ELUCIDATION OF INACTIVATED MICROBIAL WITH *Sargassum* sp. AS IMMUNOSTIMULANTS AGAINST *Vibrio parahaemolyticus* INDUCING ACUTE HEPATOPANCREATIC NECROSIS DISEASE IN *Penaeus vannamei* (BOONE, 1931)

By

AMATUL SAMAHAH BINTI MD. ALI

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

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Chair: Assoc. Prof Ina Salwany Md Yasin, PhD
Faculty: Agriculture

Acute hepatopancreatic necrosis disease (AHPND) is an emerging disease in shrimp aquaculture industry caused by *Vibrio parahaemolyticus*. It imposes a serious threat to shrimp production through mass mortality of post-larval shrimp resulting USD 1 billion loss to the shrimp industry worldwide including Malaysia. Therefore, this study aimed to characterize *V. parahaemolyticus* strains causing AHPND in shrimp from a local shrimp farm in Malaysia, to develop an inactivated microbial immunostimulant from *V. parahaemolyticus* for protection against AHPND in shrimp, to conduct immunization trials of the inactivated microbial in combination with *Sargassum* sp. as immunostimulants in shrimp infected with AHPND, and to study the effect of immunization in shrimp immune system at the transcriptional level. In this study, isolates associated with AHPND outbreak had been isolated previously from a shrimp farm in Terengganu, Malaysia, and were further characterized. Based on the phenotypic characterization and phylogenetic analysis, strain C2A and C4B were identified as *V. parahaemolyticus*, meanwhile, strain C1B, C2B, C4A, and C5 were *V. harveyi*. This study suggested that, in Malaysia, both *V. parahaemolyticus* and *V. harveyi* could be the pathogen that caused AHPND outbreak in a local shrimp farm. The most virulent AHPND positive isolate in this experiment is *V. parahaemolyticus* C4B. The draft genome sequence of C4B were also compared with *V. parahaemolyticus* P24 which is a non-causing AHPND strain. The genome assembly metrics for revealed features transposons and insertion sequences, and bacteriophages are more abundant in the *V. parahaemolyticus* VP_{AHPND} C4B genome compared to *V. parahaemolyticus* $VP_{NON\text{-}AHPND}$, P24, reflecting the organism's genome plasticity and pathogenic features. Despite these variations, all genomes exhibited greater than 98.0% average nucleotide identity (ANI), indicating they belong to the same species. Notably, *V. parahaemolyticus* AHPND strains NCKU_TV_5HP and NCKU_CV_CHN showed

ANI indices of 98.46% and 98.43%, respectively, when compared to strain C4B. Next is to develop an inactivated bacterial with *Sargassum* sp. as immunostimulants as potential protection against the AHPND in shrimp. The treatment group for this study was as follows, group 1: commercial feed, group 2: immunostimulant $1x10^3$ CFU kg/feed, group 3: immunostimulant $1x10^5$ CFU kg/feed, group 4: immunostimulant $1x10^7$ CFU kg/feed, group 5: immunostimulant $1x10^3$ CFU kg/feed + 2% *Sargassum* sp., group 6: immunostimulant $1x10_5$ CFU kg/feed + 2% *Sargassum* sp., group 7: immunostimulant $1x10^7$ CFU kg/feed + 2% *Sargassum* sp., and group 8: 2% *Sargassum* sp. After four weeks of the treatment period, immunostimulants with *Sargassum* sp. treatment groups (groups 5, 6, and 7) showed the highest percentage (>60%) of shrimp survival compared to treatment groups without *Sargassum* sp. Meanwhile, group 6 showed the highest shrimp survival after four weeks of immunization. After the challenge study, group 6 also showed the highest survival percentage, 64% compare to other treatment groups indicating that the immunostimulant of 1×10^5 CFU with *Sargassum* sp. was the best treatment in this study to increase disease against AHPND and to prevent mortalities in shrimp. The effect of the immunization using the immunostimulants had been further elucidated at the transcriptional level to find out its immune response on immunized shrimp's gene expression compared to control group. Based on the differentially expressed genes (DEGs) detected in the KEGG pathway database, several notable changes in the immune-related genes such as antimicrobial peptides (anti-lipopolysaccharide factor, penaeidin, crustin), prophenoloxidase (proPO) gene cascade and upregulation of antioxidant gene expressions were identified following the immunization. This study's findings provide recent data on AHPND-associated isolates such as *V. parahaemoluticus* and *V. harveyi,* insights into the genome and its virulence, and information on the use of inactivated microbial with *Sargassum* sp. as immunostimulants as a means of disease protection coherent for sustainable prawn global production.

Keywords: Acute Hepatopancreatic Necrosis Disease, Inactivated Microbial Immunostimulant, *Penaeus vannamei*, *Sargassum* sp., *Vibrio parahaemolyticus*

SDG: GOAL 4: Quality Education

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

PENENTUAN MIKROB TERNYAHAKTIF DAN *Sargassum* sp., SEBAGAI IMUNOSTIMULAN MELAWAN PENYAKIT NEKROSIS HEPATOPANKREATIK AKUT OLEH *Vibrio parahaemolyticus* DALAM *Penaeus vannamei* (BOONE, 1931)

Oleh

AMATUL SAMAHAH BINTI MD. ALI

Julai 2023

Pengerusi: Prof. Madya Ina Salwany Md Yasin, PhD
Fakulti: Pertanian

Penyakit nekrosis hepatopankreatik akut (AHPND) adalah penyakit yang muncul dalam industri akuakultur udang yang disebabkan oleh *Vibrio parahaemolyticus.* Penyakit ini memberi ancaman serius kepada kadar penghasilan udang akuakultur kerana menyebabkan kematian benih udang secara besar-besaran sehingga mengakibatkan kerugian sebanyak USD 1 bilion kepada industri udang di seluruh dunia termasuk di Malaysia. Oleh itu, kajian ini bertujuan untuk mencirikan strain *V. parahaemolyticus* penyebab AHPND yang dipencilkan dari udang ladang tempatan di Malaysia. Seterusnya, membangunkan imunostimulan mikrob ternyahaktif daripada *V. parahaemolyticus* dengan gabungan *Sargassum* sp. sebagai perlindungan terhadap AHPND dalam udang, mengkaji kesan imunostimulan kepada udang yang dijangkiti AHPND, dan seterusnya untuk menjalankan kajian transkriptomik bagi mengkaji kesan pemberian imunostimulan terhadap sistem keimunan udang. Dalam kajian ini, isolat yang dikaitkan dengan wabak AHPND telah dipencilkan sebelum ini daripada ladang ternakan udang di Terengganu, Malaysia dicirikan dengan lebih lanjut. Berdasarkan pencirian fenotip dan analisis filogenetik, strain C2A dan C4B dikenalpasti sebagai *V. parahaemolyticus*, manakala strain C1B, C2B, C4A, dan C5 adalah *V. harveyi.* Berdasarkan penemuan ini, di Malaysia, kedua-dua *V. parahaemolyticus* dan *V. harveyi* boleh menjadi patogen yang menyebabkan wabak AHPND di ladang udang tempatan. Berdasarkan kajian virulensi menggunakan *Artemia* sp., isolat positif AHPND yang paling virulen dalam eksperimen ini ialah *V. parahaemolyticus* C4B. Draf jujukan genom C4B yang dipencilkan dari *P. vannamei* juga dibandingkan dengan *V. parahaemolyticus* P24 yang merupakan strain yang tidak menyebabkan AHPND. Analisis metrik penjujukan genom bagi *V. parahaemolyticus* VP_{AHPND} C4B menunjukkan terdapat unsur-unsur gen transposon, gen jujukan sisipan dan bakteriofaj lebih banyak berbanding dalam genom *V. parahaemolyticus* $VP_{NON\text{-}AHPND}$, P24, yang menggambarkan keplastikan genom dan ciri-ciri patogenik isolat tersebut. Walaupun semua genom mempamerkan lebih daripada 98.0% identiti nukleotida purata (ANI), namun isolat-isolat adalah tergolong dalam spesies yang sama. Terutamanya, *V. parahaemolyticus* AHPND strain NCKU_TV_5HP dan NCKU_CV_CHN menunjukkan indeks ANI masing-masing 98.46% dan 98.43% identikal berbanding dengan strain C4B. Seterusnya adalah untuk membangunkan bakteria ternyahaktif dengan *Sargassum* sp. sebagai imunostimulan sebagai perlindungan terhadap jangkitan AHPND dalam udang. Kumpulan rawatan untuk kajian ini adalah seperti berikut, kumpulan 1: diet komersial, kumpulan 2: imunostimulan $1x10^3$ CFU kg/makanan, kumpulan 3: imunostimulan $1x10^5$ CFU kg/makanan, kumpulan 4: imunostimulan $1x10^7$ CFU kg/makanan, kumpulan 5: imunostimulan $1x10^3$ CFU kg/makanan + 2% *Sargassum* sp., kumpulan 6: imunostimulan $1x10^5$ CFU kg/makanan + 2% *Sargassum* sp., kumpulan 7: imunostimulan $1x10^7$ CFU kg/makanan + 2% *Sargassum* sp., dan kumpulan 8: 2% *Sargassum* sp. Selepas empat minggu tempoh rawatan, imunostimulan dengan *Sargassum* sp. kumpulan rawatan (kumpulan 5, 6, dan 7) menunjukkan peratusan tertinggi (>60%) kemandirian udang berbanding kumpulan rawatan tanpa *Sargassum* sp. Manakala kumpulan 6 menunjukkan kemandirian udang tertinggi selepas empat minggu imunisasi. Selepas kajian jangkitan AHPND, kumpulan 6 juga menunjukkan peratusan kemandirian udang tertinggi, 64% berbanding kumpulan rawatan lain iaitu menunjukkan bahawa imunostimulan 1×10^5 CFU dengan *Sargassum* sp. merupakan rawatan terbaik dalam kajian ini untuk meningkatkan ketahanan udang terhadap penyakit AHPND dan untuk mencegah kematian dalam udang. Kesan imunisasi menggunakan imunostimulan telah dijelaskan lebih lanjut pada peringkat transkripsi dengan mengetahui tindak balas imun udang terhadap ekspresi gen udang yang diimunisasi berbanding kawalan. Berdasarkan gen yang dinyatakan secara berbeza (DEG) yang dikesan dalam pangkalan data laluan KEGG, beberapa perubahan ketara dalam gen berkaitan imun seperti peptida antimikrob (faktor anti-lipopolisakarida, penaeidin, krustin), gen profenoloksidase (proPO) dan pengawalan antioksidan. Penemuan kajian ini memberikan data terkini tentang pencilan isolat berkaitan AHPND seperti *V. parahaemoluticus* dan *V. harveyi*, pandangan tentang genom dan virulensinya, dan maklumat tentang penggunaan imunostimulan mikrob ternyahaktif dengan *Sargassum* sp. sebagai cara perlindungan dan kerintangan penyakit yang seiring untuk pengeluaran global udang yang mampan.

Keywords: Imunostimulan Mikrob Ternyahaktif, Penyakit Nekrosis Hepatopankreatik Akut, *Penaeus vannamei*, *Sargassum* sp., *Vibrio parahaemolyticus*

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Ina Salwany binti Md. Yasin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Natrah Fatin binti Mohd Ikhsan, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Mohammad Noor Amal bin Azmai, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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%	percentage
µl	microlitre
µM	micromolar
bp	base pair
CFU	colony forming units
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
H&E	haematoxylin and eosin
His (H)	Histidine
Ig	immunoglobulin
kb	kilobase pair
LD_{50}	Median lethal dosage
LPS	Lipopolysaccharide
M	Molar
mg	milligram
mM	millimolar
NGS	Next Generation Sequencing
PBS	Phosphate Buffered saline
PCR	Polymerase chain reaction
RNA	ribonucleic acid
RPM	revolutions per minute
RPS	relative percentage survival
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background of study

Marine shrimp was among the highest demand in aquaculture, as much as USD 34.2 billion (5.51 MT) followed by freshwater crustaceans at USD 24.3 billion (2.53 MT). Shrimp contributes about 6% and 16% of the global aquaculture and value of traded seafood production respectively. However, a disease outbreak in the aquaculture system has rendered economic losses as estimated by the Food and Agriculture Organization (FAO) to be over USD 9 billion per year. Acute Hepatopancrease Necrosis Disease (AHPND) is one of the new progressive diseases in the shrimp aquaculture activities. AHPND established a serious threat to shrimp production globally and it has been recorded to cause USD 1 billion loss in the shrimp industries (Lighter et al., 2012). The outbreak of this was earliest mentioned in Southern China in 2009 by Zhang et al., (2012). Not long after that, AHPND was reported in Vietnam in 2010 (Lighter et al., 2012). Next, the disease was also reported in other countries for example Malaysia in the year 2011 (Chu et al., 2016), Thailand in the year 2012 (Flegel, 2012; Leaño and Mohan, 2012), Mexico in year 2013 (Soto-Rodriguez et al., 2015), Philippines in the year 2015 (Dabu et al., 2015) and United States of America (USA) in the year 2017 (Meza, 2017). Malaysia was also affected as outbreaks of AHPND in white-leg shrimp farms resulted in the reduction of total shrimp production from 87,000 MT in 2010 to 67,000 MT in 2011, 55,000 MT in 2012, and 50,000 MT in 2013 (Annual Fisheries Statistics, 2005-2014). Referring on the calculated shrimp production losses from 2011 to 2014, the total economic deficit from AHPND serial outbreak episodes were projected up to USD 0.49 billion (Chu et al. 2016). AHPND exerted the worst effect on the shrimp aquaculture activities, which developed as soon as 8 days after stocking and induced 100% severe mortalities within 20 to 30 days (Choi et al., 2017; Kumar et al., 2020).

Vibrio parahaemolyticus has been identified to be the agent of AHPND in shrimp (Sirikharin et al., 2015). Nonetheless, pathogens those acquiring pVA1 plasmid which codes binary toxins can cause AHPND (Lee et al., 2015). The pathogen with this specific plasmid produced and expressed toxins that are structurally similar to the *Pir* (*Photorhabdus insect-related*) binary toxin which inclusive of two subunits, $PirA_{vp}$ and $PirB_{vp}$ (Dong et al., 2019; Powers et al., 2021). Up to date, there have been reports that the plasmid also can be identified in other species such as *V. harveyi* (Kondo et al., 2015), *V. campbelli* (Dong et al., 2017) and *V. owensii* (Liu et al., 2015). AHPND affects post-larvae and juvenile shrimp within the first month (30 days) of stocking in a grow-out pond (Tran et al., 2013). Symptoms such as loose shells, slow growth, and discoloration were among the early clinical signs of this disease (Leaño and Mohan, 2012). This is how the disease which was previously known as Early Mortality Syndrome (EMS), was given a more specific name, Acute Hepatopancrease Necrosis Disease (AHPND) as the etiological cause of the disease determined (Tran et al., 2013).

Antibiotics have been commonly used in aquaculture to prevent and treat bacterial diseases in shrimp (Li et al., 2021). The emergence of antibiotic resistance strains, bioaccumulation in tissues, and potential health hazards have caused banned and restrictions imposed on the usage of antibiotics (Arsene et al., 2022; Rodriguez et al., 2007; Vidović and Vidovic 2020). Studies have shifted towards prophylaxis measures such as the usage of immunostimulants and vaccines. Patil et al. (2013) showed that the formalin-inactivated Vibrio vaccine given by an oral administration to *Fenneropenaeus mernguensis* post-larvae induces protection against *V. anguillarum* and *V. harveyi*. The enhancement of shrimp immunity has also been observed in other studies (Lin et al., 2013, Powell et al., 2011; Wongtavatchai et al., 2010). In another research, Pope et. al (2011) reported that the shrimp immune system can show a certain level of specificity after being vaccinated against *V. harveyi*. Pope et al. (2011) showed that hemocytes from vaccinated shrimp showed enhanced levels of phagocytosis after challenge *V. harveyi*, but not *Bacillus subtilis*. When shrimp were injected with *B. subtilis* rather than Vibrio, there was no significant increment in the phagocytic activity of hemocytes. This indicated that a certain level of immune specificity exists in the shrimp immune system.

Sargassum, a genus of brown macroalgae, has gained attention in recent years for its potential as an immunostimulant in aquaculture (Devoult et al., 2021). Rich in bioactive compounds, Sargassum extracts have demonstrated immunomodulatory properties that can enhance the immune response in aquatic organisms (Pratiwy and Pratiwi, 2020). The polysaccharides, polyphenols, and other bioactive molecules present in Sargassum can stimulate the production of immune-related molecules and enhance the activity of immune cells in fish and shrimp (Abbas et al., 2023). By incorporating Sargassum-based additives into aquaculture feeds, researchers aim to ameliorate the immune systems of farmed aquatic species, thus improving their resistance to diseases (Ying, 2008). This natural immunostimulant approach aligns with the growing trend in sustainable and eco-friendly aquaculture practices, offering a promising avenue to enhance the overall health and resilience of cultured marine organisms.

1.2 Problem Statement

The impact and mechanisms of infection by local strains of *V. parahaemolyticus* causing AHPND (VP_{AHPND}) on penaeid shrimps were not well-documented. Therefore, it is time for research on the establishment and pathogenesis of VP_{AHPND} infection to be carried out in penaeid shrimps in Malaysia. To control the outbreak, at the moment, effective shrimp farm management was adopted including disinfection of the water supply, use of reservoirs for microbial mature water, removing pond sludge/sediment as often as possible, use of probiotics, using clean feeds, and screening of broodstock and post-larvae from disease while some resorted to the usage of antibiotics. All of these steps taken helped in controlling the outbreak of the disease. Even so, there are still outbreak cases that occurred recently for example, in Philippines, USA and Mexico (Dabu et al., 2015; Durán-Avelar et al., 2018; Meza, 2017; Tang and Bondad-Reantaso, 2019).

Since the usage of antibiotic has been banned and restricted (Arsene et al., 2022; Vidović and Vidovic 2020), methods that can enhance shrimp's natural immune response and up-modulate disease resistance have attracted much attention recently. An alternative solution like enhancing the shrimp's natural immunity (by immunostimulants) could be used as another option to control the disease (Muahiddah and Diamahesa, 2022). Immunostimulants derived from bacteria, algae, animals, nutritional factors, and hormones/cytokines can provide protective immunity and help fight against diseases in shrimp (Kumar and Bossier, 2019). Although there is a lot of research has been conducted to use immunostimulants as an immune enhancer, a combination of the usage of immunostimulants derived from bacteria with a natural source of prebiotic ie. seaweed, *Sargassum* sp. in shrimp disease is still yet to be reported. However, thorough and careful studies are needed to ascertain the efficacy combination of immunostimulants and prebiotics and their practical usage in shrimp aquaculture systems in Malaysia.

1.3 Significance of the study

The significance of characterizing pathogen isolates from a local shrimp farm in Malaysia is to give insights into the existence and characteristics of the virulent bacterial isolates in certain shrimp culture systems in Malaysia and their potential risk to shrimp survivability. The pathogenicity of *V. parahaemolyticus* can vary among different isolates, and whether they are local or non-local can play a role in the disease infections they cause. The local environment, including water quality, temperature, and other ecological factors, can influence the prevalence and pathogenicity of *V. parahaemolyticus* isolates. Strains adapted to the local environmental conditions may have a higher likelihood of causing infections.
In addition, current global genomic data on *Vibrio* sp. in the different geographical regions are still lacking. This study will determine the whole-genome sequencing of *Vibrio* spp. isolated from a shrimp farm in Malaysia to provide a draft whole-genome sequence that would contribute to the worldwide database. This study will analyze a single genomic structure and comparative data of other *Vibrio* sp. strains isolated from other locations in the world. The draft genome of *Vibrio* sp. will be useful for further research on the influence of their virulency in different shrimp or hosts. In addition, this data will facilitate other researchers on the understanding of the metabolism genes, novel putative virulence gene clusters

as well as various pathways present in the draft genome of *Vibrio* sp. for future metabolic studies.

The significance of immunizing shrimp with inactivated microbial immunostimulant includes enhancing the immune response of shrimp to protect shrimp from AHPND. A prebiotic treatment will also be included in the immunization trials to observe its effect when combines with inactivated microbial stimulant. This study will give a proper understanding of the immunization strategy which includes the decision on which diseases to be immunized against, as well as the type of immunostimulant to be used, immunostimulant dosages, types of additives to be used, immunization method, and time sequence of immunization. Moreover, this study provides an understanding of good method administration of immunostimulants which oral administration is easier because of its non-stressful nature, a more cost-effective option, extensive usage at little expense and effort. Immunization at the early stage of shrimp life is crucial to prolong shrimp survivability and boost shrimp immune response to prevent lethal *Vibrio* sp. infection.

Additionally, the pathways involving immune-related genes on treated immunized shrimp will also be analyzed via transcriptomic analysis. A functional transcriptomic analysis is an interesting method of obtaining insights into the molecular basis of immune reactions in species, especially in shrimp, where little research has been done. In shrimp studies, expressed sequence tags (ESTs) analysis has helped to converge knowledge on genes with similarity to known immune function genes from other organisms that can respond to immune stimulation in shrimp. The data obtained provide new insights into the molecular mechanisms of the shrimp host response to AHPND disease and provide a resource for molecular marker development at the transcriptomic level.

1.4 Objectives

The objectives of this research are as follows:

a) To isolate and characterize *V. parahaemolyticus* causing AHPND from local shrimp farms in Malaysia
b) To study the whole genome structure of the *V. parahaemolyticus* causing AHPND
c) To develop and evaluate the efficacy treatment of inactivated microbial immunostimulant in combination with *Sargassum* sp. for protection against *V. parahaemolyticus* causing AHPND
d) To elucidate the effect of an administration of inactivated microbial immunostimulant in combination with *Sargassum* sp. in shrimp immune response at the transcriptomic level

1.5 Hypothesis

The hypothesis of this study are as follows:

Hypothesis 1: The local strains of *V. parahaemolyticus* causing AHPND show the same pathogenicity to the shrimp post-larvae by producing consistent pathological changes post-infection as reported.

Hypothesis 2: The genome structure of the local isolate of *V. parahaemolyticus* causing AHPND containing the *PirA* and *PirB* toxin genes in penaeid shrimps is the same as those that have been reported.

Hypothesis 3: Immunization of post-larvae shrimp with inactivated microbial immunostimulant in combination with *Sargassum* sp. at a specific dose could stimulate the immune response thus protecting against AHPND infection.

Hypothesis 4: Shrimp immune response is enhanced towards the treatment of inactivated microbial immunostimulant in combination with *Sargassum* sp. which could be observed at the transcriptomic level.

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