINAL TRACT IN BLACK TIGER SHRIMP (*Penaeus monodon*) AGAINST DYSBIOSIS

5.1. Introduction

Penaeus monodon is suffered intestinal dysbiosis, which as microbiota imbalance and lost their ability to restore balance of intestinal microbiota upon vibriosis including AHPND (Rungrassamee et al., 2016). Moreover, the indiscriminate use of antibiotic for controlling shrimp diseases can cause the emergence of antibiotic-resistant bacteria strains and shrimp gut dysbiosis, which further exacerbates the shrimp health issue (Sharma et al., 2021). Notably, the Chapter 3 demonstrated probiotic *L. plantarum* L20 exhibited robust inhibitory activity against *Vibrio* spp. and enhanced the survival of *P. monodon* during AHPND-causing *V. parahaemolyticus* infection. Moreover, Chapter 4 revealed the synbiotic diet was developed by comprising probiotic *L. plantarum* L20 and prebiotic *S. polycystum* powder which was incorporated into shrimp feeds to harness the synergistic benefits, including improved growth performance and resistance against AHPND-causing *V. parahaemolyticus* infection for *P. monodon*.

However, the effectiveness of the synbiotic against dysbiosis, an alteration of shrimp gastrointestinal microbiota caused by AHPND, remains unexplored (Holt et al., 2021). Notably, the whole community of gastrointestinal microbiota composition plays crucial roles to regulate nutrient metabolism, gastrointestinal

mucosal barrier integrity maintenance, immunomodulation, and pathogen protection for host (Jandhyala et al. 2015).

While the application of high throughput amplicon sequencing analysis has unveiled the abundance and potential metabolic functions of gastrointestinal microbiota in the *P. vannamei* and *P. monodon* (Gao et al., 2019; Kawasaki et al., 2022), no study was performed on the high throughput 16S rRNA amplicon sequencing analysis of the microbiota within the gastrointestinal tract of *P. monodon* fed with the synbiotic (combination of *L. plantarum* L20 and *S. polycystum*) and infected with Malaysia prevalent AHPND-causing *V. parahaemolyticus* S2-4. Thus, the synergistic effect of *L. plantarum* and *S. polycystum* for modulating shrimp gastrointestinal microbiota composition, network correlation, potential metabolic pathways and gut histology remains undocumented. Therefore, this study aims to investigate the dynamic modulation of the gastrointestinal microbiota abundance, community structure and their potential metabolic functions in *P. monodon* fed with the synbiotic supplemented diet, focusing on its protective efficacy against *V. parahaemolyticus* S2-4 infection via sequencing. Additionally, histological analysis of the shrimp gastrointestinal tract (hepatopancreas, stomach/foregut, midgut, hindgut) was conducted to elucidate gastrointestinal changes upon feeding with the synbiotic diet and challenge with *V. parahaemolyticus* S2-4. It was hypothesized that the synbiotic diet have the potential to reduce AHPND infection of shrimp, guarantee environmental and food safety by preventing zoonotic infection of foodborne with waterborne *V. parahaemolyticus* for

consumers and farmers (Yen et al., 2021). Besides, the conducted experimental flow charts of Chapter 5 are shown in Figure 5.1. The study aims of Chapter 5 are shown below.

1. To elucidate the gastrointestinal microbiota modification of synbiotics (*L. plantarum* L20 and *S. polycystum*) supplemented feed on the microbiota community, potential metabolic function and AHPND-associated dysbiosis resistance of *P. monodon*.
2. To evaluate the gastrointestinal structure protection of synbiotics (*L. plantarum* L20 and *S. polycystum*) supplemented feed on the gastrointestinal tissue steadiness upon AHPND-causing *V. parahaemolyticus* S2-4 infection of *P. monodon*.

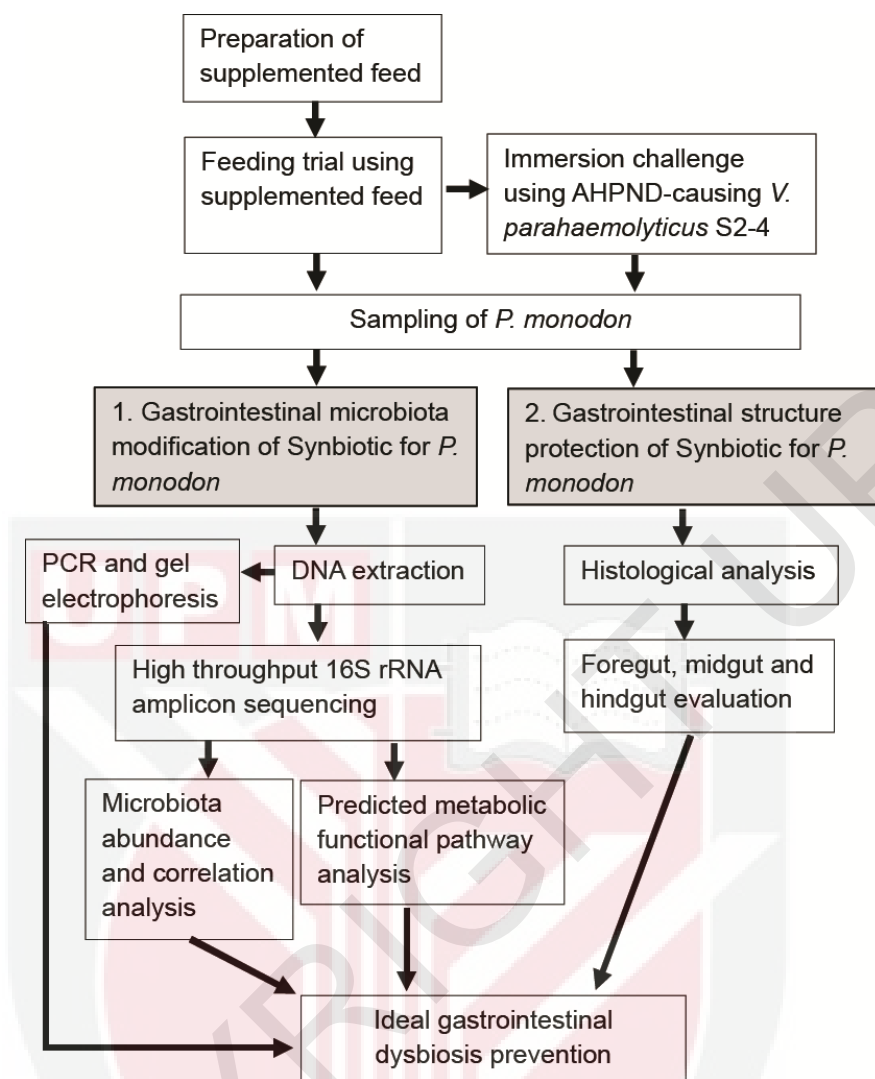


Figure 5.1: Flow charts of conducted experiments in Chapter 5

5.2. Material and Method

5.2.1. Effectiveness assessment of Synbiotic

5.2.1.1. Bacterial culture

Lactobacillus plantarum L20 and AHPND-causing *V. parahaemolyticus* S2-4 were cultured according to Chapter 4. Then, the bacterial suspension of the two bacteria were prepared as Chapter 4.

5.2.1.2. Experimental diet preparation

Two experimental diets, including control diet and synbiotic diet containing 10^8 CFU/g of *L. plantarum* L20 and 2% *S. polycystum* powder were prepared according to previous study (Chapter 4). The two experimental diets were individually preserved in transparent sterile plastic bags and kept at 4 °C before use.

5.2.1.3. Shrimp culture and feeding trial

The acclimatization of shrimp prior feeding trial was repeated as Chapter 4. The *P. monodon* postlarvae (PL15, n = 300) were purchased from a commercial hatchery in Kuantan, Pahang, Malaysia, and transported to the Institute of Bioscience, Universiti Putra Malaysia, Serdang, Malaysia. The feeding trial was adapted also from previous study (Chapter 4). The shrimps (n = 300) were distributed into two groups, which included Synbiotic group and control group only as shown in Table 5.1. On the 35th day, the two groups (Synbiotic group, control group) were sampled for DNA extraction and histological examination. Nine live shrimps were sampled (3 pooled replicates) from each group for DNA extraction. Three live shrimp were sampled from each group for histological examination (Figure 5.2). The remaining shrimps were kept for use in the immersion challenge test.

Table 5.1: Experimental design of feeding trial

Group	Supplemented feed	Feeding trial		Immersion challenge	
		Number of shrimps per tank	Replicate tank	Number of shrimps per tank	Replicate tank
Synbiotic	10 ⁸ CFU/mL of <i>L. plantarum</i> L20 & 2% (w/w) <i>S. polycystum</i> powder with PBS water	50	3	20	3
Control	PBS water	50	3	20	3

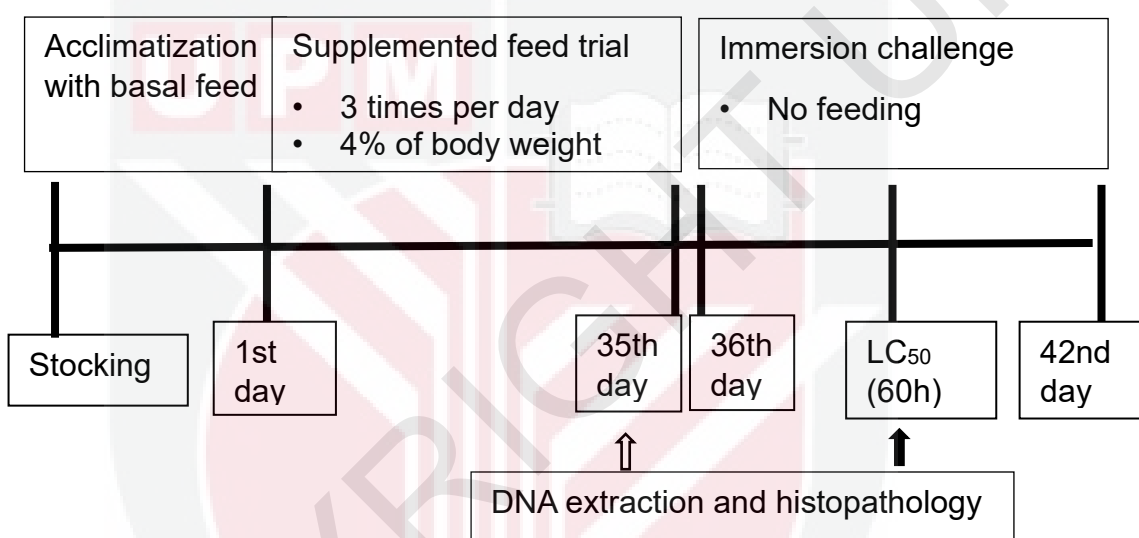


Figure 5.2: Experimental timeline of feeding trial regime and immersion challenge. Growth performance was recorded during the experiment. Survival, clinical observation, DNA extraction and histopathology assessments were performed at end of feeding trial (35th day) and immersion challenge test with LC₅₀-60h (3rd day) of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4.

5.2.1.4. *V. parahaemolyticus* S2-4 immersion challenge

The immersion challenge was adapted to previous study (Chapter 4). The shrimps (n = 60) from each group at 35th day feeding trial were collected randomly and transferred into 3 aquarium tanks as replicates as shown in Table 5.1. After a day of acclimatization (36th day), all shrimp in the tanks were challenged with LC₅₀-60 h concentration of 6×10^5 CFU/mL *V.*

parahaemolyticus S2–4 as similar with Chapter 4. The clinical signs (hepatopancreas paleness and gut transparency) of the 2 groups (3 replicates per group) were observed for 7 days (Zhang et al., 2023). At 60 h of post challenge, the two *V. parahaemolyticus* S2–4 challenged (VP-) groups (VP-Synbiotic group, VP-control group) were sampled for DNA extraction and histological examination. Nine shrimps were sampled (3 pooled replicates) from each group for DNA extraction while three shrimp were sampled from each group for histological examination.

5.2.2. Elucidation of the gastrointestinal microbiota modification

5.2.2.1. DNA extraction

Nine live shrimp were obtained from each group of the feeding trial (Synbiotic group, control group) and post challenge (VP-Synbiotic group, VP-control group). All shrimps in each group were preserved in Invitrogen™ RNAlater (Thermo Fisher Scientific, Massachusetts, USA) after anesthesia using 300 ppm of tricaine methanesulphonate, MS-222 (Sigma-Aldrich, Missouri, USA). Subsequently, the shells with flesh (muscle) of all shrimps were carefully removed using dissecting sterile scalpel and forceps immediately. Then, the entire gastrointestinal tract (Hepatopancreas, foregut, midgut, hindgut) was recognized according to Hembrom et al., (2024) and dissected out. Three shrimp gastrointestinal tracts were assembled as one replicate. Three replicates were obtained from the groups from the feeding trial (Synbiotic group, control group) and post challenge (VP-Synbiotic group, VP-control group). The entire gastrointestinal tracts of the shrimp were ground into a fine powder using

liquid nitrogen, allowing for efficient DNA extraction. Genomic DNA was extracted from the powdered samples using the FastDNA™ Spin Soil Kit (MP Biomedicals, California, USA) following the manufacturer's instructions. The quality of the purified DNA was initially assessed by electrophoresis on a 1.5 % TAE agarose gel. The DNA concentration was also determined through spectrophotometry using the NanoPhotometer® N60/N50 (Implen, Munich, Germany) and fluorometric quantification, employing the iQuant™ Broad Range dsDNA Quantification Kit (GeneCopoeia, Rockville, USA). The extracted DNA was subjected to PCR amplification and high throughput 16S rRNA amplicon sequencing.

5.2.2.2. Bacteria determination using PCR

Prior to PCR amplification, the bacterial DNA of the *L. plantarum* L20 and *V. parahaemolyticus* S2-4 were prepared as positive control (ve+). For visualizing *L. plantarum*, specific conserved protein-encoding gene, GHKL domain containing protein gene was used as indicator in Table 5.3 because they can distinguish closely related species and strains of *Lactobacillus* sp. strains (Kim et al. 2020). For visualizing AHPND-causing *V. parahaemolyticus*, Photorhabdus insect related (*pir*) toxin A (*pirA*) and toxin B (*pirB*) gene were used as indicator in Table 5.3 because they are crucial toxin genes of AHPND-causing *V. parahaemolyticus* (Han et al., 2020). The extracted DNA was subjected to PCR amplification by using specific primers as Table 5.2. The PCR mixture consisted of 100 ng of 2.5 µL template DNA, 6× of 12.5 µL PCR Master Mix (First Base Laboratories, Sri Kembangan, Malaysia), and 1 µL of 20 pmol of each primer. The total volume was brought up to 25 µL with sterile

DEPC-treated water. No DNA template was prepared as negative control (ve-). The samples were run by using Eppendorf Mastercycler Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) for PCR amplification. The amplification was performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 10 min. The PCR products were visualized using a 1.5 % TAE agarose gel electrophoresis with a 100 bp-DNA ladder (Thermo Fisher scientific, Waltham, USA).

Table 5.2: The primer sequences that used for PCR amplification

Targeted gene	Primers	Sequences (5' to 3')	Size (bp)	Reference
GHKL domain-containing protein	Forward	GCGGTATCGATTTCGATTGGT	145	Kim et al. (2020)
	Reverse	TGATGTCAATCGCCTCTTGG		
<i>PirA</i> toxin	Forward	TGACTATTCTCACGATTGGACTG	284	Han et al. (2020)
	Reverse	CACGACTAGCGCCATTGTTA		
<i>PirB</i> toxin	Forward	TGATGAAGTGATGGGTGCTC	392	Han et al. (2020)
	Reverse	TGTAAGCGCCGTTTAACTCA		

5.2.2.3. Amplicon library preparation

Prior to high throughput 16S rRNA amplicon sequencing, amplicon library preparation was conducted in strict adherence to the “16S-metagenomic sequencing library preparation guide” (Illumina, California, USA). The gene-specific primers' sequences of 16S rRNA fraction containing the V3-V4 region used in this protocol were taken from Klindworth et al. (2013). The gene-specific sequence primer pair included Forward Primer (5'-CCTACGGGNGGCWGCAG-3') and Reverse Primer (5'-

GACTACHVGGGTATCTAATCC-3'). Illumina overhang adapter nucleotide sequences were added to the gene-specific primer sequences respectively. The overhang adapter pair were Forward overhang adapter (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') and Reverse overhang adapter (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'). The DNA extracted from the shrimp samples underwent the first amplification step. These steps were carried out in 50 µL reactions, employing KOD -Multi & Epi-® (Toyobo, Osaka, Japan), with the following thermal cycling conditions: pre-denaturation at 94°C for 2 min, 30 cycles comprising denaturation at 98°C for 10 s, annealing at 55°C for 10 s, and extension at 68°C for 30 s, followed by a final extension at 68°C for 5 min, and a holding step at 4°C. The resulting amplicons were subjected to a cleanup procedure using magnetic AMPure XP beads (Beckman Coulter, California, USA) to remove free primers and primer dimer species following the manufacturer's specifications. Subsequently, a second amplification step was performed to introduce dual indices and sequencing adapters, employing the Nextera XT Index Kit v2 (Illumina, California, USA) per the manufacturer's protocols. A second round of cleanup using AMPure XP beads (Beckman Coulter, California, USA) was conducted for library purification. To assess the quality of the libraries, measurements were conducted using the Agilent Bioanalyzer 2100 System with the Agilent DNA 1000 Kit (Agilent Technologies, California, USA). Additionally, quantification was performed fluorometrically using the Helixyte Green Quantifying Reagent (AAT Bioquest, California, USA).

5.2.2.4. High throughput 16S rRNA amplicon sequencing

Initially, each library was adjusted to a concentration of 4 nM, utilizing 10 mM Tris (pH 8.5) as the diluent. Subsequently, these libraries were pooled in the same proportion. To facilitate denaturation, libraries were treated with 0.2N NaOH. Additionally, a standard PhiX Control Library (Illumina, California, USA) underwent denaturation and was employed as an internal control. The denatured library and the PhiX control were adjusted to a concentration of 8 pM and then mixed, with the final mixture comprising 95% library and 5 % PhiX control. The mixture was then heated at 96°C for 2 min and immediately cooled on ice for 5 min. The prepared libraries were loaded into a MiSeq v3 Reagent Tray (Illumina, California, USA), which housed a MiSeq v3 Flow Cell (Illumina, California, USA), and the sequencing results were obtained after 300 cycles. The sequences were deposited with Bioproject number: PRJNA1023040 in the NCBI database.

5.2.2.5. Microbial taxonomy, diversity, and functional prediction analysis

The quality assessment of the raw reads was conducted using FastQC (Andrews 2010), followed by the removal of primers and adaptors using Cutadapt 3.5 (Martin 2011). Paired-end reads were processed and merged utilizing DADA2 V1.18 (Callahan et al. 2016), chimera screening and taxonomy assignment were performed using the SILVA ribosomal RNA database (Quast et al., 2013) Version 138.1 (updated March 10, 2021). The ASVs with taxonomic assignments were exported as ASV and taxonomic classification

count tables for subsequent analyses. The statistical meta-analyses by their analytical platforms were performed subsequently as shown in Table 5.3.

Table 5.3: The list of statistical meta-analyses

Platform	Data analysis	Statistical method	References
ggVennDiagram	Venn diagram clustering	Jaccard similarity	Gao et al., (2021)
MicrobiomeAnalyst 2.0	Taxonomic profiling	Taxonomic bar plot	Lü et al., (2023)
	Dendrogram clustering	Unweighted UniFrac distance	
	Alpha diversity	Richness indices (ACE, Chao1)	
		Diversity indices (Shannon, Simpson)	
	Beta diversity	Permutational multivariate analysis of variance (PERMANOVA)	Weiss et al., (2016)
		Principal coordination analysis (PCoA) using Bray-cutis index	
	Relative abundance of microbial family	Linear discriminant analysis effect size (LEfSe)	
	Network correlations of microbiota community	Pearson correlation coefficient	
PICRUST2	Relative abundance of predicted metabolic pathways	Linear discriminant analysis effect size (LEfSe)	Douglas et al., (2020)

5.2.3. General statistical analysis

The significant difference of the growth performance parameters, the proximate analysed value, and the shrimp survival rate were calculated with Student T-test using IBM SPSS Statistics version 22.0. The significant difference of Alpha diversities value was calculated with one-way ANOVA using IBM SPSS Statistics version 22.0.

5.2.4. Evaluation of the gastrointestinal structure protection

5.2.4.1. Histological sample preparation

Three live shrimp were sampled from each group of the feeding trial (Synbiotic group, control group) and post-challenge (VP-Synbiotic group, VP-control group). The shrimps were first anesthetized with 300 ppm MS-222 (Sigma-Aldrich, Massachusetts, USA). The shells with flesh (muscle) of all shrimps were carefully removed using dissecting sterile scalpel and forceps. The entire gastrointestinal tract (Hepatopancreas, foregut, midgut, hindgut) of shrimp were recognized according to Hembrom et al., (2024) and dissected out (Figure 2). Then, the entire gastrointestinal tracts were fixed in 10% neutral buffered formalin for 24 h. Subsequently, the samples were transferred to 70% ethanol and subjected to a dehydration process involving a series of increasing ethanol concentrations (ranging from 50% to 100%). Following dehydration, the tissue samples were oriented with longitudinally cut section to accurately assess alterations in the overall structure of gastrointestinal tract tissue (Erben et al., 2014). The samples were embedded in paraffin wax and cut into 4 μ m thickness. These sections were subsequently stained with haematoxylin and eosin (Robert et al., 2014).

5.2.4.2. Histopathological observation

Microscopic examination and microphotography were conducted along the gastrointestinal tract (foregut, midgut, hindgut) at magnifications of 200 $\times$ using the Olympus BX51 digital light microscope (Olympus, Tokyo, Japan). Firstly, the digestive gland (hepatopancreas) of anterior midgut which originally from

shrimp cephalothorax were examined microscopically (Figure 5.3) to evaluate AHPND infectious stage. Then, the proventriculus (stomach) of foregut which originally from shrimp cephalothorax were examined microscopically (Figure 5.3); The middle of midgut which originally from the 2nd - 4th segment of shrimp abdomen were examined microscopically (Figure 5.3); The posterior caecum of anterior hindgut which originally from the 6th segment of shrimp abdomen were examined microscopically (Figure 5.3). These examinations were used to evaluate effect of AHPND related dysbiosis on the gastrointestinal tract structure.

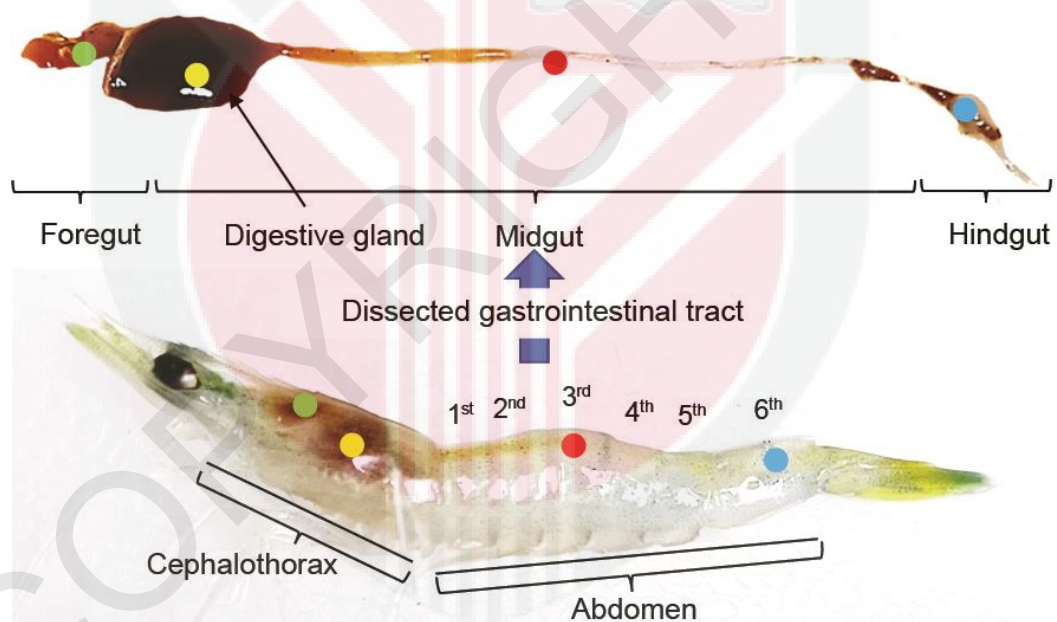


Figure 5.3: Recognition of dissected fresh gastrointestinal tract's portions originally from *P. monodon* body. The same colour of dots pinpoint portions of gastrointestinal tract which originally from respected shrimp portions according to according to Hembrom et al., (2024). The foregut is originally from shrimp cephalothorax. The digestive gland (hepatopancreas) of anterior midgut is originally from shrimp cephalothorax. The middle portion of midgut is originally from the 2nd – 4th segments of shrimp abdomen. The hindgut is originally from the 6th segment of shrimp abdomen.

Ethical statement

All procedures in this study involving animals were approved by the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee, approval number: UPM/IACUC/AUP-R061/2022 (Appendix 5).

5.3. Result

5.3.1. Gastrointestinal microbiota modification in *P. monodon*

5.3.1.1. Validation of *L. plantarum* L20 and AHPND-causing *V. parahaemolyticus*

The PCR was performed on the extracted DNA samples from feeding trial and challenge test. GHKL domain-containing protein were detected in all samples of Synbiotic group and VP-Synbiotic group. However, no GHKL domain-containing protein were observed in all samples of control group and VP-control group (Figure 5.5). This confirmed the presence of *L. plantarum* L20 in shrimp of Synbiotic group and VP-Synbiotic group from feeding trial and challenge test (Figure 5.4).

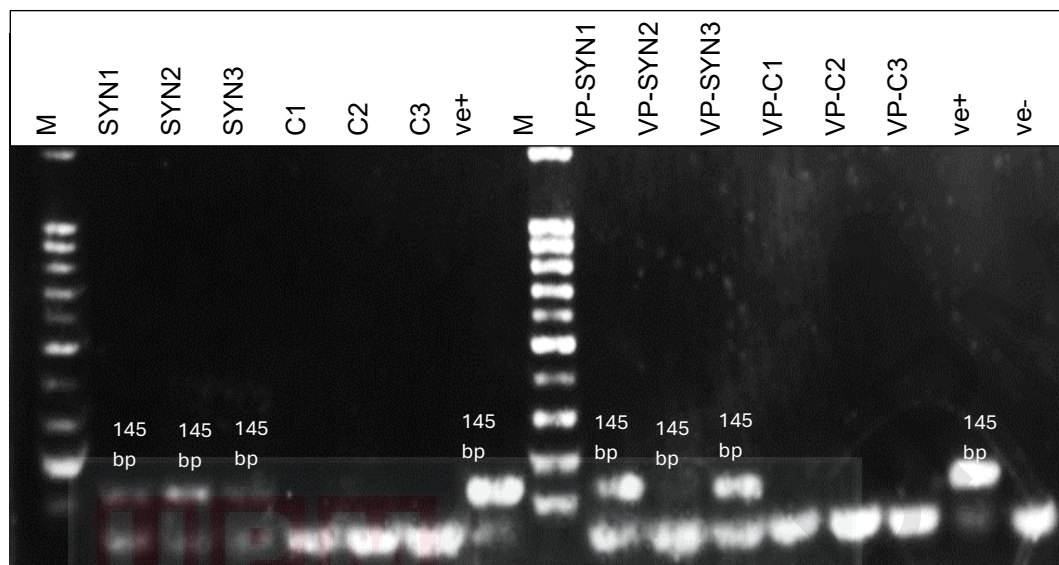


Figure 5.4: The presence of *L. plantarum* L20 was visualized using gel electrophoresis with GHKL domain-containing protein genes respectively after PCR amplification. Figure 5.5 shows Lane M (100-bp DNA ladder), SYN1 - SYN3 (Synbiotic group from 35th day feeding trial), C1 - C3 (control group from 35th day feeding trial), ve+ (Bacterial PCR amplicon as positive control), VP-SYN1 – VP-SYN3 (Synbiotic group from 60 h post challenge), VP-C1 – VP-C3 (control group from 60 h post challenge), ve- (No template as negative control).

No *pirA* and *pirB* toxins were detected in all samples of Synbiotic group and control group from the feeding trial. However, during the challenge test, low intensity *pirA* and *pirB* toxins were observed for VP-SYN1 and VP-SYN2, with only *pirA* toxins detected for VP-SYN2 from VP-Synbiotic group (Figure 5.5). High intensity of *pirA* and *pirB* toxins were observed from VP-control group (Figure 5.5). This could indicate presence of AHPND-causing *V. parahaemolyticus* S2-4 in shrimp of VP-control group but minimal presence of the *V. parahaemolyticus* S2-4 in shrimp of VP-Synbiotic group from challenge test (Figure 5.5).

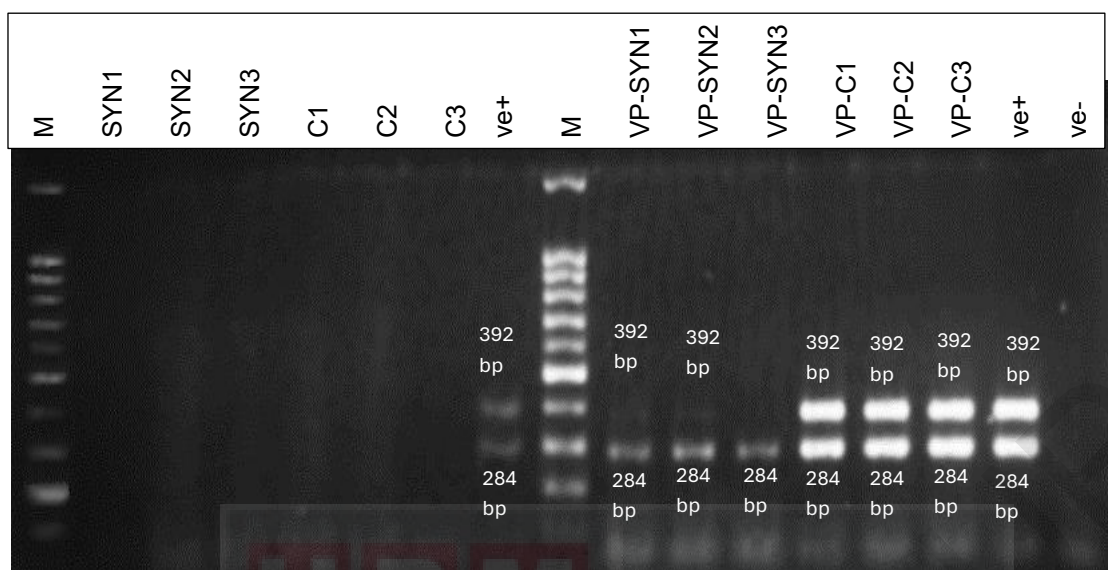


Figure 5.5: The presence of AHPND-causing *V. parahaemolyticus* S2-4 was visualized using gel electrophoresis with *pirAB* toxins genes respectively after PCR amplification. Figure 5.6 shows Lane M (100-bp DNA ladder, Thermo Fisher scientific, Waltham, USA), SYN1 - SYN3 (Synbiotic group from 35th day feeding trial), C1 - C3 (control group from 35th day feeding trial), ve+ (Bacterial PCR amplicon as positive control), VP-SYN1 – VP-SYN3 (Synbiotic group from 60 h post challenge), VP-C1 – VP-C3 (control group from 60 h post challenge), ve- (No template as negative control).

5.3.1.2. Alpha diversity indices and Venn diagrams

In comparing groups, the Chao1, ACE, Shannon and Simpson indices of Synbiotic group did not significant difference ($p>0.05$) compared to the control group during the feeding trial. However, during the challenge test, while the Chao1 and ACE indices of VP-Synbiotic group displayed slightly significant difference ($p<0.05$) compared to the VP-control group (Figure 5.6A & 5.6B), the Shannon and Simpson indices of VP-Synbiotic group exhibited significant difference ($p<0.05$) compared to the VP-control group (Figure 5.6C & 5.6D). Additionally, when comparing the feeding trial with the post challenge trial, the Synbiotic group showed slightly significant differences ($p<0.05$) with VP-Synbiotic group for Chao1 and ACE indices (Figure 5.6A & 5.6B). However,

control group show significant difference ($p<0.05$) compared to the VP-control group for all indices (Figure 5.6). In this context, the richness and diversity of VP-Synbiotic group's gut microbiota were higher than those of the VP-control group, but not significantly different from Synbiotic group. In contrast, the richness and diversity of VP-control group's gut microbiota was lower than the control group.

In the Venn diagrams in Figure 5.7, the highest Jaccard similarity index percentage of ASVs (about 26 %) are shown by pair of 35th day Synbiotic group with 35th day control group (Figure 5.7A) and pair of 35th day Synbiotic group with 60 h VP-Synbiotic group (Figure 5.7C). The pair of 60 h VP-Synbiotic group with 60 h VP-control group (Figure 5.7B) and pair of 35th day control group with 60 h VP-Synbiotic group (Figure 5.7F) shows Jaccard similarity index percentage of ASVs (about 19 %). The lowest Jaccard similarity index percentage of ASVs (about 9 %) are shown by both pair of 35th day control group with 60 h VP-control group (Figure 5.7D) and pair of 35th day Synbiotic group with 60 h VP-control group (Figure 5.7E). In some way, the results of Venn diagram were aligned with the Alpha diversity results.

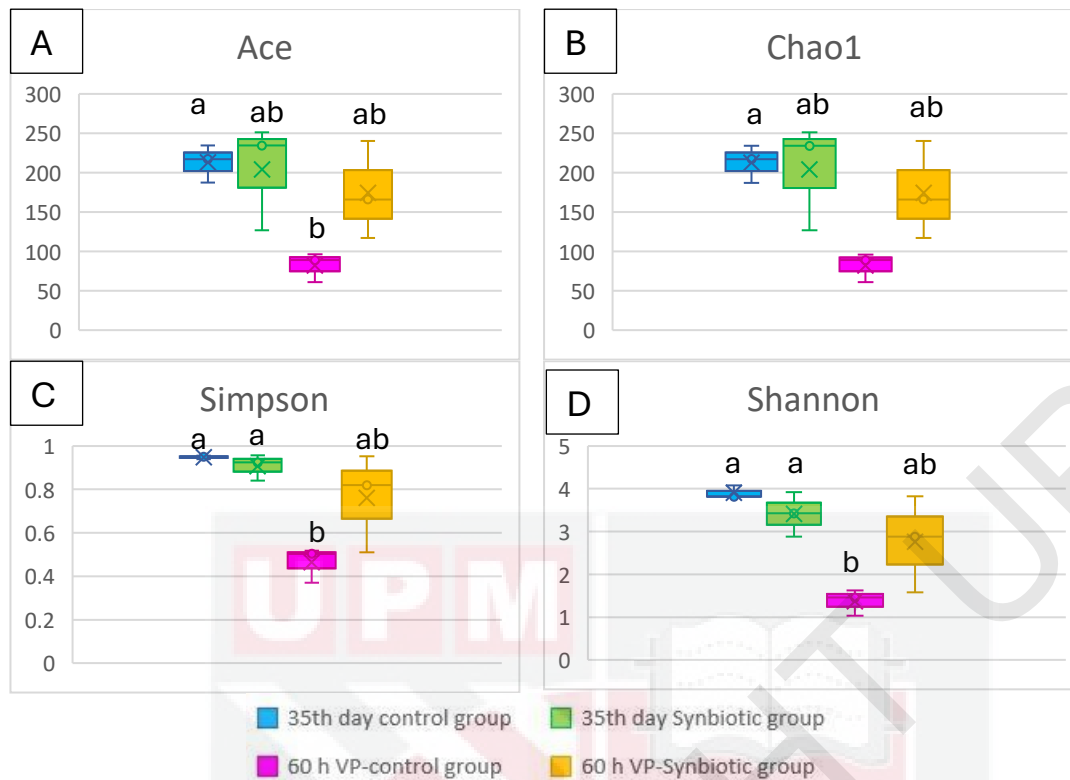


Figure 5.6: Alpha diversity metrics of bacterial community in *P. monodon*'s gastrointestinal tracts. The significant difference of the Alpha diversity indices' value was calculated with Student T-test. (A), (B), (C) and (D) indicates ACE, Chao1, Shannon and Simpson indices respectively. Different superscript letter above the boxplot indicates significant different (p<0.05) between groups.

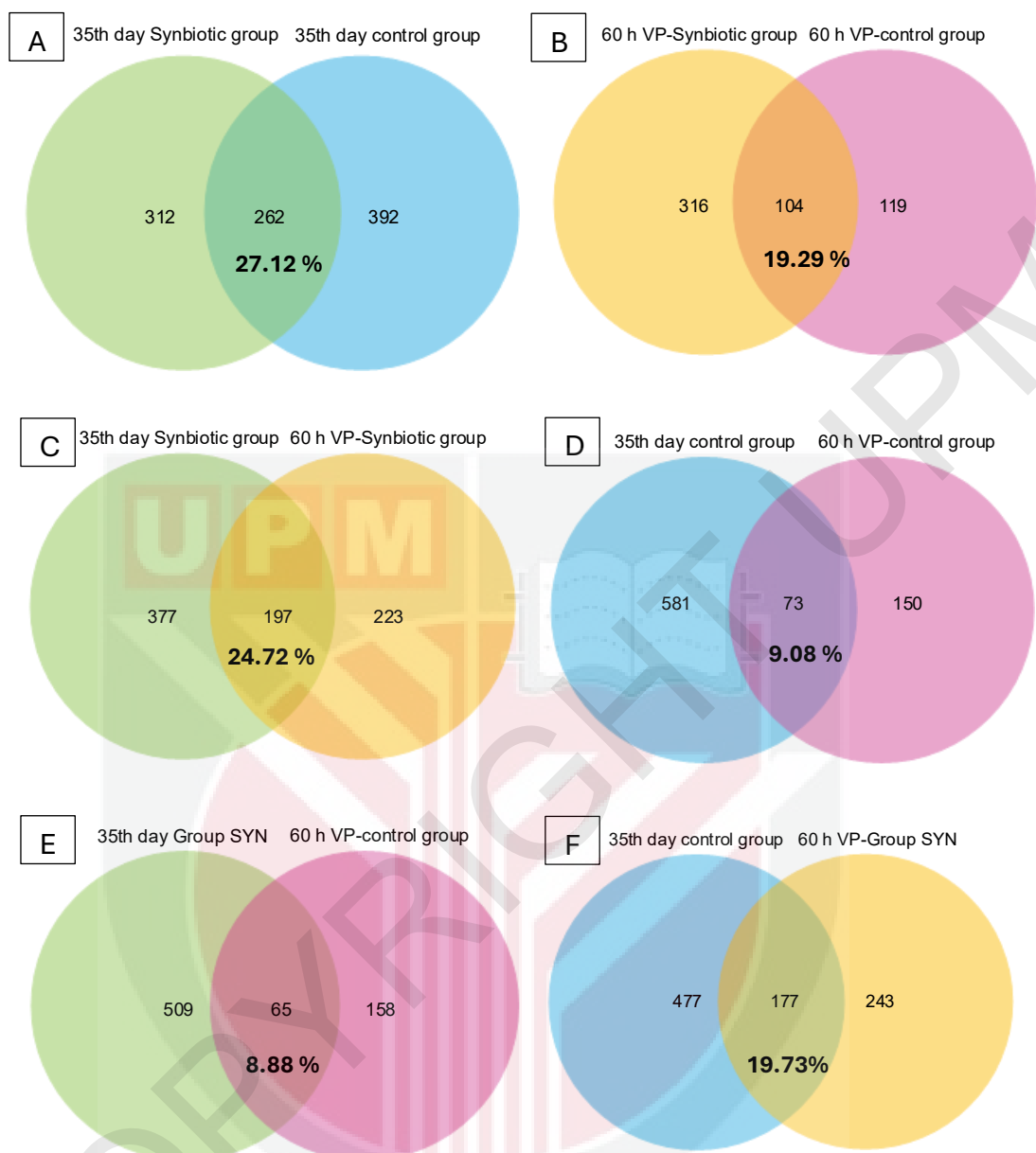


Figure 5.7: Venn diagram of shared and unique ASVs. The Jaccard similarity index percentage was shown between two groups of each pair. “Synbiotic group” and “control group” from the feeding trial. “VP-Synbiotic group” and “VP-control group” from the post-challenge.

5.3.1.3. Beta diversity outcomes and dendrogram

However, there were no significant differences ($p > 0.05$) observed in all pairwise comparisons of the groups by PERMANOVA (Table 5.4). These results indicate that shrimp gastrointestinal microbiota dispersion or variability

of the groups was not significant different ($p>0.05$) with each other. Otherwise, the PERMANOVA result (R^2 : 0.400, p : 0.008) demonstrates a statistically significant difference ($p<0.05$) for 35th day feeding trial groups (35th day Synbiotic group & 35th day control group) with VP-challenged groups (60 h VP-Synbiotic group & 60 h VP-control group) (Table 5.4). Conversely, there was no significant difference for Synbiotic supplemented diet-fed groups (35th day Synbiotic group & 60 h VP-Synbiotic group) with control diet-fed groups (35th day control group & 60 h VP-control group) (Table 5.4). Therefore, the results exhibit the shrimp gastrointestinal microbiota was highly influenced by environmental factors.

Principal Coordinate Analysis (PCoA) plots reveal substantial variations among samples of the shrimp gastrointestinal microbiota using the Bray-Curtis Index. The cluster of the 35th day Synbiotic group notably overlapped with the 35th day control group (Figure 5.8). The cluster of 60 h VP-Synbiotic group overlapped with 35th day Synbiotic group and 60 h VP-control group (Figure 5.8). The cluster of the control group was notably separated from the 60 h VP-control group's cluster (Figure 5.8). The dendrogram of Unweighted UniFrac analysis shows 35th day Synbiotic group was closely related with 35th day control group and 60 h VP-Synbiotic group (Figure 5.9). 60 h VP-control group was dispersed with other groups (Figure 5.9).

Table 5.4: PERMANOVA analysis with Bray-Curtis index of the different diet fed *P. monodon*'s gastrointestinal microbiota upon feeding trial and *V. parahaemolyticus* immersion challenge

Comparison		PERMANOVA	
Group	Counterpart group	Statistic values	Significant difference
35th day Synbiotic group	35th day control group	F: 0.219, R ² : 1.174	<i>p</i> -value: 0.227 (<i>p</i> > 0.05)
60 h VP-Synbiotic group	60 h VP-control group	F: 0.356, R ² : 3.34	<i>p</i> -value: 0.455 (<i>p</i> > 0.05)
35th day Synbiotic group	60 h VP-Synbiotic group	F: 0.387, R ² : 1.805	<i>p</i> -value: 0.311 (<i>p</i> > 0.05)
35th day control group	60 h VP-control group	F: 1.082, R ² : 13.8	<i>p</i> -value: 0.775 (<i>p</i> > 0.05)
35th day feeding trial groups (35th day Synbiotic group & 35th control group)	60 h VP-challenged groups (60 h VP-Synbiotic group & 60 h VP-control group)	F: 6.663, R ² : 0.400	<i>p</i> -value: 0.008 (<i>p</i> < 0.05)
SYN diet-fed groups (35th day Synbiotic group & 60 h VP-Synbiotic group)	Control diet-fed groups (35th day control group & 60 h VP-control group)	F: 1.211, R ² : 0.108	<i>p</i> -value: 0.278 (<i>p</i> > 0.05)

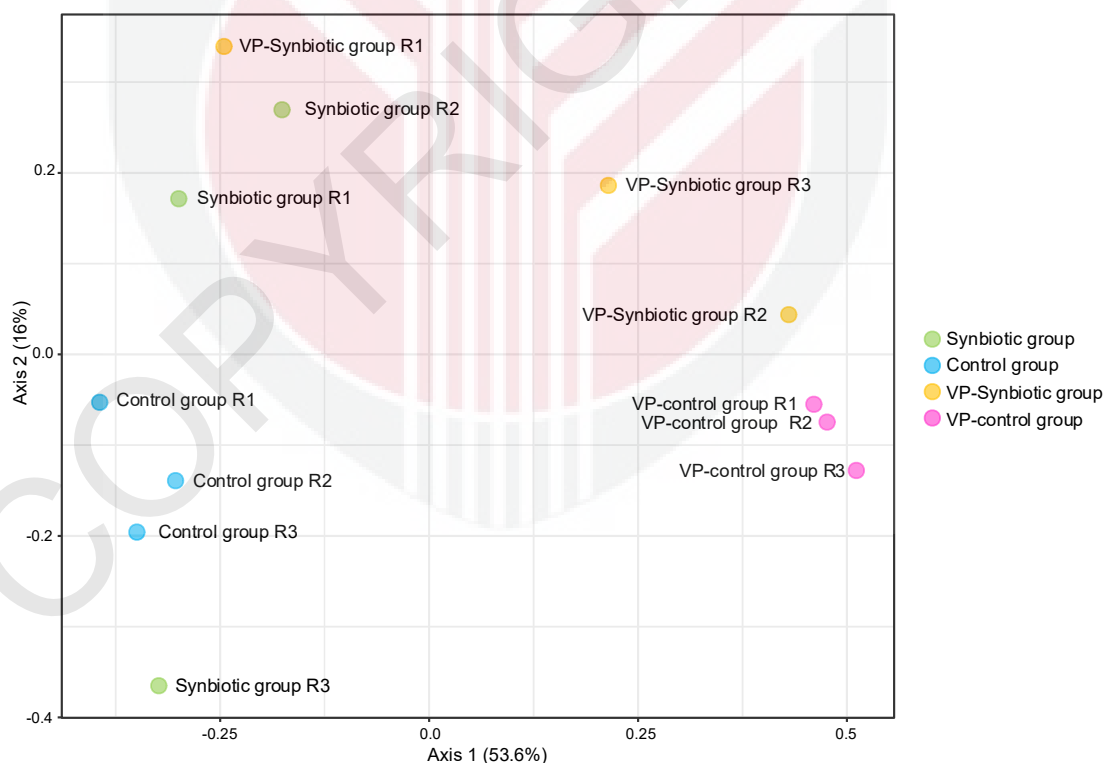


Figure 5.8; Beta diversity of Principal coordination analysis (PCoA) with Bray-Curtis Index for different diet fed *P. monodon* groups gastrointestinal microbiota upon feeding trial and *V. parahaemolyticus* immersion challenge. “Synbiotic group” and “control group” from the feeding trial while “VP-Synbiotic group” and “VP-control group” from the post-challenge.

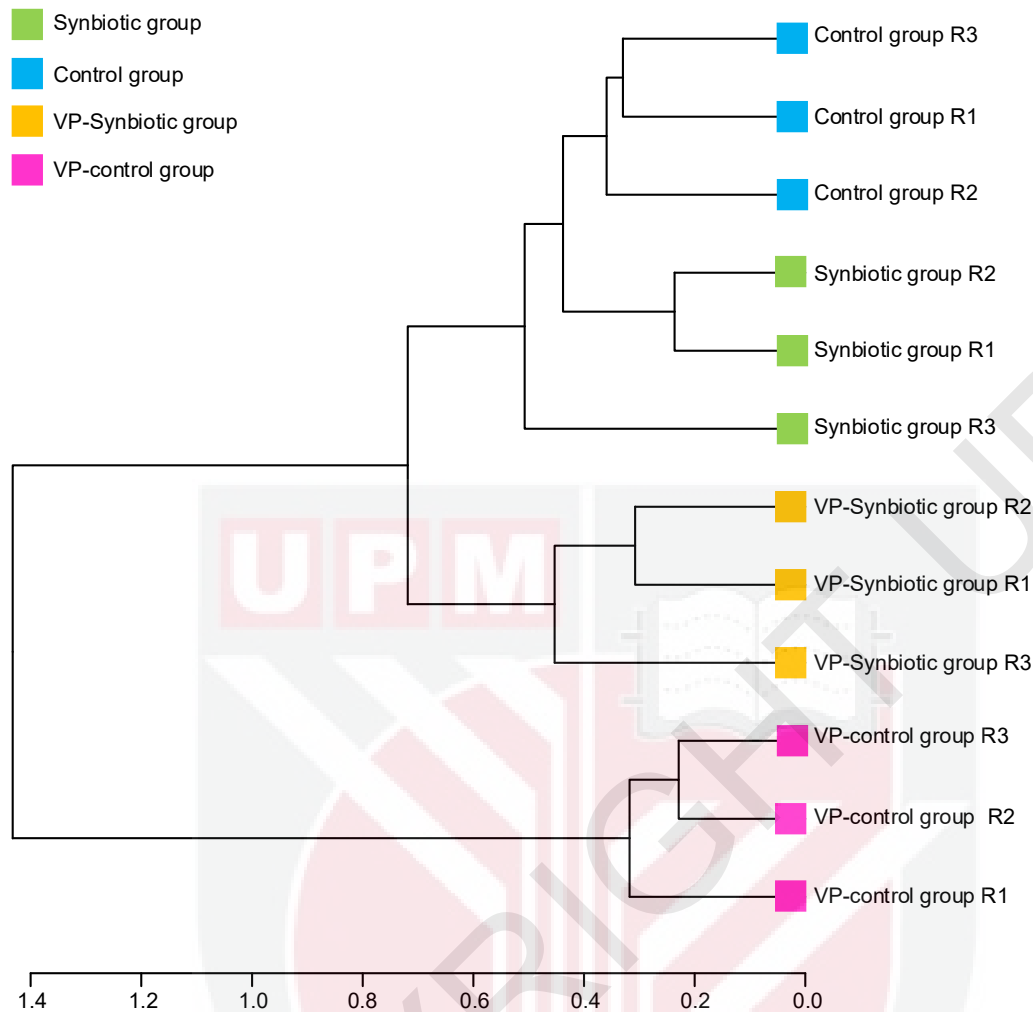


Figure 5.9: Dendrogram (hierarchical tree) of unweighted UniFrac distance analysis. The samples in the plots were tagged by groups of the feeding trial and post-challenge. “Synbiotic group” and “control group” from the feeding trial while “VP-Synbiotic group” and “VP-control group” from the post-challenge.

5.3.1.4. Comparison of gastrointestinal microbiota taxa

The gastrointestinal microbiota structure of groups were explored. In bacterial phylum (Figure 5.10A), the Proteobacteria was predominant in 35th day Synbiotic group (35.26 % to 67.20 %), 35th day control group (51.73 % to 58.07 %), 60 h VP-Synbiotic group (72.78 % to 92.04 %) and 60 h VP-control group (95.21 % to 98.98 %). Although Bacteroidota and Actinobacteriota were

presence in all groups of 35th day feeding trial and 60 h VP-challenge test, Bacteroidata was second dominant in 60 h VP-Synbiotic group (5.75 % to 13.77 %) and 60 h VP-control group (0.81 % to 3.31 %) whereas Actinobacteriota was second dominant in 35th day Synbiotic group (5.81 % to 50.96 %) and 35th day control group (20.62 % to 30.31%). Notably, Firmicutes was presence in 35th day Synbiotic group (2.60 % to 12.39 %), 35th day control group (0.29 % to 2.45 %) and 60 h VP-Synbiotic group (0.13 % to 0.29 %), but almost absence in 60 h VP-control group (0 % to 0.04 %). In bacterial family (Figure 5.10B), Vibrionaceae was predominant in 60 h VP-Synbiotic group (9.53 % to 77.40 %) and 60 h VP-control group (86.72 % to 96.19 %) but subordinate in 35th day Synbiotic group (3.54 % to 19.90 %) and 35th day control group (3.73 % to 11.45 %). Rhodobacteraceae was second dominant in 35th day Synbiotic group (6.82 % to 16.75 %), 35th day control group (12.21 % to 26.77 %), 60 h VP-Synbiotic group (2.39 % to 30.37 %) and 60 h VP-control group (0.81 % to 4.12 %).

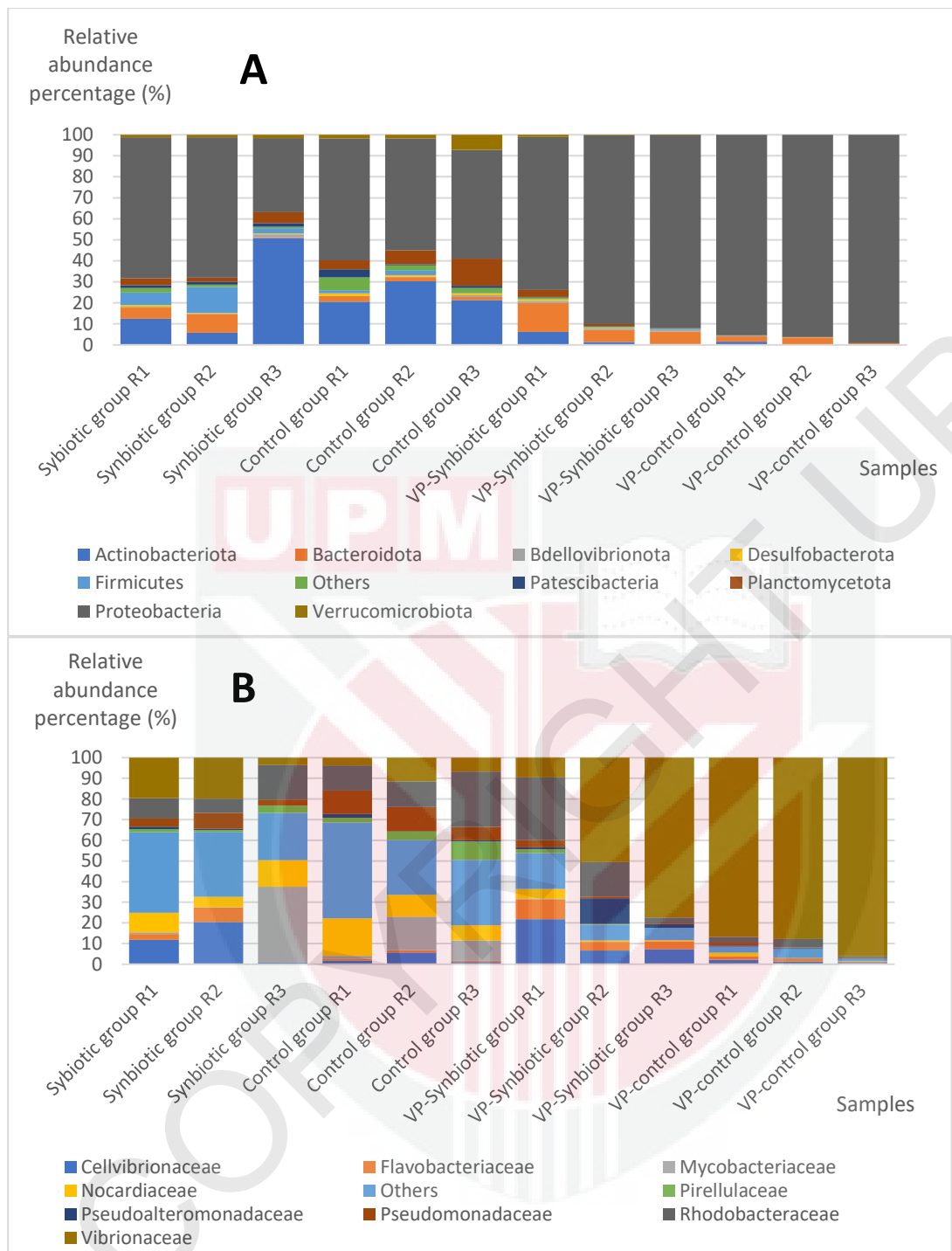


Figure 5.10: Relative abundances of *P. monodon*'s gastrointestinal microbiota samples among the feeding trial and *V. parahaemolyticus* S2-4 immersion challenge. (A) shows the relative abundance percentage of bacterial phyla. (B) shows the relative abundance percentage of bacterial families. "Synbiotic group" and "control group" from the feeding trial while "VP-Synbiotic group" and "VP-control group" from the post-challenge.

5.3.1.5. Differential abundance of gastrointestinal microbiota community composition

The linear discriminate analysis (LDA) shows differential abundance of family taxa with significant level ($p < 0.05$) in the shrimp gastrointestinal microbiota of different groups. In the feeding trial, the Synbiotic group exhibited LDA score above 3.0 for family Mycoplasmataceae, Lactobacillaceae, and Devosiaceae (Figure 5.11A). In contrast, the control group displayed LDA score of above 3.0 for family Hyphomicrobiaceae, Halomonadaceae, Paracaedibacteraceae, Rhizobiaceae, and Chlamydiaceae (Figure 5.11A).

In the *V. parahaemolyticus* immersion challenge, the VP-Synbiotic group displayed LDA score of above 3.0 for families Cellvibrionaceae, Flavobacteriaceae, Pseudoalteromonadaceae, NS9 marine group, Halomonadaceae, Pirellulaceae, Bacteriovoracaceae, Rhizobiaceae, Chlamydiaceae, Rubinisphaeraceae, Mycoplasmataceae, Legionellaceae, Stappiaceae, Saccharospirillaceae, Phycisphaeraceae, Kiloniellaceae, Granulosicoccaceae, Gimesiaceae, and Hyphomonadaceae (Figure 5.11B). Conversely, the VP-control group exhibited LDA score of above 3.0 for families Vibrionaceae, Crocinitomicaceae, Thiotrichaceae, and Idiomarinaceae (Figure 5.11B).

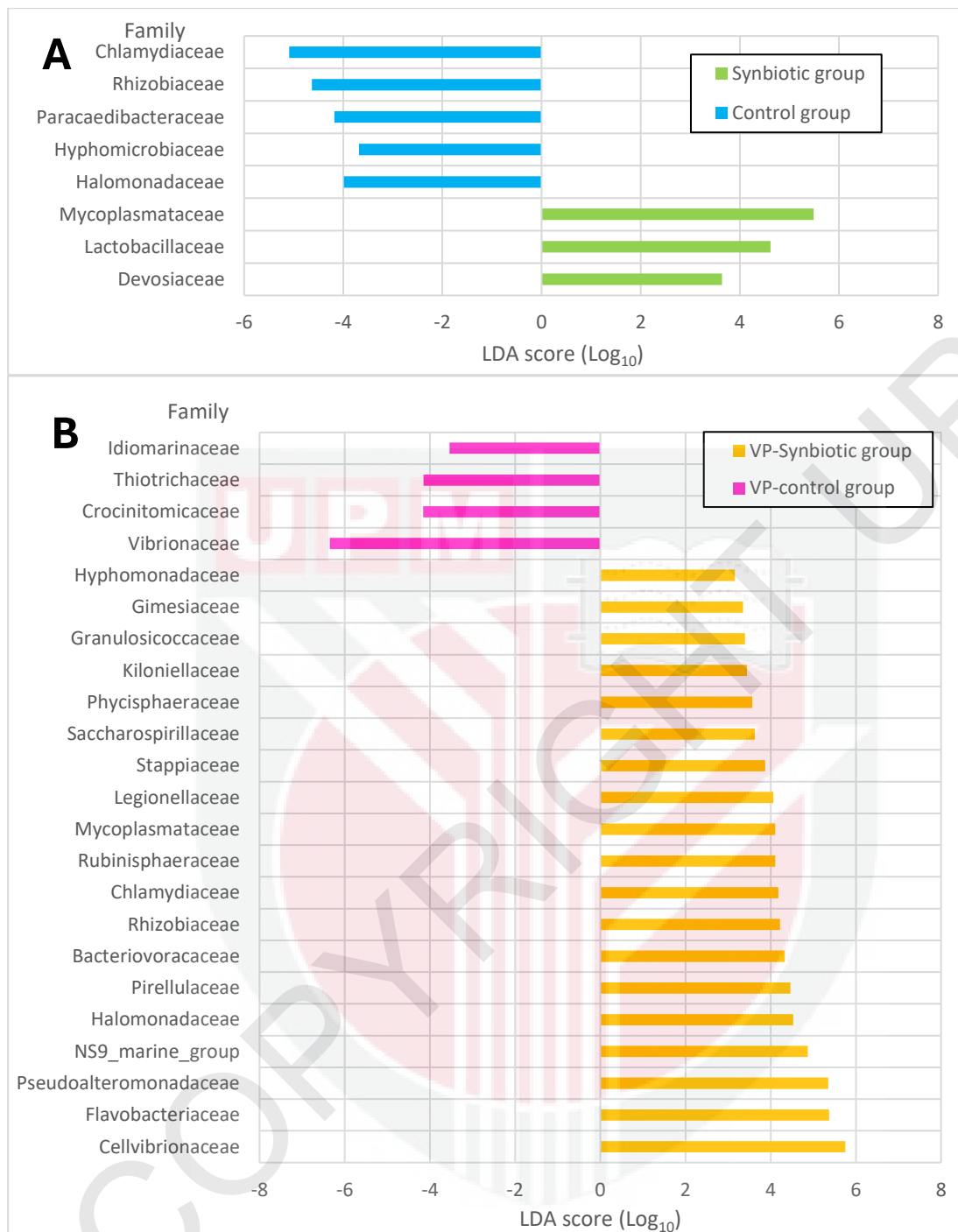


Figure 5.11: Linear discriminate analysis (LDA, $p < 0.05$) of gastrointestinal bacterial community composition of *P. monodon* upon the feeding trial and *V. parahaemolyticus* S2-4 immersion challenge. (A) shows comparison of Synbiotic group with control group in the feeding trial. (B) shows comparison of VP-Synbiotic group with VP-control group in the challenge test.

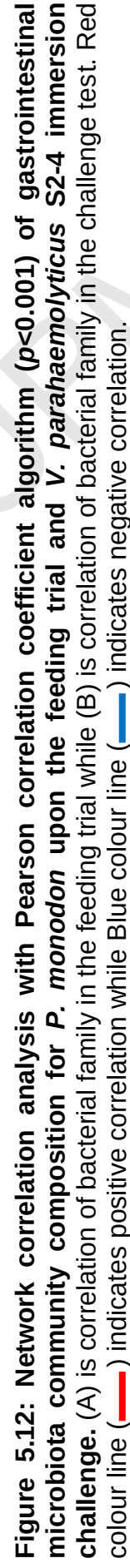
5.3.1.6. Correlation between gastrointestinal microbiota community composition

In this study, synbiotic diet and *V. parahaemolyticus* S2-4 were the experimental factors for the feeding trial and the challenge test. Therefore, LDA score of above 3.0 ($p < 0.05$) for family Lactobacillaceae, Saccharimonadaceae, Mycoplasmataceae, and Devosiaceae were selected as indicative of synbiotic diet (comprising *L. plantarum* L20 and *S. polycystum*) among the microbiota community of the feeding trial based on result in Figure 5.11A. On the other hand, the presence of Vibrionaceae is indicative of the presence of *V. parahaemolyticus* S2-4 within the microbiota community during the challenge test in Figure 5.11B. Hence, we explored the correlation of family Lactobacillaceae, Mycoplasmataceae, and Devosiaceae during the feeding trial, while focusing on Vibrionaceae for the challenge test.

In the feeding trial, family Lactobacillaceae showed highly significant ($p < 0.001$) positive correlation with families Legionellaceae (0.991), Saccharimonadaceae (0.994), Comamonadaceae (0.946), Bacillaceae (0.9443), and Chroococcidiopsaceae (0.973) (Figure 5.12A). Devosiaceae showed a highly significant ($p < 0.001$) positive correlation with Cyclobacteriaceae (0.954) and Sandaracinaceae (0.954) (Figure 5.12A). Mycoplasmataceae was highly significantly ($p < 0.001$) positively correlated with Flavobacteriaceae (0.979), Nitrospiraceae (0.965), Lentisphaeraceae (0.998), Cellvibrionaceae (0.967), Flammeovirgaceae (0.991), and UBA12411 (Order Bacteroidales) (0.968) (Figure 5.12A).

In the challenge test, family Vibrionaceae exhibited a highly significant ($p < 0.001$) negative correlation with families Nannocystaceae (-0.983), Bdellovibrionaceae (-0.984), Myxococcaceae (-0.975), Chlamydiaceae (-0.987), Rubinisphaeraceae (-0.982), Halomonadaceae (-0.996), Flavobacteriaceae (-0.979), Phycisphaeraceae (-0.977), Legionellaceae (-0.986), Stappiaceae (-0.983), Rhizobiaceae (-0.991), Geminococcaceae (-0.978), Rhodobacteraceae (-0.987), and Schlesneriaceae (-0.984) (Figure 5.12B).





5.3.1.7. Differential abundance of predicted MetaCyc metabolic pathway

The linear discriminate analysis (LDA) showed differential abundance among predicted MetaCyc pathway (Child class) with significant level ($p < 0.05$) in the shrimp gastrointestinal microbiota of different groups. In the feeding trial, Synbiotic Group exhibited LDA score of about 4 for “Glycolysis”, while control group exhibited LDA score of about 4 for “interconversion” (Figure 5.13A). In the challenge test, VP- Synbiotic group exhibited LDA score of above 3 for “Glycolysis”, “C1 compounds Utilization and Assimilation”, “Electron Transfer”, “Respiration”, “Cell Structures Biosynthesis” and “Nucleosides and Nucleotides Biosynthesis”. Meanwhile, VP-control group exhibited LDA score of above 3 for “Polymeric compounds Degradation”, “Nucleosides and Nucleotides Degradation”, “Siderophore Biosynthesis”, “Secondary Metabolites Degradation”, “Pentose Phosphate Pathways” and “Metabolic Regulators Biosynthesis” (Figure 5.13B).

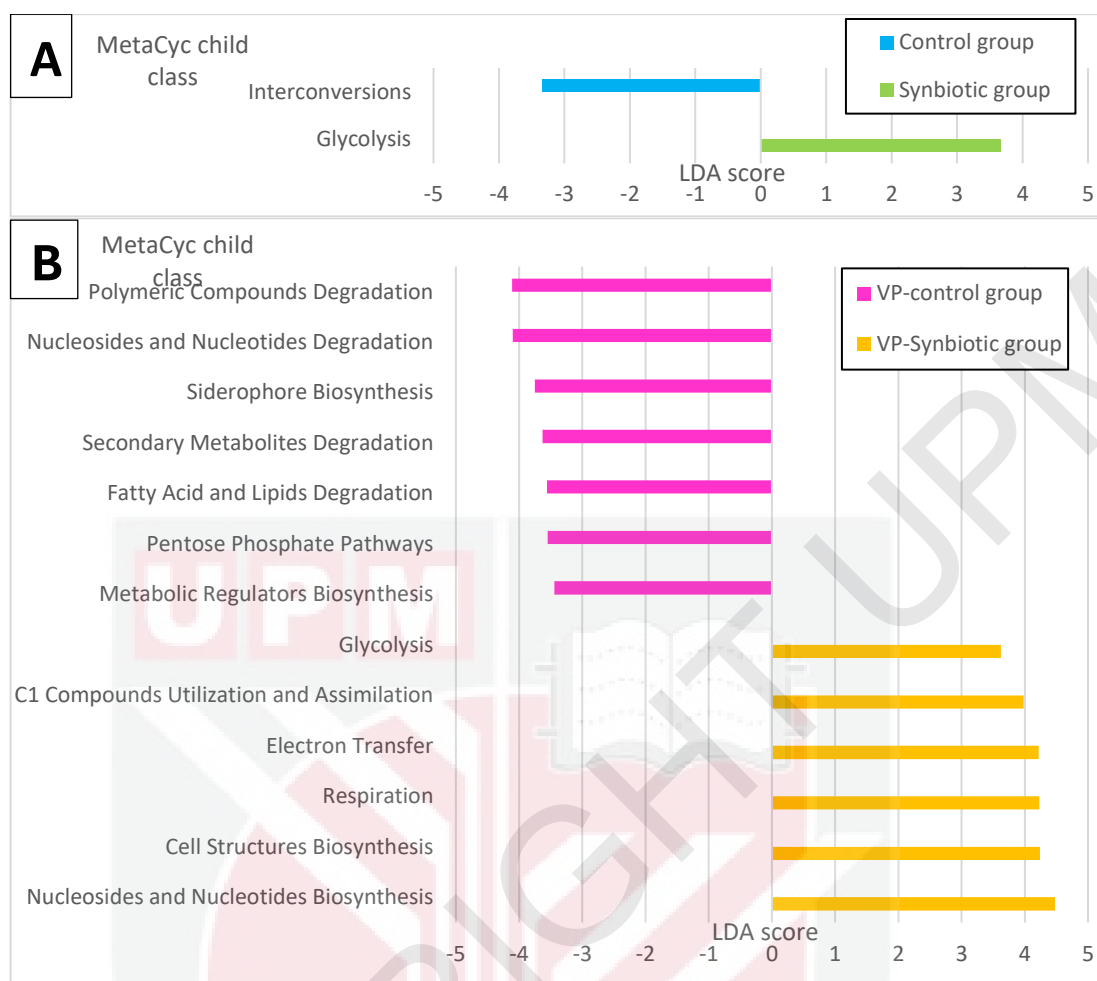


Figure 5.13: Linear discriminate analysis (LDA, $p < 0.05$) for predicted MetaCyc metabolic pathway (Child class) of gastrointestinal bacterial community composition of *P. monodon* upon the feeding trial and *V. parahaemolyticus* S2-4 immersion challenge. Figure 5.13A shows comparison of Synbiotic group with control group in the feeding trial. Figure 5.13B shows comparison of VP-Synbiotic group with VP-control group in the challenge test.

5.3.2. Clinical symptoms

Normal hepatopancreas and gastrointestinal tract (brownish line above shrimp) were observed as evident in the live shrimp of Synbiotic group and control group during the feeding trial (Figure 5.14A & 5.14B). The normal hepatopancreas and gastrointestinal tract (brownish line above shrimp) were observed also from shrimp from the VP-Synbiotic group (Figure 5.14C). However, pale empty hepatopancreas and transparent (empty) intestinal tract

(paleness line above shrimp) were observed from shrimp from the VP-control group (Figure 5.14D).

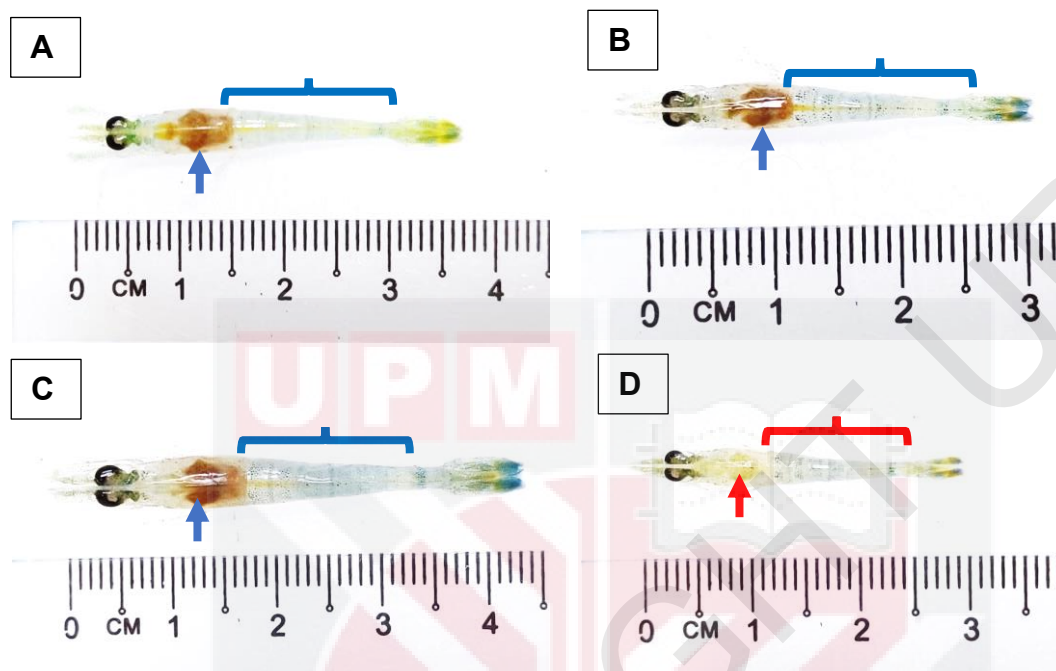


Figure 5.14: Clinical sign observation of different supplemented diets administered *P. monodon* upon feeding trial and *V. parahaemolyticus* S2-4 immersion challenge. (A) and (B) show 35th day of feeding trial. (C) and (D) shows 60 h of 6×10^5 CFU/mL *V. parahaemolyticus* S2-4 immersion challenge. (A – C) show normal hepatopancreas (→) and intestine (—). (D) shows pale white hepatopancreas (→) and empty intestine (—).

5.3.3. Histological observations

5.3.3.1. Histopathological evaluation of hepatopancreas in *P. monodon*

Normal structures of digestive gland (hepatopancreas) included resorptive cell (R-cell), blister-like cell (B-cell), fibrillar cell (F-cell) and hepatic lumen were shown in live shrimp from Synbiotic group (Figure 5.15A) and control group (Figure 5.15B). However, hepatic tissue lesion and hemocyte inflation were shown in shrimp from VP-Synbiotic group (Figure 5.15C). Acute hepatic tissue

necrosis and hemocyte congestion were shown in shrimp from VP-control group (Figure 5.15D).

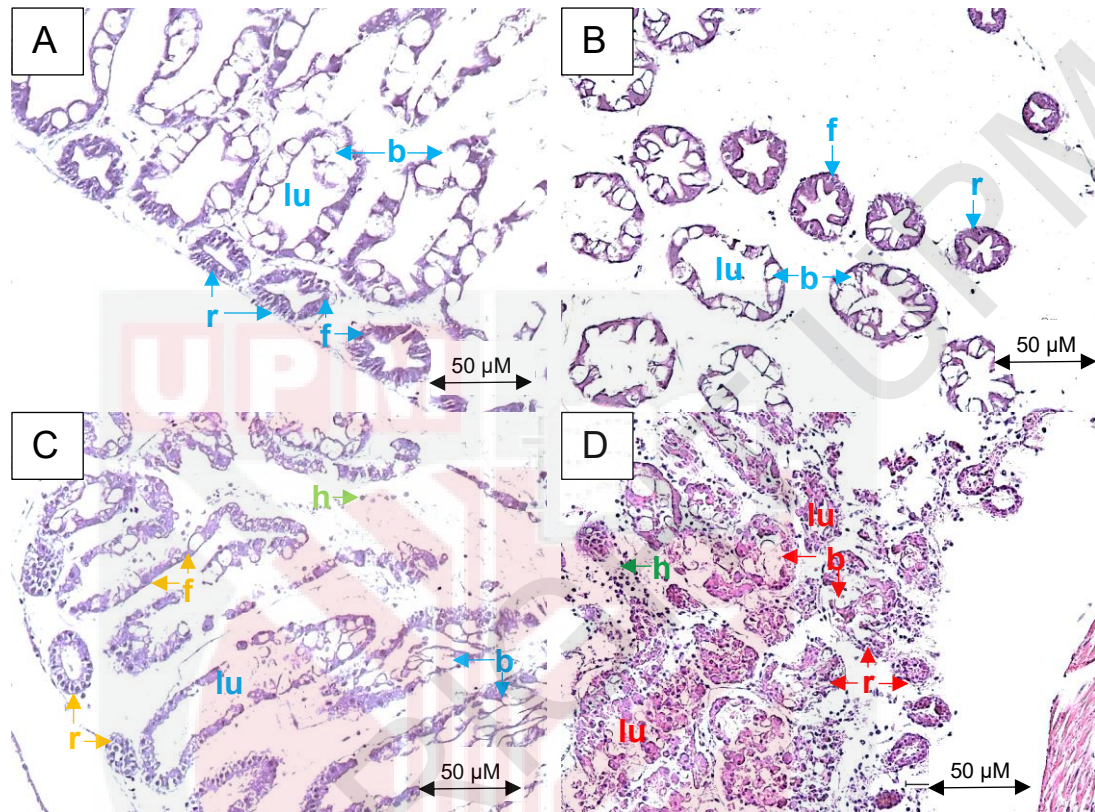


Figure 5.15: Histopathology for foregut digestive gland (hepatopancreas) of different diet fed *P. monodon* fed upon the feeding trial and *V. parahaemolyticus* S2- 4 immersion challenge. The foregut digestive gland (Hepatopancreas) was originally from the shrimp cephalothorax. (A) and (B) were the hepatopancreas from Synbiotic group and control group of the feeding trial respectively. (C) and (D) were the hepatopancreas from VP-Synbiotic group and VP-control group of the challenge test respectively. (A) and (B) show resorptive cell (R-cell: **r**), blister-like cell (B-cell: **b**), fibrillar cell (F-cell: **f**), hepatic lumen (**lu**) ($\times 200$). (C) shows hepatic tissue lesion with mild-vacuolated R-cell (**r**) and F-cell (**f**), and hemocyte inflation with presence of hemocytes (**h**) ($\times 200$). (D) shows acute hepatic tissue necrosis with ruptured karyomegaly R-cell (**r**), F-cell (**f**), B-cell (**b**) and sloughed hepatic lumen (**lu**), and congestion with high amount of hemocytes (**h**) ($\times 200$).

5.3.3.2. Histopathological evaluation of foregut proventriculus in *P. monodon*

Normal structure of foregut proventriculus (stomach) included cuticle, lumen, cuticular epithelium and connective tissue were observed in live shrimp from Synbiotic group (Figure 5.16A) and control group (Figure 5.16B). Interestingly, plant fiber with ingested food was found in live shrimp from Synbiotic group (Figure 5.16A), while only ingested food was observed in live shrimp from control group (Figure 5.16B). However, lesion of cuticular epithelial tissue was observed in shrimp from VP-Synbiotic group (Figure 5.16C). Cuticular epithelial tissue necrosis and hypertrophy of connective tissue were evident in shrimp from VP-control group (Figure 5.16D).

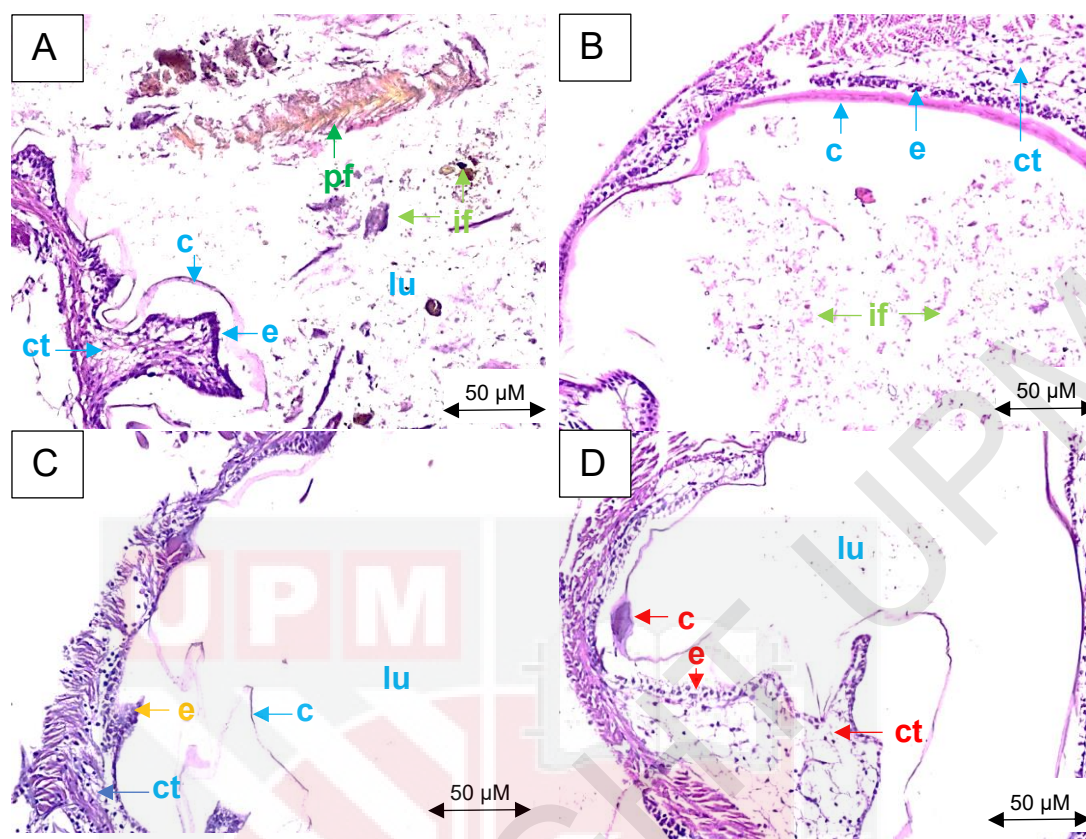


Figure 5.16: Histopathology for foregut proventriculus (stomach) of different diet fed *P. monodon* fed upon the feeding trial and *V. parahaemolyticus* S2-4 immersion challenge. The foregut proventriculus (stomach) was originally from shrimp cephalothorax. (A) and (B) were stomach from Synbiotic group and control group of the feeding trial respectively. (C) and (D) were the stomach from VP-Synbiotic group and VP-control group of the challenge test respectively. (A) and (B) show cuticle (**cu**), lumen (**lu**), ingested food (**if**), cuticular epithelium (**e**) and connective tissue (**ct**) ($\times 200$) but only (A) shows plant fiber (**pf**) ($\times 200$). (C) shows epithelial tissue lesion with mild vacuolated cuticular epithelium (**e**) ($\times 200$). (D) shows epithelial tissue necrosis with damaged cuticle (**cu**) and vacuolated cuticular epithelium (**e**), and hypertrophy of connective tissue (**ct**) ($\times 200$).

5.3.3.3. Histopathological evaluation of midgut trunk in *P. monodon*

Normal structure of midgut trunk included lumen, mucosal epithelium and connective tissue were shown in live shrimp from Synbiotic group (Figure 5.17A) and control group (Figure 5.17B). However, mucosal epithelial tissue lesion and hypertrophy of connective tissue were observed in shrimp from VP-Synbiotic group (Figure 5.17C). Mucosal epithelial tissue necrosis and atrophy

of connective tissue were shown in shrimp from VP-control group (Figure 5.17D).

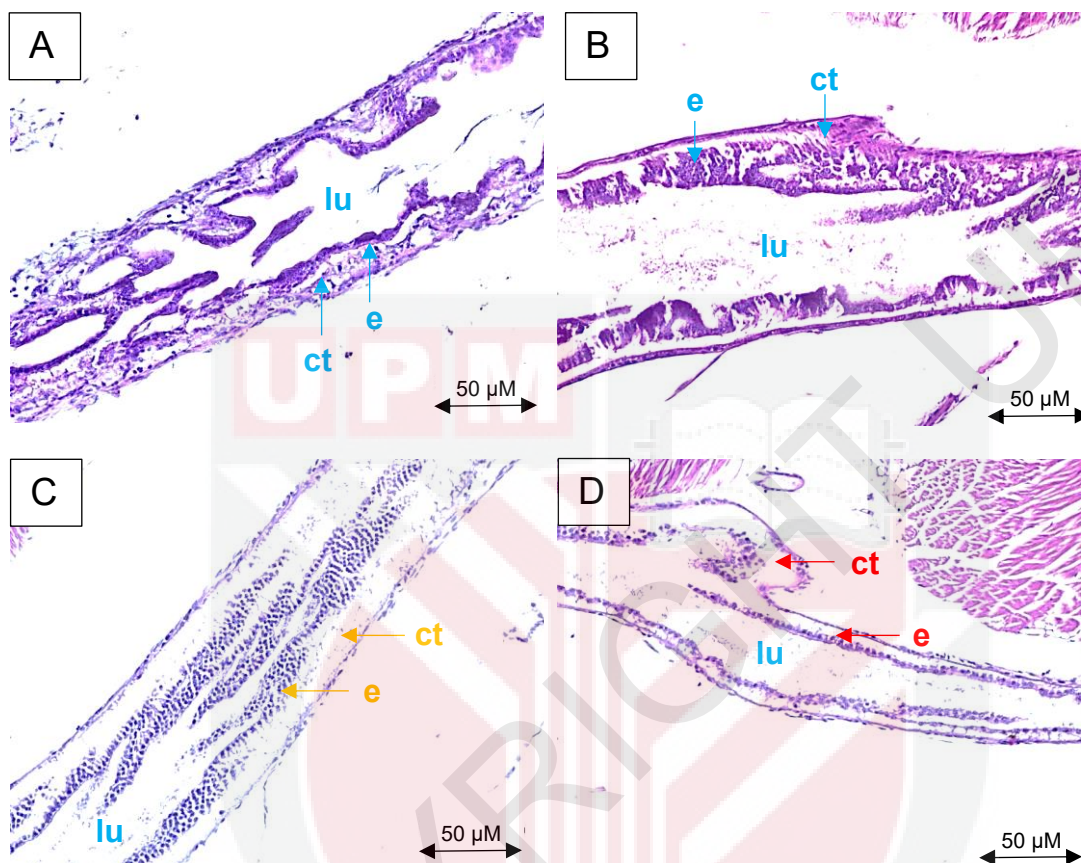


Figure 5.17: Histopathology for midgut trunk of different diet fed *P. monodon* fed upon the feeding trial and *V. parahaemolyticus* S2-4 immersion challenge. The midgut was originally from middle area (2nd – 4th segments) of shrimp abdomen. (A) and (B) were midgut from Synbiotic group and control group of the feeding trial. (C) and (D) were midgut from VP-Synbiotic group and VP-control group of the challenge test. (A) and (B) show lumen (**lu**), mucosal epithelium (**e**) and connective tissue (**ct**) ($\times 200$). (C) shows epithelial tissue lesion with mild-vacuolated mucosal epithelium (**e**) and hypertrophy of connective tissue (**ct**) ($\times 200$). Figure 5.17D shows epithelial tissue necrosis with vacuolated mucosal epithelium (**e**) and atrophy of connective tissue (**ct**) ($\times 200$).

5.3.3.4. Histopathological evaluation of hindgut posterior caecum in *P. monodon*

Normal structure of hindgut posterior caecum included lumen, caecum epithelium and connective tissue were shown in live shrimp from Synbiotic

group (Figure 5.18A) and control group (Figure 5.18B). However, caecum epithelial tissue lesion was shown in shrimp from VP-Synbiotic group (Figure 5.18C). Caecum epithelial tissue necrosis and atrophy of connective tissue were shown in shrimp from VP-control group (Figure 5.18D).



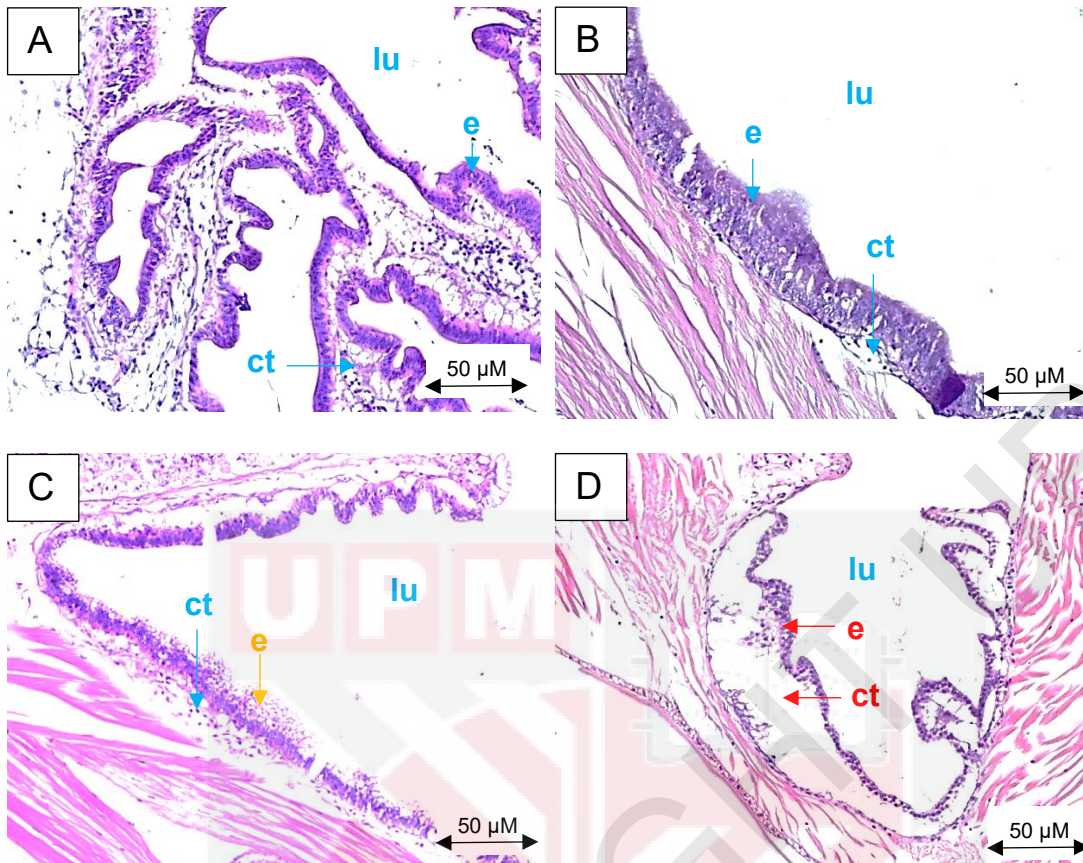


Figure 5.18: Histopathology for hindgut posterior caecum of different diet fed *P. monodon* fed upon the feeding trial and *V. parahaemolyticus* S2-4 immersion challenge. The hindgut posterior caecum was originally from 6th (last) segment of shrimp abdomen near telson tail part. (A) and (B) were posterior caecum from Synbiotic group and control group of the feeding trial. (C) and (D) were posterior caecum from VP-Synbiotic group and VP-control group of the challenge test. (A) and (B) show lumen (lu), caecum epithelium (e) and connective tissue (ct) ($\times 200$). (C) shows epithelial tissue lesion with mild-vacuolated caecum epithelium (e) ($\times 200$). (D) shows epithelial tissue necrosis with vacuolated caecum epithelium (e) and atrophy of connective tissue (ct) ($\times 200$).

5.4. Discussion

In this study, there was no significant difference on the Alpha diversity and Beta diversity of shrimp gastrointestinal microbiota for Synbiotic group compared with control group from the feeding trial. This finding aligned with previous study that also reported no significant difference on the Alpha

diversity (Chao1, Ace and Shannon indices) and Beta diversity (PCoA based on unweighted-UniFrac) of shrimp intestinal microbiota of *P. vannamei* supplemented with mixed probiotic (*Bacillus subtilis*, *B. licheniformis*, *Lactobacillus* sp.) as compared with control group (Xie et al., 2019). We inferred that similar proportions of the dietary protein and fat contents between the two diet groups could account for the slight differences in microbial diversity of shrimp gastrointestinal microbiota (He et al., 2022). The outbreak of AHPND-causing *V. parahaemolyticus* in water environment caused AHPND infection for shrimp, directly impacting the gastrointestinal microbiota diversity of the infected shrimp because a fraction of marine bacterial species, including *Vibrio* spp from the environment could inhabit the gastrointestinal environment through shrimp physiological activities (Chen et al., 2017). Besides, the changes of water environmental variables may also affect the low feed intake, high energy utilization for osmoregulation, high stress responses, and increased disease susceptibility due inhibition of immune responses, resulting in poor growth and high mortality of shrimp (Zheng et al., 2017). Consequently, significant differences were observed in the Alpha diversity and Beta diversity of shrimp gastrointestinal microbiota between the VP-control group, compared with control group and Synbiotic group in this study.

In the feeding trial, Proteobacteria, Planctomycetota, Actinobacteriota and Firmicutes are commonly found in the intestines of cultured shrimp (Vargas-Albores et al., 2017), we noted a higher abundance of Firmicutes and Bacteroidota in shrimp gastrointestinal microbiota of Synbiotic group. We inferred that Firmicutes could have function of plant-based fibre and cellulose

degradation, while Bacteroidota may be involved in the degradation of carbohydrates and proteins, thereby improving shrimp digestion (Mtshali et al., 2022). Moreover, the Synbiotic supplemented diet improved the relative abundance of Lactobacillaceae, Mycoplasmataceae and Devosiaceae. These Lactobacillaceae can evoke shrimp innate immune system, gastrointestinal carbohydrate metabolism and produce beneficial organic acids (lactic acid), which can influence the composition of shrimp gastrointestinal microbiota (Dekham et al., 2023). The presence of *L. plantarum* L20 (positive GHKL domain-containing protein) also may have contributed to the increased abundance of Lactobacillaceae and Firmicutes in the shrimp gastrointestinal tract. Additionally, Mycoplasmataceae in intestinal microbiota of marine animal have been associated with carbohydrate metabolic pathways and induced energy for the host (Zhou et al., 2022), while Devosiaceae may be involved with cellulose decomposition (Zhao et al., 2023). High-throughput sequencing targeting the V3-V4 region of the 16S rRNA gene, commonly utilized for quantifying *Vibrio* spp. including AHPND-causing *V. parahaemolyticus* S2-4 (Zhang et al. 2021). In the post-challenge, we revealed that the predominance of AHPND-causing *V. parahaemolyticus* S2-4, indicated by presence of binary *pirAB* genes, resulted in Proteobacteria and Vibrionaceae becoming the most abundant taxa in the shrimp gastrointestinal tract of the VP-control group. This alteration of the shrimp gastrointestinal microbiota by AHPND is often referred to as dysbiosis (Holt et al., 2021). In contrast, other study reported lack of dysbiosis in *P. vannamei* infected with non-AHPND-causing *V. parahaemolyticus* (Prathiviraj et al., 2021). Interestingly, our study found that the Alpha diversity and Beta diversity of shrimp gastrointestinal microbiota for

VP-Synbiotic group exhibited slight but significant differences compared to the VP-control group, while showing no significant difference compared with Synbiotic group and control group. This inferred that the Synbiotic supplemented diet was effective in mitigating AHPND-related shrimp dysbiosis in *P. monodon*. Therefore, we further explored the comparison between the VP-Synbiotic group and the VP-control group. The high concentration of *V. parahaemolyticus* S2-4 in the water environment resulted in the predominant presence of Proteobacteria and a reduction in other beneficial phyla (including Firmicutes and Bacteroidota), by colonizing the shrimp gastrointestinal microbiota of VP-Synbiotic group and VP-control group (Fan et al., 2019). However, VP-Synbiotic group exhibited lower abundance of Proteobacteria and higher abundance of other phyla than the VP-control group. We hypothesized that the Synbiotic supplemented diet enhanced gastrointestinal microbiota's resistance to the colonization of *V. parahaemolyticus* S2-4-related Proteobacteria (Chang et al., 2023).

In the feeding trial, the increased abundance of gastrointestinal Lactobacillaceae in the Synbiotic group positively correlated with Bacillaceae and Comamonadaceae. These bacteria can metabolize plant-derived monosaccharides, disaccharides, and polysaccharides of *S. polycystum* through their cellulose-degrading capabilities (Li et al. 2019; Jinno et al. 2022; Nurliana et al. 2022). Despite function of Legionellaceae still remain unknown for Synbiotic group, we suggested the Lactobacillaceae positively correlated with Legionellaceae due to *Lactobacillus* sp. metabolite (l-cysteine) and *S. polycystum* component (iron) supported the growth of Legionellaceae (Matoba

et al., 2020; Nazarudin et al. 2020). However, the reasons for the positive correlation between Lactobacillaceae, Chroococcidiopsaceae and Saccharimonadaceae remain unclear. Furthermore, the increased abundance of gastrointestinal Devosiaceae demonstrated positive correlation with Cyclobacteriaceae and Sandaracinaceae, suggesting potential interactions involved in polysaccharide-cellulose degradation and biofilm formation, which may contribute to the digestion of brown algae, *S. polycystum* (Mohr et al. 2012; Pinnaka & Tanuku 2014; Kesy et al. 2019). Additionally, the enrichment of Mycoplasmataceae displayed positive correlations with various bacterial families such as Flavobacteriaceae, Nitrospiraceae, Lentisphaeraceae, Cellvibrionaceae, Flammeovirgaceae, and UBA12411 (order Babeliales), known for their ability to degrade and utilize carbohydrates (Lucena et al. 2019; Gavriilidou et al. 2020; Liu et al. 2022; Yoshida et al. 2022; Thiele et al. 2023). In the post challenge, the VP-Synbiotic group showed enriched microbiota, including Cellvibrionaceae, Mycoplasmataceae, Pirellulaceae, Granulosicoccaceae, Rubinisphaeraceae, Saccharospirillaceae, NS9 marine group (Flavobacteriales), Phycisphaerae, Kilonellaceae, Gimesiaceae, and Hyphomonadaceae, which are known for their cellulose and various carbohydrate hydrolyzing abilities (Park et al. 2014; Si et al. 2017; Lucena et al. 2019; Kallscheuer et al. 2020; Feng et al. 2021; Hervé et al. 2021; Pei et al. 2021; Garate et al. 2022; Leadbeater et al. 2022). Additionally, the enriched microbiota, including Flavobacteriaceae, Stappiaceae, Pseudoalteromonadaceae, Halomonadaceae, and Rhizobiaceae can produce various antibacterial compounds (Selvarajan et al. 2019; Gavriilidou et al. 2020; Zhu et al. 2022). Microbiota-secreted bacteriocins and bacteriocin-like proteins

may play a role in killing pathogenic *V. parahaemolyticus* (Nayak et al., 2021). The enriched Bacteriovoraceae could prey on *V. parahaemolyticus* strains associated with AHPND (Kongrueng et al. 2017). Despite that, the interaction of shrimp gastrointestinal microbiota between Chlamydiaceae and Legionellaceae were still poorly understood. However, we hypothesized that these enriched bacteria may potentially inhibit the growth of Vibrionaceae, predominantly represented by *V. parahaemolyticus* S2-4, by competing for nutrients (Ochoa-Romo et al., 2022). Previous studies have shown negative correlations between Vibrionaceae and several bacterial families included Legionellaceae, Bdellovibrionaceae, Stappiaceae, Rhizobiaceae, Rhodobacteraceae, Flavobacteriaceae, Halomonadaceae and Rubinisphaeraceae in experimental challenge test (Richards et al., 2012; Callac et al., 2022; Deris et al., 2022; Zheng et al., 2022; Almeida et al., 2023; Ghonimy et al., 2023; Wang et al., 2023; Xie et al., 2023). Although Lactobacillaceae was absent in shrimp gastrointestinal microbiota of VP-Synbiotic group, but the presence of *L. plantarum* L20 (positive GHKL domain-containing protein) was detected in the genomic DNA samples of VP-Synbiotic group's shrimp gastrointestinal tract through highly accurate and specific PCR-based methods (Kim et al., 2020). A study showed that the high-throughput amplicon sequencing of 16S rRNA gene's V3-V4 region was inaccurate and unable to detect *Lactobacillus* spp. due to a mismatch with *Lactobacillus* spp. by using 5' termini end of the 16S rRNA gene's 337F primer (Park et al. 2021). Therefore, this is due to the *V. parahaemolyticus* S2-4 inhibited other bacteria of Lactobacillaceae, *L. plantarum* L20 was able to resist this pathogen.

In the feeding trial, further supporting the role of these bacteria in carbohydrate metabolism within the shrimp gastrointestinal tract. High abundance of MetaCyc child class (level 2) pathway, “glycolysis” was predicted from Synbiotic group’s gastrointestinal microbiota, suggesting the breakdown of carbohydrate into simple glucose by Lactobacillaceae for lactic acid biosynthesis, which may contribute to energy production and growth enhancement in *P. monodon* (Wang et al., 2021; Li et al., 2022). In this circumstance, we suggested that the enzymatic digestion of shrimp gastrointestinal microbiota, coupled with additional dietary nutrition provided by the Synbiotic supplemented diet significantly improved the growth performance of *P. monodon* during the feeding trial. VP-Synbiotic group shows high abundance of few MetaCyc child class (level 2) pathways, including “Glycolysis”, “C1 Compound Utilization and Assimilation”, “Electron transfer” and “Respiration”. This inferred that shrimp gastrointestinal microbiota of VP-Group Synbiotic supplemented feed likely had an enhanced capacity for nutrient conversion, utilizing carbon sources to provide additional energy and metabolites for *P. monodon* (Wang et al., 2021; Zhou et al., 2022). This enhanced metabolic activity could contribute to improved resistance to the *V. parahaemolyticus* S2-4 colonization and increased survivability of shrimp in harsh environment. In the post-challenge, VP-Synbiotic group also showed high abundance of MetaCyc child class (level 2) pathways, including “Cell structures Biosynthesis” and “Nucleosides and Nucleotides Biosynthesis”. We hypothesized that shrimp gastrointestinal microbiota of VP-Synbiotic group was capable of microbial cell proliferation to maintain or restore the balance of gastrointestinal microbiota against dysbiosis (Alderwick et al., 2015; Lipowska

et al., 2019). In these situations, the survival rate of shrimp of VP-Synbiotic Group was higher than VP-control group in the challenge test. This result was in concordance with our previous study, in which shrimp of Group SYN exhibited highest survival among multiple different supplemented diet groups (probiotic, prebiotic, and synbiotic) during AHPND challenge (Chapter 4).

In the feeding trial, we confirmed that shrimp ingested synbiotic diet by the presence of plant fiber-like substances in shrimp proventriculus (stomach) of Synbiotic group in feeding trial. This observation aligned with a study that reported high fiber content in the proximate composition of whole shrimp, *P. vannamei*, that was fed with *S. polycystum* supplemented diet (Abdel-Rahim et al., 2021). Moreover, no adverse effect for the structure of gastrointestinal tract, including the digestive gland (hepatopancreas), proventriculus (stomach), midgut and hindgut's posterior caecum observed in Synbiotic group during feeding trial, consistent with findings from other studies investigating different shrimp synbiotics (Yao et al., 2021). Thus, we can infer that the synbiotic diet had no harmful effects on the shrimp gastrointestinal tract during the feeding trial. In the post-challengee, epithelial tissue necrosis along with hypertrophy and atrophy of connective tissue was observed along the gastrointestinal tract structure in the VP-control group during the challenge test. This damage likely occurred through the reduction in the viability of epithelial cells, the disruption of tight junctions, and the destabilization of mucosal mucins within the gastrointestinal tract of the shrimp (Duan et al., 2018). Recent study found that *V. parahaemolyticus* released endotoxin and induced diamide oxidase activity, damaging gastrointestinal epithelial tissue and increasing permeability,

particularly into deeper parts of shrimp tissue, such as the hepatopancreatic epithelial tissue (Jiao et al., 2020). In the hepatopancreas of the VP-control group, acute hepatic tissue necrosis and hemocyte congestion were observed, indicating the severe impact of AHPND-causing *V. parahaemolyticus* S2-4, which leads to the sloughing of tubule epithelial cells and ultimately shrimp mortality (Kumar et al., 2020). The occurrence of shrimp epithelial tissue necrosis in the gastrointestinal tract was likely associated with secondary bacterial infections following *V. parahaemolyticus* infection (Khimmakthong and Sukkarun, 2017). Therefore, we hypothesize that the presence of opportunistic infectious bacteria, resulting from dysbiosis in the VP-control group, caused subsequent infection. However, only mild clinical sign (tissue lesion) was found in shrimp gastrointestinal epithelial tissue of VP-Synbiotic group during the challenge test. We hypothesized that *L. plantarum* L20 and other beneficial microbiota may exhibit transient distinct adhesion to the host's intestinal mucosa and potentially possess immunomodulatory effects on the host (Santarmaki et al., 2017). Moreover, probiotic and beneficial bacteria can inhibit opportunistic bacteria in shrimp gastrointestinal microbiota by competing for nutrient and producing various antimicrobial substances such as lactic acid and acetic acid (Imaizumi et al., 2021). Therefore, we further hypothesized that the gastrointestinal microbiota of the VP-Synbiotic group delayed and reduced the pathogenesis of the *V. parahaemolyticus* S2-4 in shrimp.

5.5. Conclusion

Although no significant different between synbiotic group with control group in shrimp gastrointestinal microbiota, our findings suggested that the diet that

comprising *S. polycystum* and *L. plantarum* L20, improved some of beneficial microbial populations in the shrimp's gastrointestinal microbiota during the feeding trial and enhance *V. parahaemolyticus* infection resistance of shrimp. Furthermore, our study explains the complex metabolic interactions among bacterial families, shedding light on the mechanisms by which dietary synbiotic supplementation may against the impact of pathogenic infections. However, the SYN diet insignificantly defended the shrimp gastrointestinal microbiota community against dysbiosis during the immersion challenge of the *V. parahaemolyticus*. Hence, we suggest shrimp feeding of the SYN diet should be applied to *V. parahaemolyticus* S2–4 immersion challenge also in future studies for further examining its disease resistance and shrimp gastrointestinal microbiota protection.

****This chapter was published and entitled as “Elucidation of synbiotic diet comprising of *Lactobacillus plantarum* L20 and *Sargassum polycystum* on gastrointestinal microbiota, tissue structures and AHPND associated dysbiosis susceptibility in black tiger shrimp (*Penaeus monodon*)” in *Aquaculture* at year 2025 with DOI: 10.1016/j.aquaculture.2024.741339.**

CHAPTER 6

SUMMARY AND RECOMMENDATION

6.1. Summary

Lactobacillus spp. is the most effective and widely used probiotic as a feed supplement for aquatic animals' health by controlling microbial diseases in aquaculture. Among *Lactobacillus* spp., all *L. plantarum* strains inhibited *V. parahaemolyticus* strain robustly in well diffusion assay and coculture assay of their experiment because effective producers of lactic acid. Among *L. plantarum* strains, *L. plantarum* L20 improved growth performances and enhanced survival of *P. monodon* against infection of AHPND-causing *V. parahaemolyticus* S2-4. The result proved that the *L. plantarum* L20 has adhesive ability for colonizing host intestinal mucosa and potential immunomodulatory effect for *P. monodon*. Therefore, *L. plantarum* L20 was chosen as a probiotic. The *Sargassum polycystum* were recommended as a prebiotic because high nutritional contents, cheap market price and improving growth performance of *P. monodon*. The fermentable carbohydrates and other nutrient compositions of *S. polycystum* can potentially modulate innate immunity and improve host gastrointestinal microbiota to confer health for the *P. monodon*. Thus, the synergistic effect of incorporated *L. plantarum* L20 with *S. polycystum* improved growth performances of *P. monodon* because improved nutrient absorption and supplied additional peptides and free essential amino acids. Moreover, the synergistic effect of combined *L. plantarum* L20 with *S. polycystum* also provided various antimicrobial

compounds and immunostimulant substances to evoke immune responses and disease resistance of *P. monodon* against infection of AHPND-causing *V. parahaemolyticus* S2-4. The synergistic effect of combined *L. plantarum* L20 with *S. polycystum* improve gastrointestinal microbiota diversity and their potential metabolic pathway of *P. monodon* because *L. plantarum* L20 colonization and utilization of various additional carbohydrate. The improved gastrointestinal microbiota resisted dysbiosis by inhibiting the growth of AHPND-causing *V. parahaemolyticus* S2-4.

6.2. Future prospects

In addition, the synbiotic diet can be prepared for shrimp farming practical scalability. The two crucial components of synbiotic, such as *L. plantarum* L20 as probiotic and *S. polycystum* as prebiotic can be obtained in large scale. In fact, the probiotic, *L. plantarum* L20 can be cultured in mass production using fed-batch culture technique with bioreactor (Coghetto et al., 2016), whereas the prebiotic, *S. polycystum*, which are abundant and under-utilized along the coast, especially east coast of Peninsular Malaysia, offer a promising source for application (Alallam et al., 2025).

Furthermore, there were several suggestions for improving crucial components of synbiotic. The prebiotic, *S. polycystum* which is one of the main components of the synbiotic can be further improved. In this circumference, the *S. polycystum* hydrolysate, which is known as partial product from alkaline-heat hydrolysis of extraction process by using *S. polycystum* is recommended. This substance contains partial extracted alginate which can easily encapsulate

chosen probiotic, especially polysaccharide-utilizing lactic acid bacteria as another component of synbiotic. Moreover, It contains partial extracted carbohydrate also which can be absorbed by digestive systems of aquatic animals upon dietary supplementation, especially farmed carnivorous aquatic animal species, such as Asian seabass (*Lates calcarifer*), Tiger grouper (*Epinephelus fuscoguttatus*) and Red snapper (*Lutjanus erythropterus*).

The synbiotic are suggested also for other famous farmed aquatic animal species to improve their growth performance, especially early growth stages that incomplete developed adaptive immunity. Importantly, application of this synbiotic potentially against also their specific aquatic bacterial diseases for other famous farmed aquatic animal species. In example, aeromoniasis that caused by *Aeromonas veronii* and *A. hydrophilia* on farmed freshwater aquatic species, such as Giant freshwater prawn (*Macrobrachium rosenbergii*) and Nile tilapia (*Oreochromis niloticus*); vibriosis that caused by *V. harveyi* and *V. parahaemolyticus* on farmed marine aquatic species, such as Tiger grouper (*E. fuscoguttatus*) and Golden snapper (*L. johnii*).

Besides *in vitro* and *in vivo* experiments of synbiotic in laboratory, field validation is also suggested to evaluate the *in-situ* effectiveness of the synbiotic for shrimp rearing in pond system at farm site. Moreover, the economic feasibility, productivity and profitability of synbiotic for aquaculture industry can be determined during the field validation. Therefore, the field validation provides crucial result for commercializing the synbiotic as product at the local and international market of aquafeed supplement in the future.

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APPENDICES

Appendix 1. The biochemical characteristics of gram-positive bacteria that considered as presumptive lactic acid bacteria (LAB)

No.	Sources	Isolate	Oxidase	Catalase	Sulfide	Indole	Motility
1.	Quoy's garfish (<i>H. quoyi</i>)	T1	-	-	-	-	-
2.		T2	-	-	-	-	-
3.		T31	-	-	-	-	-
4.		T4	-	-	-	-	-
5.		T51	-	-	-	-	-
6.		T61	-	-	-	-	+
7.		T72	-	-	+	-	-
8.		T8	-	-	-	-	+
9.		T81	-	-	+	+	-
10.		T9	-	-	+	+	-
11.	Blackspot Snapper (<i>L. ehrenbergii</i>)	KD1	-	-	-	-	-
12.		KD2	-	-	-	-	-
13.		KD22	-	-	-	-	+
14.		KD3	-	-	-	-	+
15.		KD4	-	+	-	-	-
16.		KD52	-	-	-	+	-
17.		KD6	-	-	-	-	+
18.		KD71	-	-	-	+	+
19.		KD8	-	-	-	+	-
20.		KD9	-	-	-	+	-
21.	Streaked spinefoot (<i>S. javus</i>)	DJ1	-	-	-	-	-
22.		DJ2	-	-	-	-	-
23.		DJ3	-	-	-	-	+
24.		DJ4	-	-	-	-	-
25.		DJ5	-	-	-	-	+
26.		DJ6	-	-	-	+	-
27.		DJ7	-	-	-	+	-
28.		DJ8	+	-	-	-	-
29.		DJ81	+	-	-	-	-
30.		DJ9	+	-	-	-	-
31.	Largescale tonguesole (<i>Cynoglossus arel</i>)	LT1	-	-	-	-	-
32.		LT2	-	-	-	+	-
33.		LT21	-	-	-	-	-
34.		LT3	-	-	-	+	+
35.		LT41	-	-	-	-	-
36.		LT51	-	-	+	-	-
37.		LT6	-	-	+	+	-
38.		LT7	-	-	-	-	+
39.		LT8	-	-	+	-	-
40.		LT9	-	-	-	+	+
41.	Half-smooth golden	PF1	-	-	-	-	-
42.		PF2	-	-	-	-	+
43.		PF3	-	-	-	+	-

44.	pufferfish (<i>L. spadiceus</i>)	PF4	-	+	-	-	-
45.		PF5	-	-	-	-	-
46.		PF6	-	-	-	-	+
47.		PF7	-	+	-	-	-
48.		PF8	-	-	+	+	-
49.		PF9	-	-	-	-	+
50.		PF91	-	-	-	-	+
51.	Fourfinger threadfin (<i>E. tetradactylum</i>)	SN1	-	-	-	-	-
52.		SN12	-	-	-	+	-
53.		SN2	-	-	-	-	-
54.		SN21	-	-	-	+	-
55.		SN3	-	-	-	+	+
56.		SN4	-	-	-	-	+
57.		SN5	-	-	-	+	-
58.		SN6	-	-	-	-	+
59.		SN8	-	-	-	+	-
60.		SN9	-	-	-	+	-
61.	Moonfish (<i>M. maculate</i>)	KP1	-	-	-	-	-
62.		KP2	-	-	-	-	-
63.		KP3	-	-	-	-	+
64.		KP4	-	-	-	-	-
65.		KP5	-	-	+	-	-
66.		KP61	-	-	+	-	+
67.		KP7	-	-	-	+	-
68.		KP8	-	-	-	+	-
68.		KP81	-	-	+	-	+
70.		KP9	-	-	-	-	+
71.	Threadfin bream (<i>N. japonicus</i>)	KL1	-	+	-	-	-
72.		KL12	-	+	-	+	-
73.		KL21	+	+	-	-	-
74.		KL32	-	+	+	-	-
75.		KL41	-	+	-	-	-
76.		KL51	-	+	-	-	+
77.		KL62	-	+	-	-	+
78.		KL71	+	+	-	-	-
79.		KL8	-	+	-	-	-
80.	Silver pomfret (<i>P. argenteus</i>)	BW1	-	-	-	-	-
81.		BW2	-	-	-	-	-
82.		BW3	-	-	-	-	+
83.		BW31	-	-	-	+	-
84.		BW4	-	-	-	+	-
85.		BW5	-	-	-	-	+
86.		BW6	-	-	-	-	+
87.		BW71	-	-	-	-	+
88.		BW8	-	-	-	-	+
89.		BW91	-	-	-	+	-
90.		BW9	-	-	-	+	-
91.		KF1	-	-	-	-	-

92.	Malabar trevally	KF2	-	-	-	-	-
93.	(C.	KF3	-	-	-	+	+
94.	<i>malabaricus</i>)	KF4	-	-	-	-	+
95.		KF5	-	-	-	+	-
96.		KF6	-	-	-	+	+
97.		KF7	-	-	-	+	-
98.		KF8	-	-	-	-	+
99.		KF8	-	-	-	-	+
100.		KF10	-	-	-	-	+
101.	Common	KS1	-	-	-	-	-
102.	ponyfish (L.	KS2	-	-	-	+	+
103.	<i>equulus</i>)	KS3	-	-	-	-	+
104.		KS4	-	-	-	+	-
105.		KS5	-	-	+	+	-
106.		KS6	-	-	+	-	+
107.		KS7	-	-	+	-	+
108.		KS8	-	-	-	+	-
109.		KS9	-	-	+	-	+
110.		KS10	-	-	+	-	+
111.	Yellowtail scad	SK1	-	-	-	-	-
112.	(A. <i>mate</i>)	SK2	+	-	-	-	+
113.		SK3	+	-	-	+	-
114.		SK4	-	+	-	-	-
115.		SK5	-	-	-	+	-
116.		SK6	-	+	-	-	-
117.		SK7	-	-	-	-	+
118.		SK8	+	-	-	-	-
119.		SK9	-	+	-	-	-
120.		SK10	-	-	-	+	-
121.	Bigeye scad (S.	MT1	-	-	-	-	-
122.	<i>crumphthalmus</i>)	MT2	+	+	-	-	-
123.		MT31	-	+	-	-	-
124.		MT4	-	-	-	+	-
125.		MT51	+	-	-	-	-
126.		MT6	-	-	-	+	-
127.		MT7	+	-	-	-	-
128.		MT81	+	-	-	-	-
129.		MT9	-	+	-	-	-
130.		MT10	-	-	-	-	+
131.	Herring scad	SP1	-	-	-	-	-
132.	(A. <i>vari</i>)	SP2	-	-	-	-	-
133.		SP31	-	-	-	+	-
134.		SP41	-	-	-	-	+
135.		SP5	-	-	+	-	-
136.		SP6	-	-	-	+	-
137.		SP71	-	+	-	-	-
138.		SP8	+	-	-	-	+
139.		SP9	-	-	+	-	-

140.		SP91	-	-	+	+	-
141.	Belanger's	GL1	-	-	-	-	-
142.	croaker (<i>J.</i>	GL2	-	-	-	-	+
143.	<i>belangerii</i>)	GL3	-	-	-	+	+
144.		GL41	-	-	-	-	+
145.		GL4	-	-	-	+	-
146.		GL5	-	-	+	-	+
147.		GL6	-	-	-	+	-
148.		GL7	-	-	-	-	+
149.		GL8	-	-	-	+	-
150.		GL9	-	-	-	-	+
151.	Yellowtail	TF1	-	-	-	+	-
152.	fusilier	TF11	-	-	+	-	+
153.	(<i>C. cuning</i>)	TF2	-	-	-	+	+
154.		TF21	-	-	+	+	+
155.		TF3	-	-	-	+	-
156.		TF4	-	-	-	+	-
157.		TF5	-	-	+	-	+
158.		TF6	-	-	+	-	-
159.		TF7	-	-	-	-	-
160.		TF8	-	-	-	-	+
161.	Milkfish (<i>C.</i>	SS1	-	-	-	-	-
162.	<i>chanos</i>)	SS2	-	-	-	-	+
163.		SS3	-	-	-	-	-
164.		SS4	-	-	-	-	+
165.		SS5	-	-	-	-	+
166.		SS6	-	-	-	-	-
167.		SS7	-	-	-	-	+
168.		SS8	-	-	-	-	+
169.		SS9	-	-	-	-	+
170.		SS10	-	-	-	-	+
171.	Orange-spotted	BG1	-	-	-	-	-
172.	Grouper (<i>E.</i>	BG2	-	-	-	+	+
173.	<i>coioides</i>)	BG3	-	-	-	-	-
174.		BG4	-	-	-	+	-
175.		BG5	-	-	-	+	+
176.		BG6	-	-	-	-	+
177.		BG7	-	-	-	+	-
178.		BG8	-	-	-	-	+
179.		BG9	-	-	-	+	-
180.		BG10	-	+	-	-	-
181.	Red Snapper	MR1	-	-	-	-	-
182.	(<i>L.</i>	MR2	+	-	-	+	-
183.	<i>erythropterus</i>)	MR3	+	-	-	-	+
184.		MR4	-	+	-	-	-
185.		MR5	+	-	-	-	-
186.		MR6	-	-	-	+	-
187.		MR7	-	+	-	-	+

188.		MR8	-	+	-	-	+
189.		MR9	-	-	-	+	-
190.		MR10	-	+	-	+	-
191.	Asian seabass (<i>L. calcarifer</i>)	SK1	-	-	-	-	-
192.		SK2	+	-	+	-	-
193.		SK3	-	+	-	-	-
194.		SK4	-	-	-	+	-
195.		SK5	-	-	-	-	+
196.		SK6	-	-	-	+	-
197.		SK7	-	-	+	-	-
198.		SK8	-	-	+	-	-
199.		SK9	+	-	-	-	-
200.		SK10	-	+	-	+	-
201.	Pacific white shrimp (<i>P. vannamei</i>)	UP1	-	+	-	-	-
202.		UP2	-	-	+	-	+
203.		UP3	+	-	-	+	-
204.		UP4	-	-	-	-	-
205.		UP5	-	-	-	-	-
206.		UP6	-	-	-	-	-
207.		UP7	-	-	+	-	-
208.		UP8	-	-	-	+	-
209.		UP9	-	+	-	-	+
210.		UP10	-	+	-	+	-
211.	Black tiger shrimp (<i>P. monodon</i>)	UH1	-	-	+	-	-
212.		UH2	-	-	-	-	-
213.		UH3	-	-	+	-	+
214.		UH4	-	-	-	-	-
215.		UH5	-	-	-	-	-
216.		UH6	-	-	-	-	-
217.		UH7	-	-	-	+	+
218.		UH8	-	-	-	+	-
219.		UH9	-	-	-	-	+
220.		UH10	-	-	-	-	+
221.	Giant freshwater prawn (<i>M. rosenbergii</i>)	UG1	-	-	-	-	-
222.		UG2				+	
223.		UG3	-	-	-	-	-
224.		UG4	-	-	-	+	-
225.		UG5	+	-	-	-	-
226.		UG6	-		-	-	+
227.		UG7	-	+	-	-	-
228.		UG8	-	+	-	+	+
229.		UG9	-	-	+	-	-
230.		UG10	+	-	-	+	+
231.	Banana shrimp (<i>P. merguensis</i>)	UM1	-	-	-	-	-
232.		UM2	-	-	-	-	-
233.		UM3	-	-	-	+	-
234.		UM4	-	-	-	+	-
235.		UM5	-	+	-	-	-

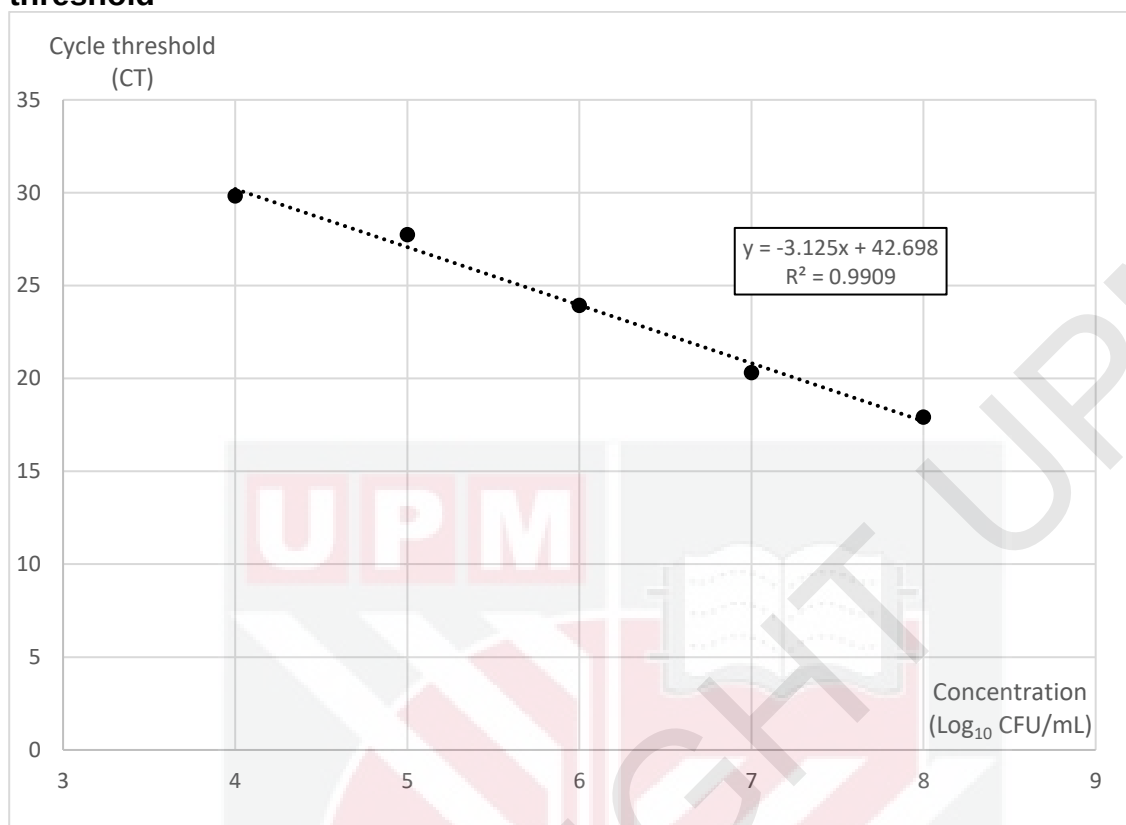
236.		UM6	+	-	-	-	+
237.		UM7	-	-	-	-	-
238.		UM8	-	+	-	-	+
239.		UM9	-	-	-	-	+
240.		UM10	-	-	-	-	+
241.	Lab collection	L8	-	-	-	-	-
242.		L20	-	-	-	-	-



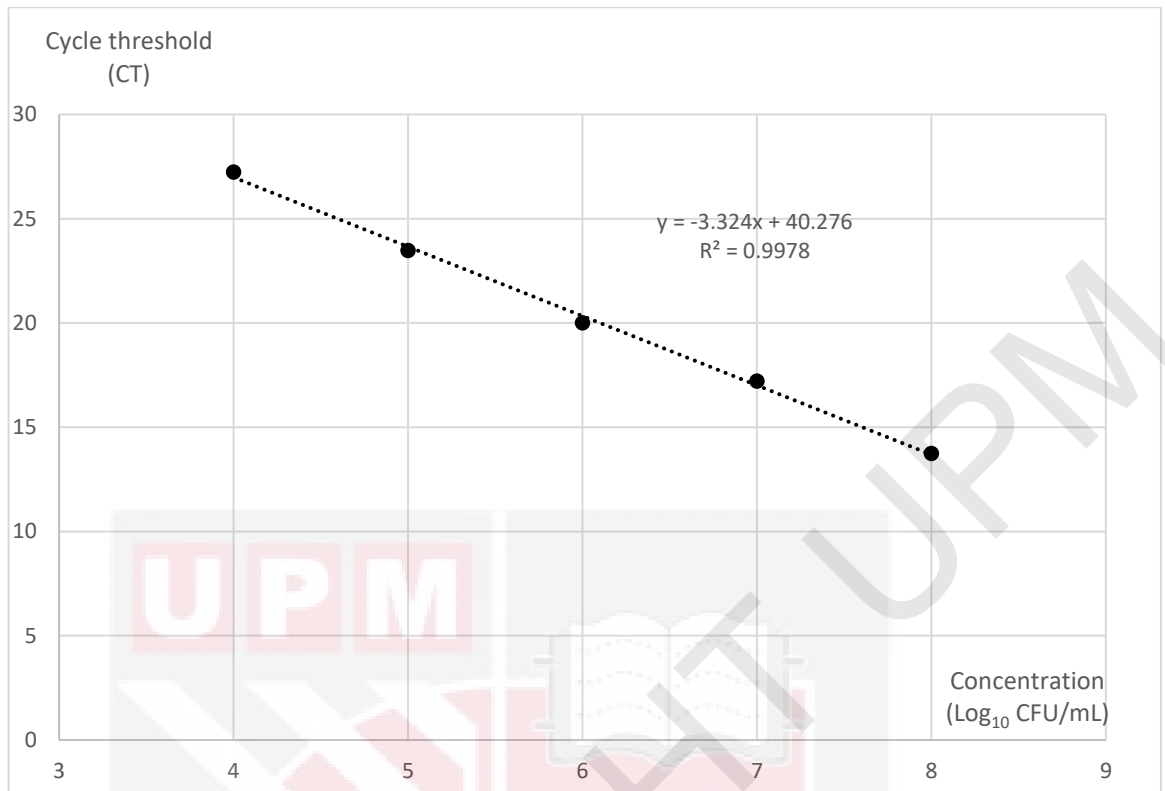
Appendix 2. List of 16S rRNA gene sequence names from database of GenBank.

No	Reference sequences name	Accession number
1.	<i>Weissella cibaria</i> 6253	MT463653.1
2.	<i>W. cibaria</i> 1255	MT573699.1
3.	<i>W. ceti</i> IMP-BG-B057	MN860269.2
4.	<i>W. paramesenteroides</i> 5140	MT512146.1
5.	<i>Leuconostoc citreum</i> HDB3022	MH844902.1
6.	<i>L. citreum</i> TR116	MN135986.1
7.	<i>L. pseudomesenteroides</i> S-2	KF263165.1
8.	<i>L. pseudomesenteroides</i> L12028	KT952382.1
9.	<i>L. mesenteroides</i> 4459	MT544985.1
10.	<i>Lactobacillus curvatus</i> 1123	MT573613.1
11.	<i>L. curvatus</i> 1TP02-BL07	MG031210.1
12.	<i>L. sakei</i> 6668	MT515987.1
13.	<i>L. sakei</i> 1783	MT597629.1
14.	<i>L. plantarum</i> LG1.1	MZ208265.1
15.	<i>L. plantarum</i> F5S5	KF245560.1
16.	<i>L. plantarum</i> 7984	MT538887.1
17.	<i>Vagococcus lutrae</i> m1134/87/1	NR_026467.1
18.	<i>Enterococcus faecalis</i> pDX6b	MZ636608.1
19.	<i>E. faecium</i> BXC18	OQ103373.1
20.	<i>Staphylococcus saprophyticus</i> 55QC2CO	OP067970.1
21.	<i>S. edaphicus</i> 2202	MT604700.1
22.	<i>S. saprophyticus</i> cqsmh2	MN826552.1
23.	<i>S. epidermidis</i> KS4H17	KJ571206.1
24.	<i>Macrococcus caseolyticus</i> ZY02	MG996517.1
25.	<i>Bacillus thuringiensis</i> NS18	ON045804.1
26.	<i>B. subtilis</i> Bs24	OQ423159.1
27.	<i>B. cereus</i> NIBSM OsG2	KY930333.1
28.	<i>B. cereus</i> F4a	MK088302.1
29.	<i>B. weidmannii</i> D59-6	MT256065.1
30.	<i>Streptococcus parauberis</i> 271	OM837060.1
31.	<i>Lactococcus chungangensis</i> R25-61	MN330516.1
32.	<i>L. petauri</i> TA-U-100	OK326448.1
33.	<i>L. garvieae</i> CMGB-L25	MF348237.1
34.	<i>L. garvieae</i> 5529	MT463487.1
35.	<i>L. taiwanensis</i> HBUAS53376	MK818762.1
36.	<i>L. lactis</i> A5908	MN466963.1
37.	<i>L. lactis</i> YL-5	MW007836.1
38.	<i>L. lactis</i> subsp <i>lactis</i> FFNL2424	MT196908.1
39.	<i>L. lactis</i> 4472	MT544997.1
40.	<i>L. lactis</i> subsp. <i>Hordniae</i> S-3	MT416432.1

Appendix 3. Standard curve for bacterial enumeration against cycle threshold

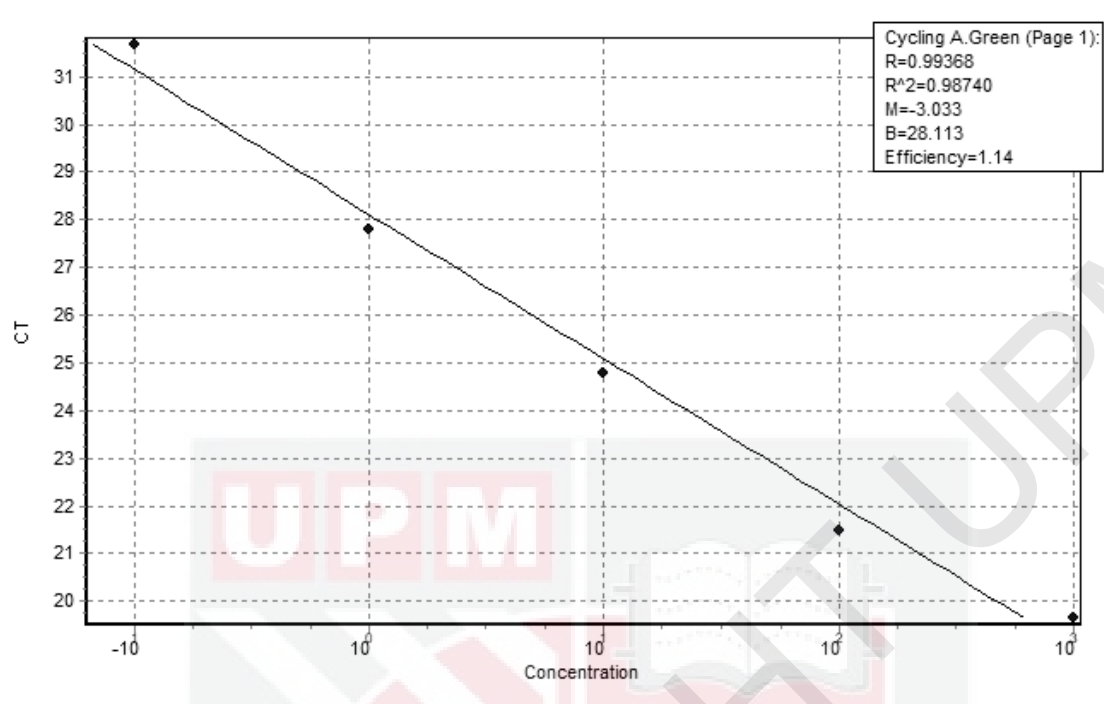


Appendix 3A. Absolute standard curve of GHKL domain containing protein gene which *Lactobacillus plantarum* concentration (Log₁₀ CFU/mL) against cycle threshold.

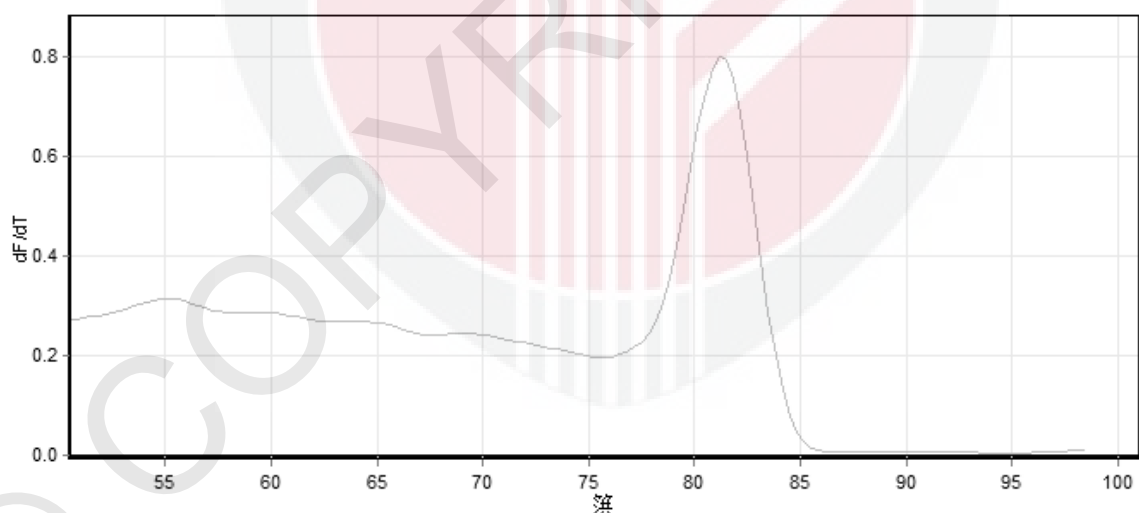


Appendix 3B. Absolute standard curve of *pirA* gene which *Vibrio parahaemolyticus* positive AHPND concentration (Log₁₀ CFU/mL) against cycle threshold.

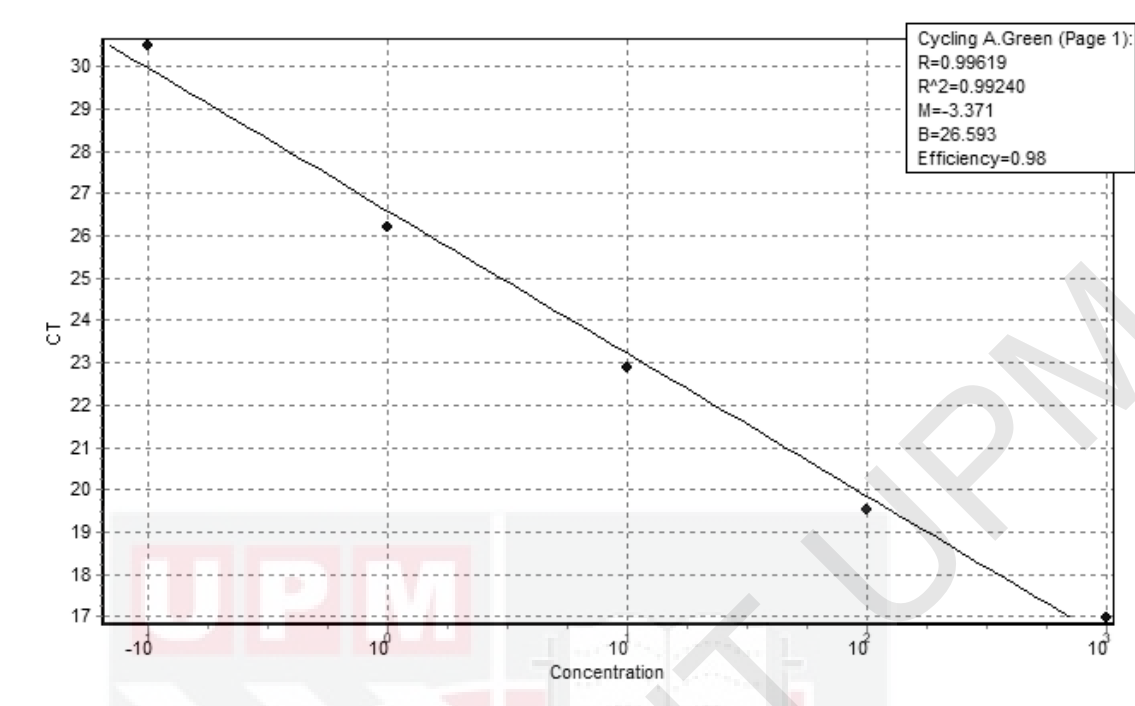
Appendix 4. Standard curve for shrimp immune gene expression



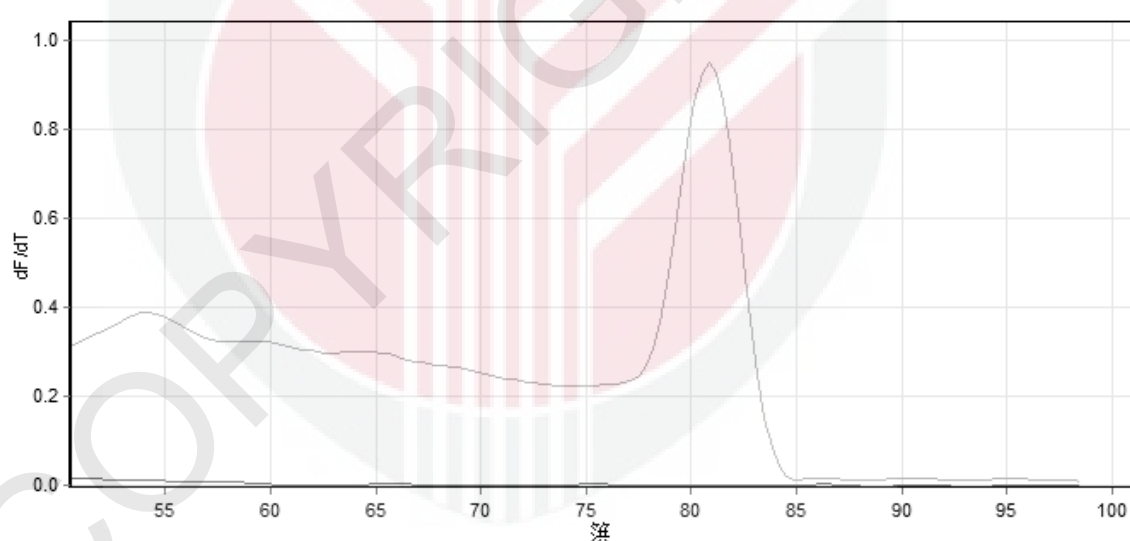
Appendix 4A. Lipopolysaccharide and Beta-1,3-glucan binding protein (LGBP) standard curve



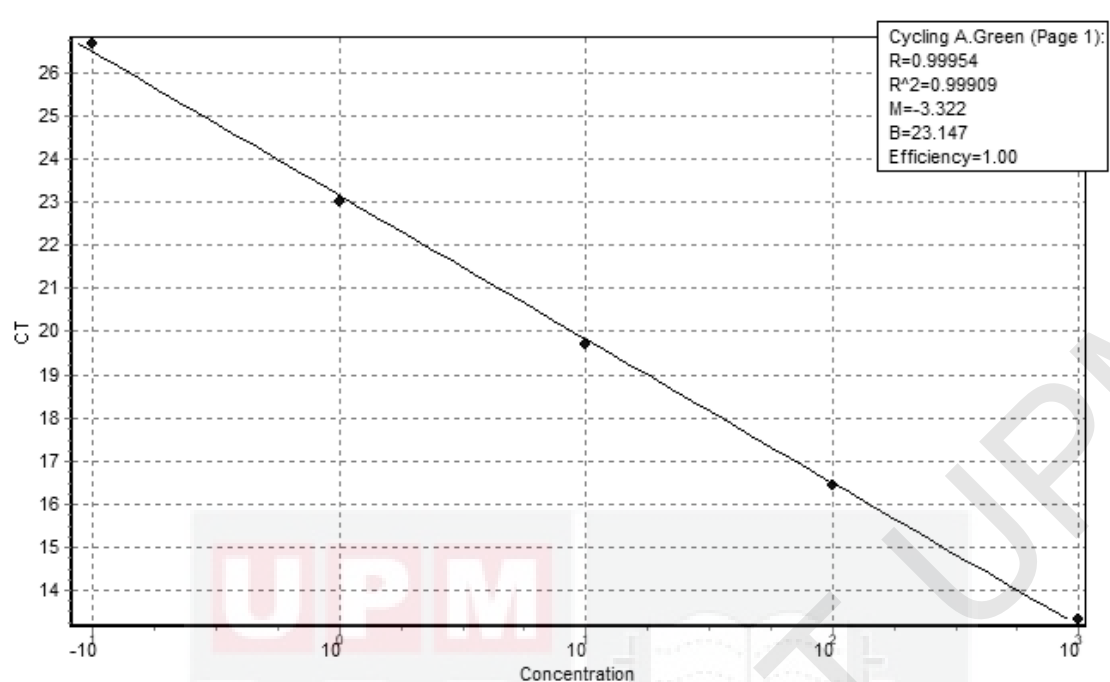
Appendix 4B. Lipopolysaccharide and Beta-1,3-glucan binding protein (LGBP) melting curve



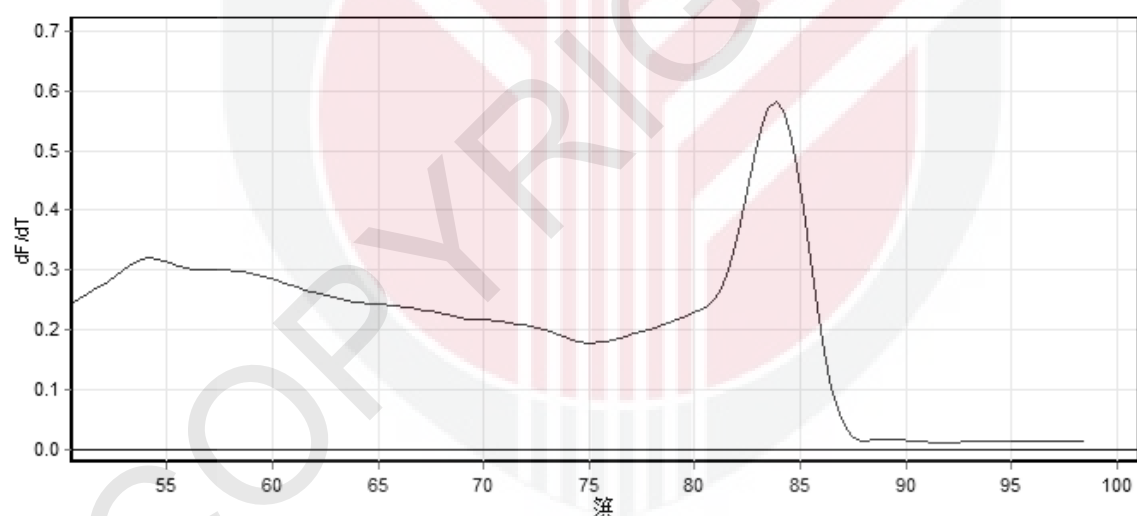
Appendix 4C. Peroxinectin standard curve



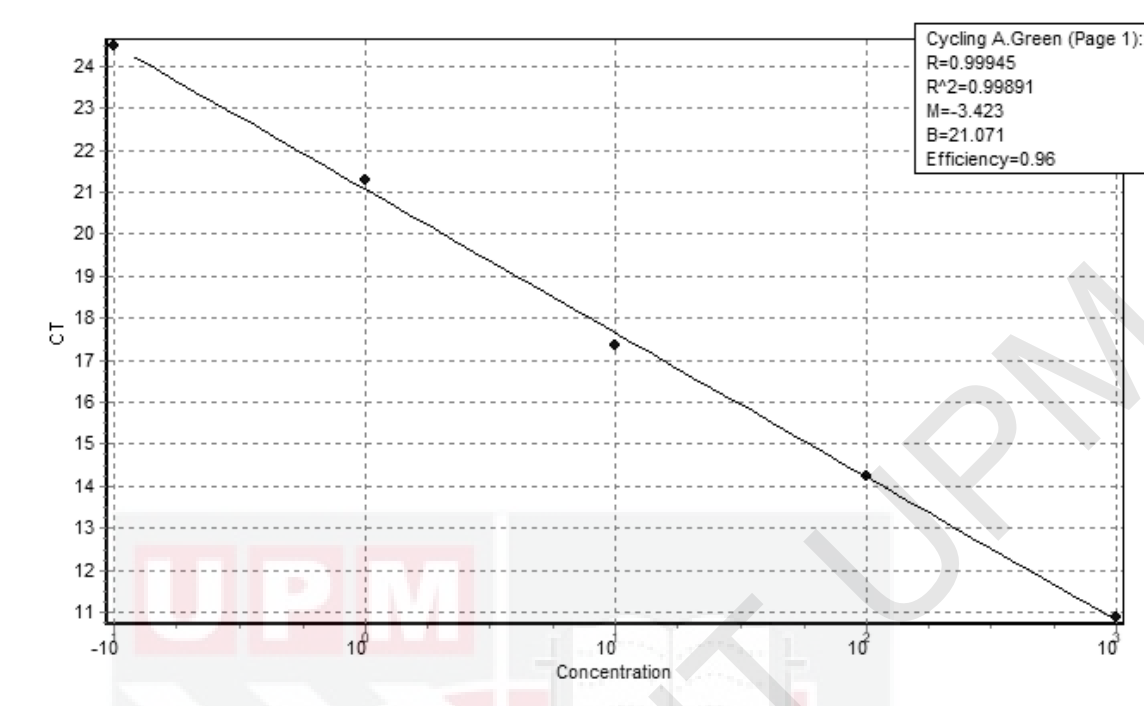
Appendix 4D. Peroxinectin melting curve



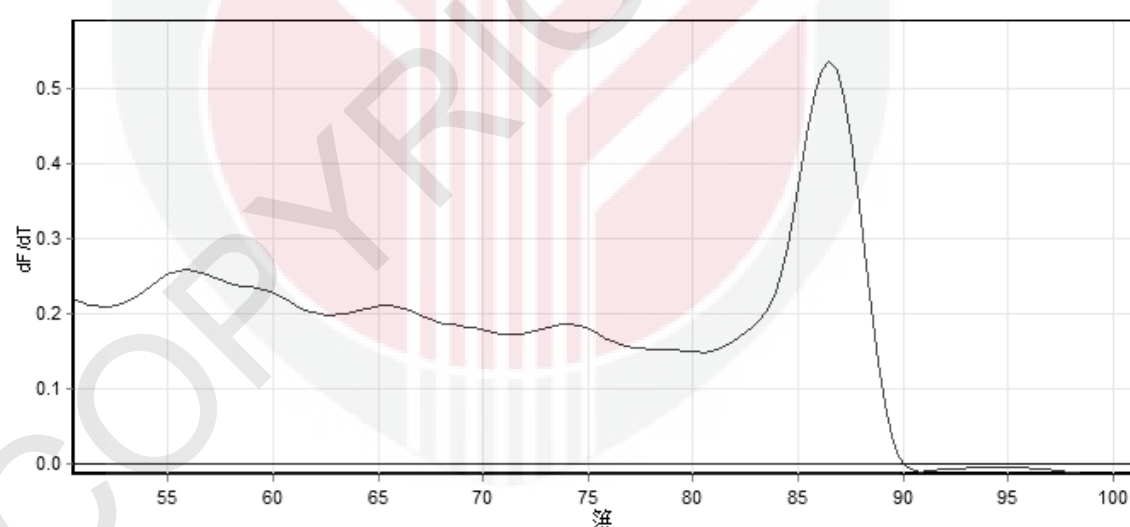
Appendix 4E. Prophenoloxidase standard curve



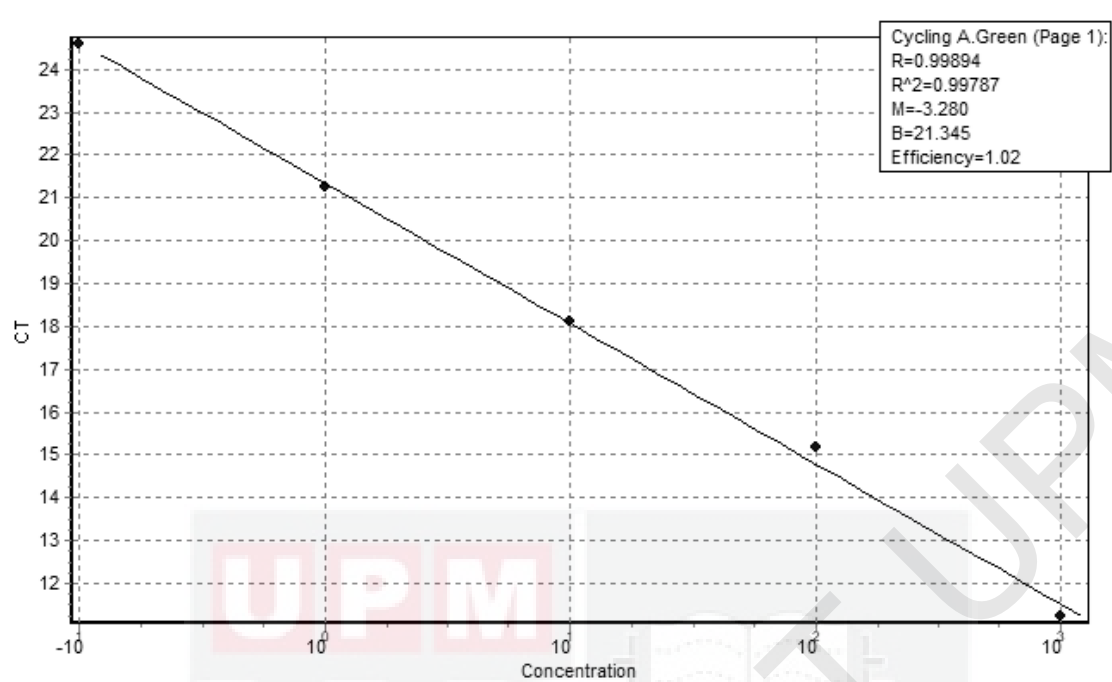
Appendix 4F. Prophenoloxidase melting curve



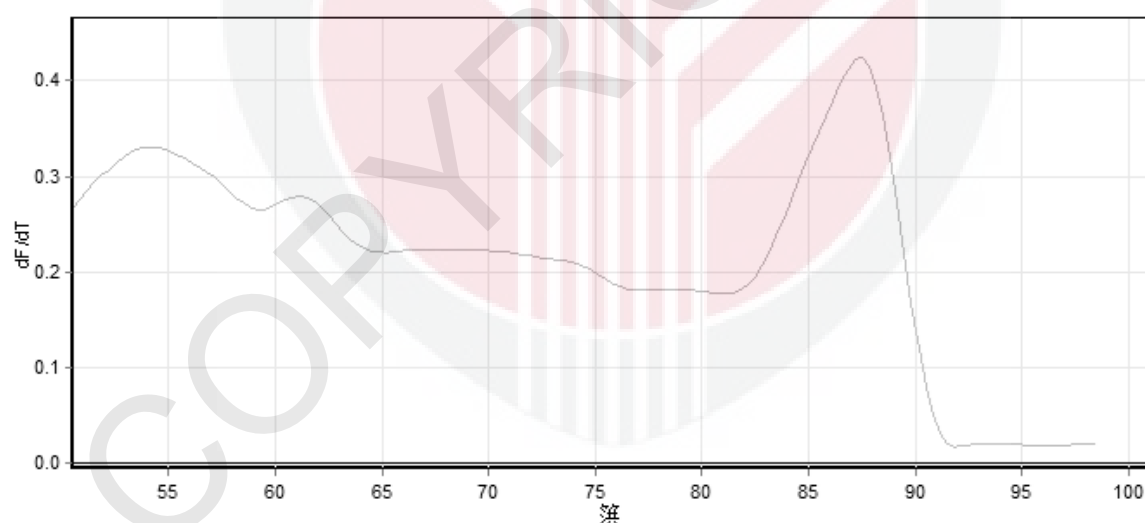
Appendix 4G. Heat shock protein (HSP70) standard curve



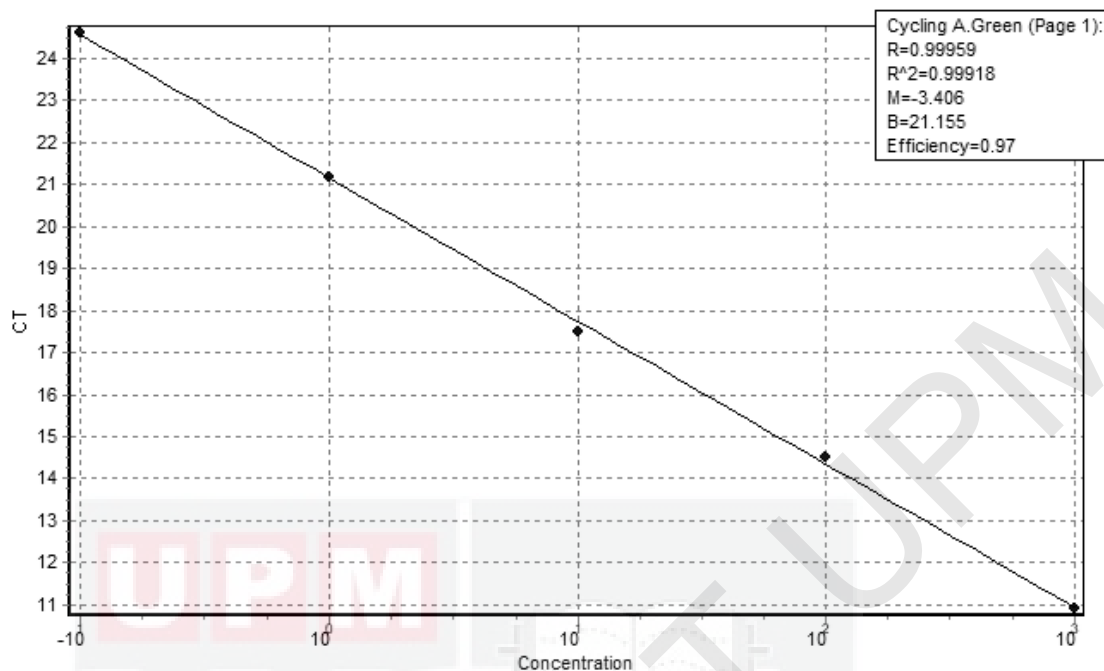
Appendix 4F. Heat shock protein (HSP70) melt curve



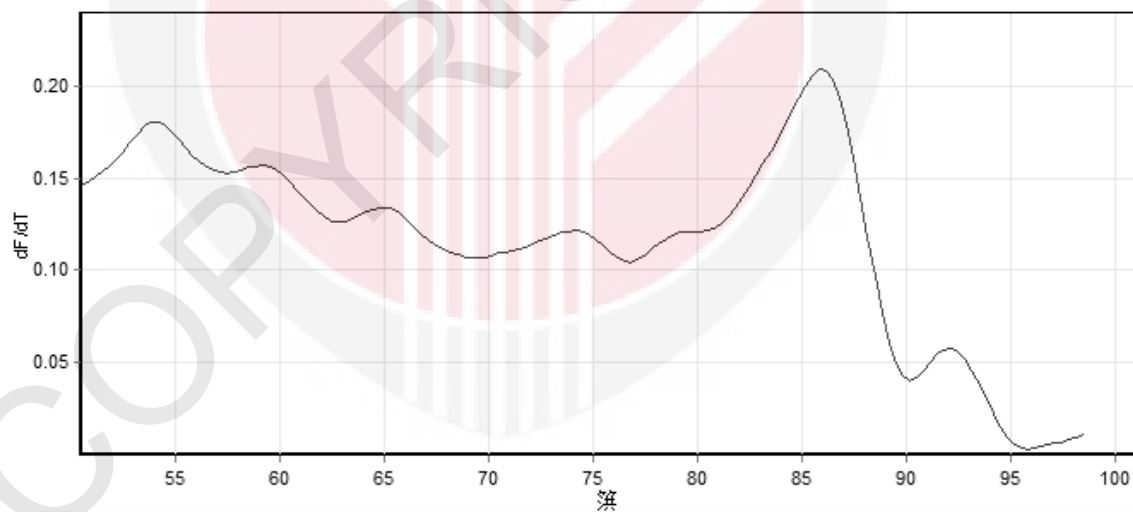
Appendix 4H. Lysozyme standard curve



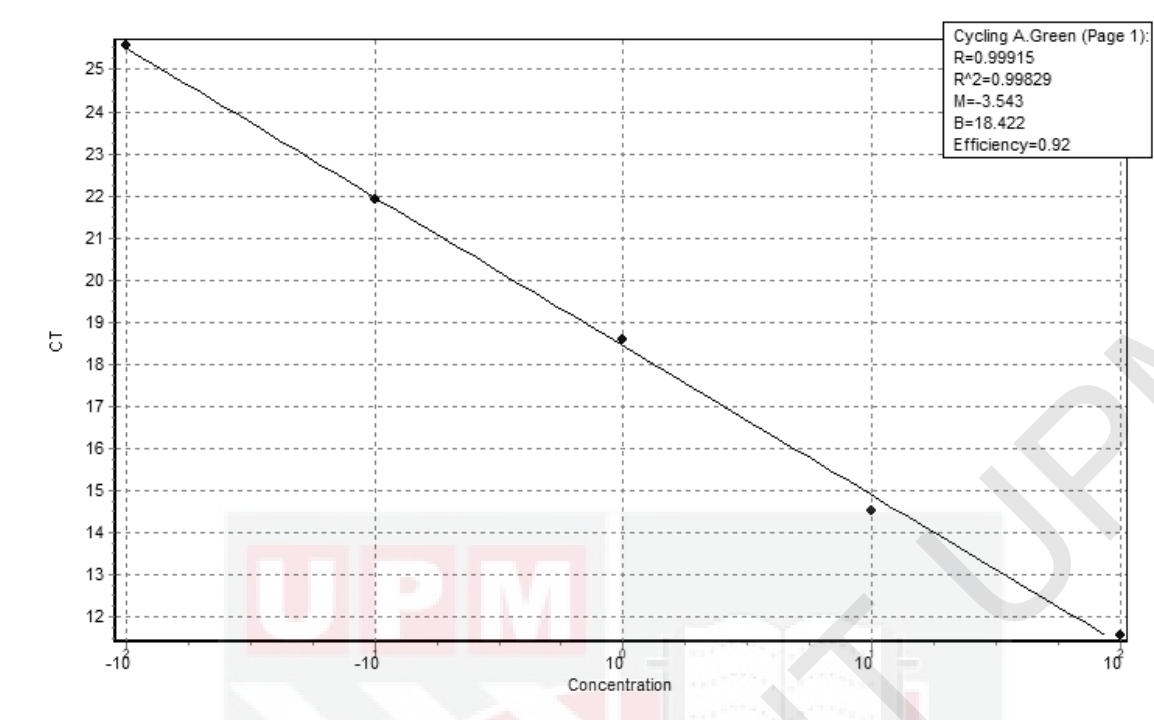
Appendix 4I. Lysozyme melting curve



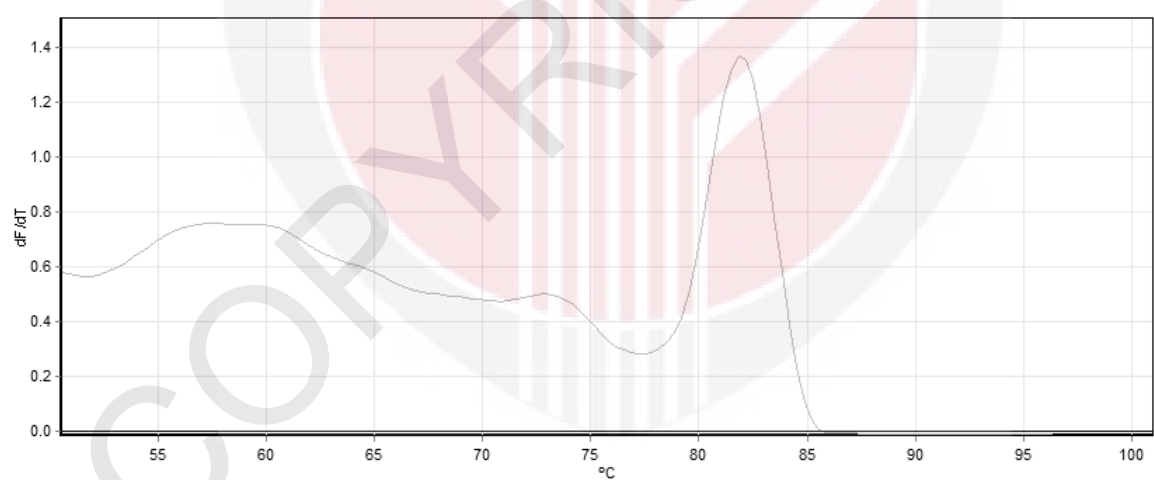
Appendix 4J. Crustin standard curve



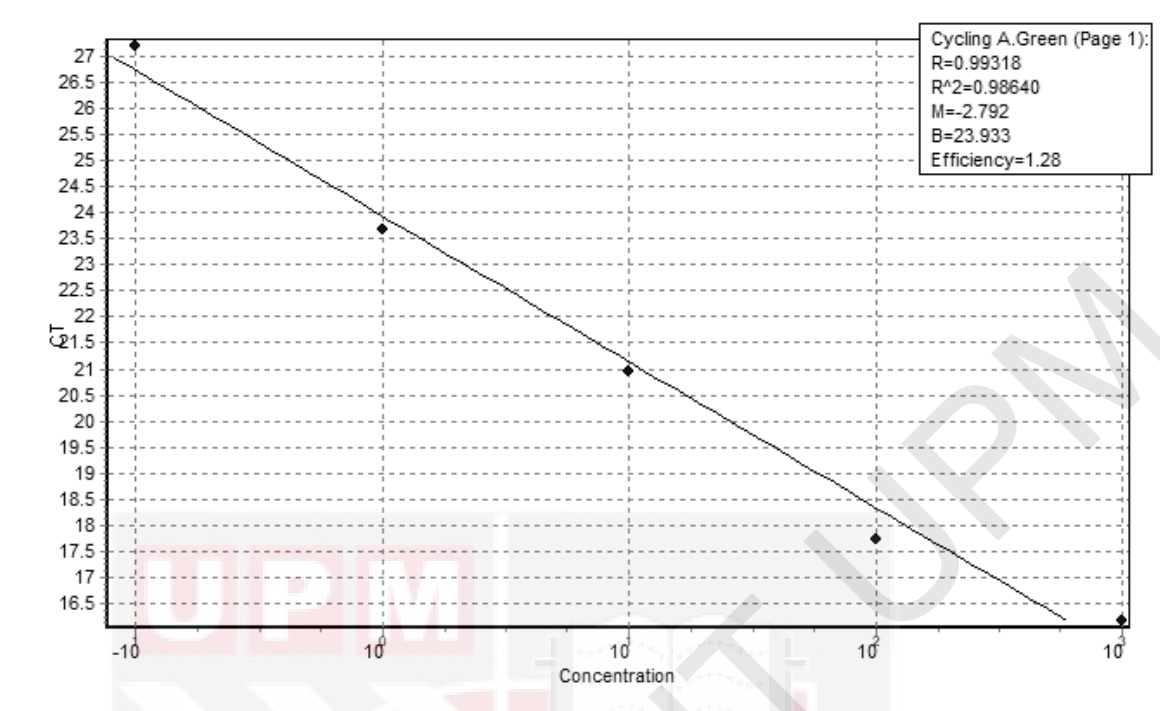
Appendix 4K. Crustin melting curve



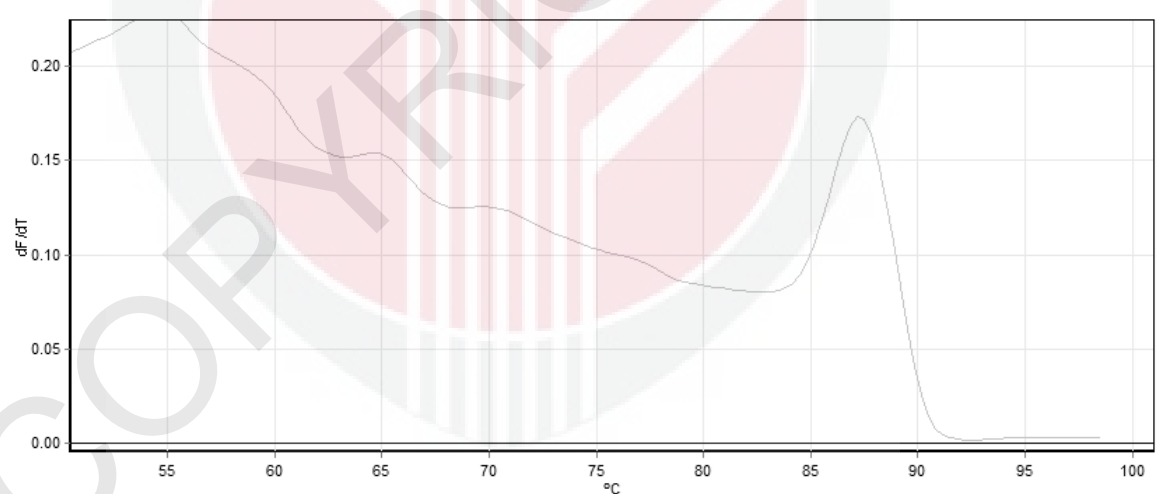
Appendix 4L. Toll-like receptor (TLR) standard curve



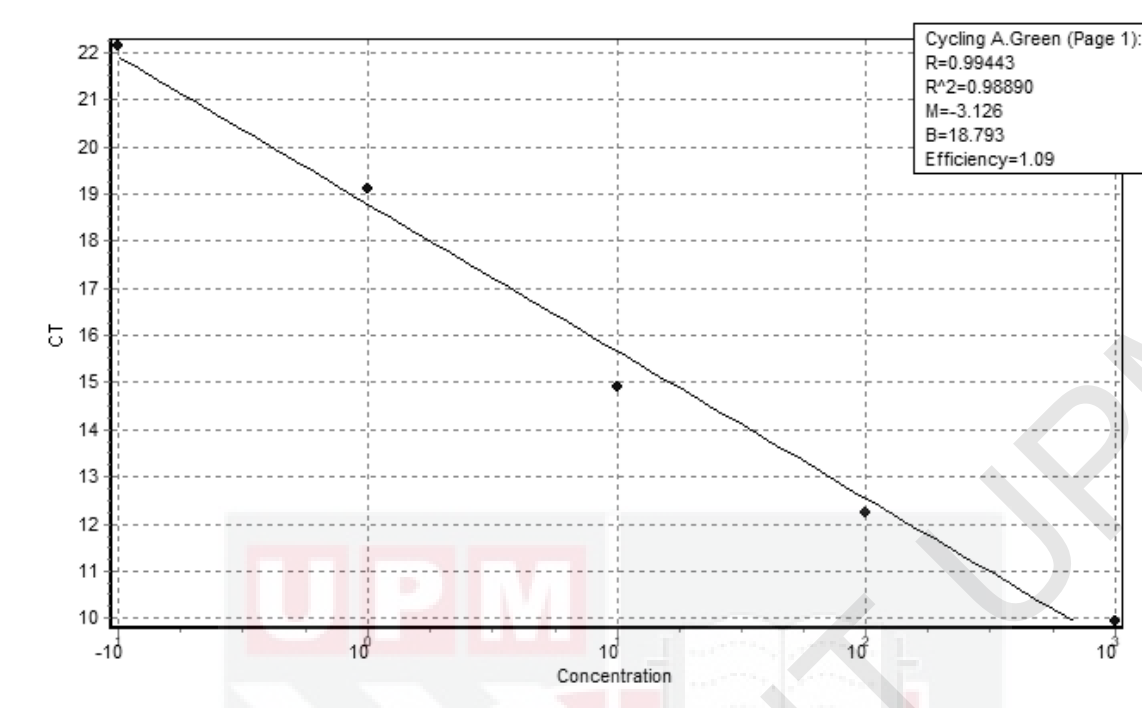
Appendix 4M. Toll-like receptor (TLR) melting curve



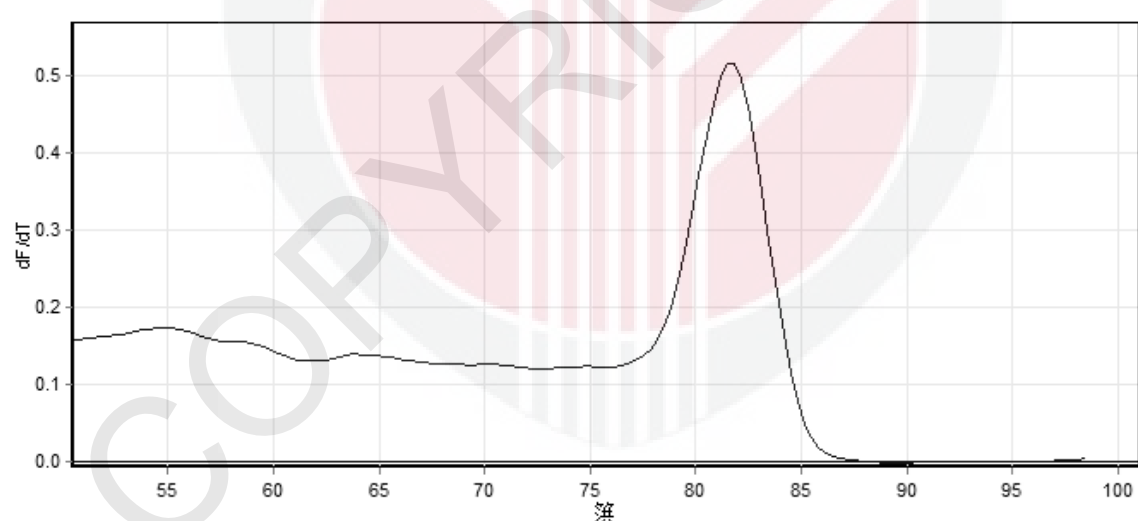
Appendix 4O. Penaeidin standard curve



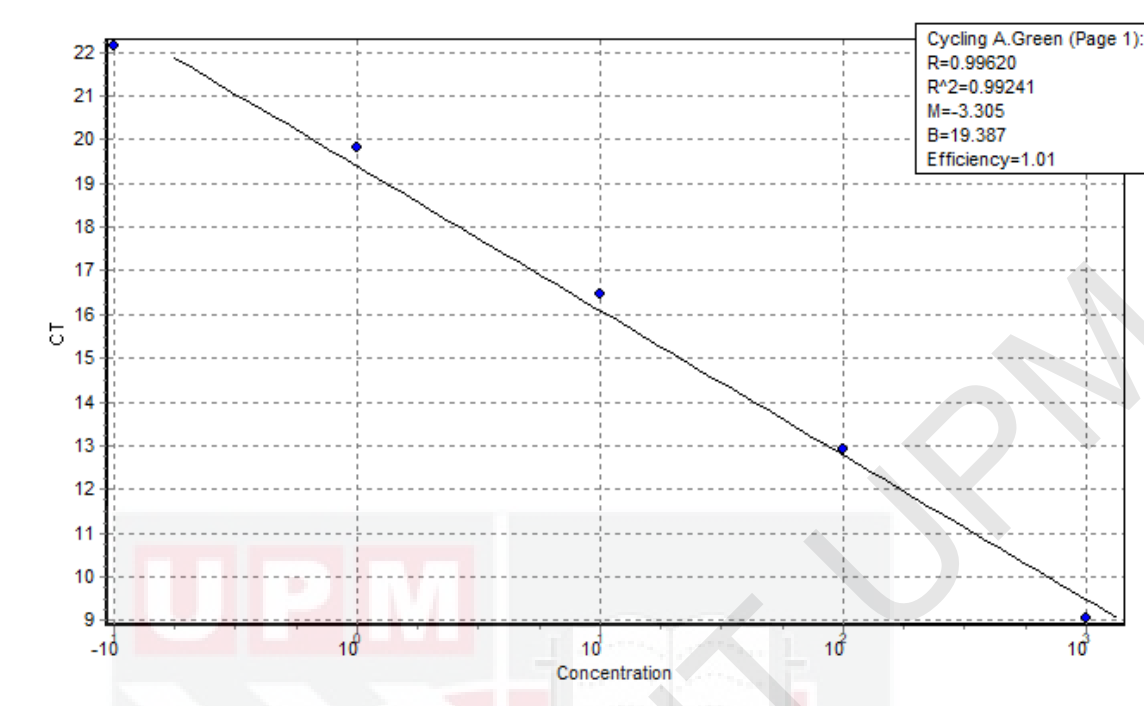
Appendix 4P. Penaeidin melting curve



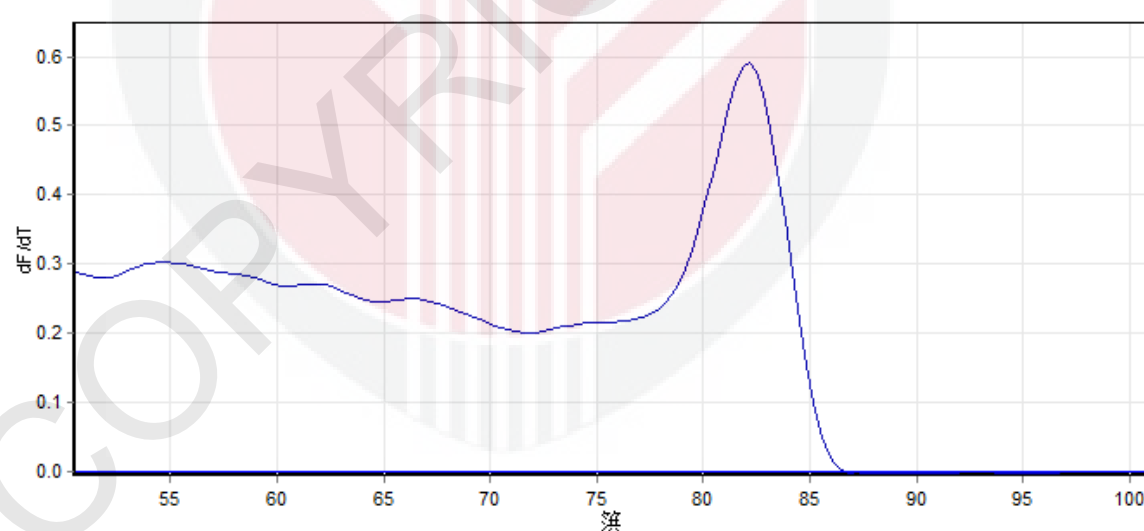
Appendix 4Q. Elongation factor-alpha standard curve



Appendix 4R. Elongation factor-alpha melting curve



Appendix 4S. Beta actin standard curve



Appendix 4T. Beta actin melting curve

Appendix 5

Institutional Animal Care And Use Committee (IACUC)



PEJABAT TIMBALAN NAIB CANSOLOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date: 6th January 2023

AUP No.: UPM/IACUC/AUP-R061/2022

Project Title: Assessment of Synbiotic Feed Against Acute Hepatopancreas Necrosis Disease (AHPND) in Black Tiger Shrimp (*Penaeus Monodon*)

Principal Investigator: Assoc. Prof. Dr. Ina Salwany Md Yasin

Members: Chin Yong Kit, Wan Haifa Haryani Wan Omar, Amatul Samahah Md. Ali, Mohd Ihsanuddin Ahmad.

Attending Veterinarian: Assoc. Prof. Dr. Annas Salleh

Committee Decision: The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.

Project Classification: Chronic

Category of Invasiveness: B

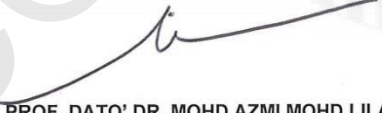
Source of Animals: **Unibionesto Sdn Bhd.**
Jalan Teluk Senangin,
32200 Lumut, Perak

Number of Animals Approved: 1,020 Shrimp

Housing: AquaHealth Hatchery, Institute of Bioscience, UPM

Duration: 6th January 2023 – 6th January 2024

Ethical approval is required in the case of amendments to the approved animal utilisation protocol (AUP). Please apply using Form 105. Kindly submit a final/annual report (Form 106) upon study completion, or before expiry of approval.


PROF. DATO' DR. MOHD AZMI MOHD LILA
Chairman
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

✉ Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) ☎ 603-9769 1002, Pejabat Pentadbiran TNCPi ☎ 603-9769 1608, Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) ☎ 603-9769 1610, Pejabat Pengarah, Putra Science Park (PSP) ☎ 603-9769 1291
🌐 <http://www.tncpi.upm.edu.my>

BIODATA OF STUDENT



Chin Yong Kit was born in Ipoh, Perak in 1993 and grew up in Puchong, Selangor. He received his primary and secondary education at Sekolah Jenis Kebangsaan Han Ming Puchong Batu 14, Sekolah Menengah Kebangsaan Puchong Perdana and Sekolah Menengah Kebangsaan Pusat Bandar Puchong (1). Then, he study at Universiti Malaysia Terengganu (UMT), Gong Badak, Terengganu for First degree in Bachelor of Agrotechnology Science (Aquaculture). He graduated in Master degree (Aquatic Biotechnology) from Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) under supervision of Prof. Dr Ina Salwany Md Yasin and co-supervised with Dr. Chong Chou Min and Dr. Zarirah Mohamed Zulperi at year 2019. Currently, He graduated also in PhD degree (Microbial Biotechnology) from Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) under supervision of Prof. Dr Ina Salwany Md Yasin and co-supervised with Prof. Dr. Mohammad Noor Amal Azmai, Prof. Dr. Murni Marlina Abd Karim and Dr. Norhariani Mohd Nor at year 2025.

LIST OF PUBLICATIONS

Published papers:

- Chin, Y. K., Haifa-Haryani, W. O., Nazarudin, M. F., Ahmad, M. I., Azzam-Sayuti, M., Ali, N. S. M., Ngalmat, M. S., Mohamad, A., Ida-Muryany, M. Y., Karim, M., Salleh, A., Norhariani, M. N., Amal, M. N. A., & Ina-Salwany, M. Y. (2024a). In vivo assessment of *Lactobacillus plantarum* strains in black tiger shrimp (*Penaeus monodon*): implications for growth performance, probiotic-pathogen interaction, and defense against AHPND infection. *Aquaculture International*. DOI: 10.1007/s10499-023-01366-3
- Chin, Y. K., Haifa-Haryani, W. O., Nazarudin, M. F., Ahmad, M. I., Azzam-Sayuti, M., Mohamad, A., Ida-Muryany, M. Y., Karim, M., Salleh, A., Amal, M. N. A., Norhariani, M. N., & Ina-Salwany, M. Y. (2024b). The synergistic *Lactobacillus plantarum* L20 and *Sargassum polycystum* - added diet for improvement of Black tiger shrimp, *Penaeus monodon* 's growth, immune responses, bacterial profiles, and resistance against *Vibrio parahaemolyticus* associated Acute hepatopancreatic necrosis disease (AHPND) infection. *Aquaculture Reports*, 34: 101903. DOI: 10.1016/j.aqrep.2023.101903
- Chin, Y. K., Azzam-Sayuti, M., Mohamad, A., Haifa-Haryani, W. O., Ahmad, M. I., Nazarudin, M. F., Ali, N. S. M., Ida-Muryany, M. Y., Karim, M. M. A., Salleh, A., Nor, N. M., Azmai, M. N. A., & Ina-Salwany, M. Y. (2025). Elucidation of synbiotic diet comprising of *Lactobacillus plantarum* L20 and *Sargassum polycystum* on gastrointestinal microbiota, tissue structures and AHPND associated dysbiosis susceptibility in black tiger shrimp (*Penaeus monodon*). *Aquaculture*, 594: 741339. DOI: 10.1016/j.aquaculture.2024.741339