UNIVERSITI PUTRA MALAYSIA

ORAL ADMINISTRATION ASSESSMENT OF OUTER MEMBRANE PROTEINS OF Vibrio alginolyticus ON THE GROWTH OF GIANT FRESHWATER PRAWNS (Macrobrachium rosenbergii De Man)

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By

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Chairman : Associate Professor Sabri Bin Mohd Yusoff, PhD
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The production of prawns is unarguably a fast-growing global aquaculture. This is evident in the culture of freshwater prawns which is rapidly gaining momentum in terms of productions and values in Malaysia and the world at large. *Vibrio alginolyticus* is one of the most pathogenic species of *Vibrio* that cause high mortality in freshwater prawns. The conventional use of antibiotics in the treatment of this disease has remained ineffective and resulted in an exponential increase in virulence and pathogenicity of the microbes. Hence, the need for better and practicable measures of disease prevention and treatment. This was the first study to investigate the effects of oral administration of outer membrane protein of vibrio in *Macrobrachium rosenbergii* experimentally infected with *V. alginolyticus*. Prawns were divided into three groups A, B, and C of 10 prawns each with replicates in 6 (150 L) glass aquaria. Group A was fed with OMPs-mixed diet, group B with OMPs-FIA (Freund's incomplete adjuvants) mixed diet while group C was fed with OMPs or adjuvant-free diet (control diet). Groups A and B were fed for seven days, alternated with control diet for seven days and a booster dose for another seven days. All prawns were weighed weekly, and haemolymph was collected to examine the total haemocytes counts (THC), phenoloxidase activity (PO) and the presence of OMPs in the haemolymph. All prawns were challenged intramuscularly with 50 µL of 10^7 CFU of *V. alginolyticus*. The results of the analysis revealed significance difference in mean weight gain and THC ($P < 0.05$) between the treated groups and the control but not with PO activity. Although there was no significant difference ($P > 0.05$) in the level of mortality in all the groups after 24 h, this was not unconnected to pains from the injection coupled with stress, as this was also observed in blank control (not challenged with *V. alginolyticus*). In the second experiment, 45 prawns were divided into three different groups of 15 prawns each. Group A was treated with formalin killed *Vibrio* cell by immersion only, B with OMPs by intramuscular injection and boosted by immersion and C with PBS by immersion only at days 0, 3, 5 and 7. A bacterial challenge was carried out by immersion on day 9 and observed for mortality

for seven days. The total haemocyte count (THC) increased in the treatment groups more than the control but no significant difference in the level of THC increment between the treatment groups. There was no mortality in the treatment groups, but the mortality rate in the control group was 55% over the period of seven days. Haemolymph (both coagulated and non-coagulated) that was also collected to detect the presence of OMPs in the system using SDS-PAGE revealed no bands of OMPs but only those of the plasma proteins, this could be as a result of natural clearance activity of the prawns to get rid of foreign agents. Gross examination of the experimentally challenged prawns was carried out following the mortality and tissues were processed for histopathological lesions and immuno-histochemical reaction. The untreated group showed more pronounced lesions than the treatment groups. This study, however, concluded that oral administration of OMPs with or without Freund's incomplete adjuvant is a good growth promoter and has the potential for protection against vibriosis in *Macrobrachium rosenbergii* when administered with unique antigen protection vehicle and at appropriate dosages, but the protection may be for a short period of time.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENILAIAN PENGAMBILAN PROTEIN MELALUI MULUT MEMBRAN LUAR *Vibrio alginolyticus* TERHADAP PERTUMBUHAN UDANG GALAH (*Macrobrachium rosenbergii* DE MAN)

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Pengeluaran udang dengan jelasnya merupakan global akuakultur yang berkembang dengan pesat. Ini dibuktikan dengan kultur udang air tawar di mana ianya mendapat momentum yang besar dari segi pengeluaran dan harga di Malaysia dan di dunia secara amnya. *Vibrio alginolyticus* ialah salah satu daripada sepsis *Vibrio* yang paling patogenik yang menyebabkan kematian yang banyak di kalangan udang air tawar. Penggunaan antibiotik secara konvensional dalam rawatan penyakit ini masih kekal tidak berkesan dan menyebabkan peningkatan eksponen dalam kemudaratan dan kepatogenan mikrob ini. Oleh itu, langkah yang lebih baik dan praktikal untuk menangani penyakit ini dan rawatannya sangat diperlukan. Kajian ini adalah menjadi yang pertama mengkaji kesan-kesan pemberian selaput luar protein *Vibrio* secara oral dalam *Macrobrachium rosenbergii* yang dijangkiti oleh *V. alginolyticus*. udang dibahagikan kepada tiga kumpulan A, B, dan C yang terdiri daripada 10 udang setiap satu dengan 6 replikasi dalam (150 L) akuarium kaca. Kumpulan A diberi makan dengan diet campuran-OMPs, Kumpulan B dengan diet campuran OMPs-FIA (adjuvant tak lengkap Freund), manakala Kumpulan C diberi makan OMPs atau diet bebas adjuvant (diet kawalan). Kumpuan A dan B diberi makan selama 7 hari, diselangi dengan diet kawalan selama 7 hari dan satu dos penggalak untuk 7 hari lagi. Kesemua udang ditimbang setiap minggu dan hemolimfa diambil untuk pemeriksaan jumlah kiraan hemosit (THC), aktiviti fenoloksidas (PO) dan kehadiran OMPs di dalam hemolimfa. Kesemua udang dicabar secara intraotot dengan 50 µL 10^7 CFU *V. alginolyticus*. Keputusan analisa menunjukkan perbezaan yang signifikan bagi min penambahan berat badan dan THC ($P < 0.05$) di antara kumpulan rawatan dan kumpulan kawalan tetapi tidak dengan aktiviti PO. Walaupun tiada perbezaan yang signifikan ($P > 0.05$) bagi tahap kematian dalam semua kumpulan selepas 24 jam, ini bukan berkaitan dengan kesakitan disebabkan suntikan beserta tekanan, kerana ini turut diperhatikan di dalam kawalan blank (tidak dicabar dengan *V. alginolyticus*). Dalam percubaan kedua, 45 udang telah dibahagikan kepada tiga kumpulan yang berbeza daripada 15 udang setiap satu. Kumpulan A telah dirawat dengan formalin

membunuh sel Vibrio oleh rendaman sahaja, B dengan Omps melalui suntikan intramuskular , dirangsang oleh keasyikan dan C dengan PBS oleh rendaman hanya pada hari 0 , 3, 5 dan 7. Cabaran bakteria telah dijalankan oleh rendaman pada hari 9 dan diperhatikan untuk kematian selama tujuh hari. Total perkiraan haemocyte (THC) meningkat dalam kumpulan rawatan lebih daripada kawalan tetapi tiada perbezaan yang signifikan dalam tahap THC kenaikan di antara kumpulan rawatan. Tidak ada kematian dalam kumpulan rawatan, tetapi kadar kematian dalam kumpulan kawalan adalah 55% sepanjang tempoh tujuh hari.

Hemolimfa (bergumpal dan tidak bergumpal, kedua-duanya) yang turut diekstrak untuk mengesan kehadiran OMPs di dalam sistem menggunakan SDS-PAGE menunjukkan ketiadaan jalur OMPs tetapi jalur yang dihasilkan oleh protein plasma, ini mungkin disebabkan oleh aktiviti pemugaran semulajadi udang bagi menghapuskan agen-agen asing. Pemeriksaan secara kasar terhadap udang-udang yang dicabar secara eksperimen dilakukan selepas kematian dan tisu diproses untuk lesi-lesi histopatologi dan dan reaksi imunohistokimia. Kumpulan yang tidak dirawat menunjukkan lesi yang lebih ketara berbanding dengan kumpulan yang dirawat. Kajian ini, bagaimanapun, menyimpulkan bahawa pemberiaan OMPs secara oral dengan atau tanpa adjuvan tidak lengkap Freund merupakan penggalak pertumbuhan yang bagus dan mempunyai potensi untuk perlindungan terhadap vibriosis di dalam *Macrobrachium rosenbergii*, apabila diberi dengan perantara perlidungan antigen yang unik dan pada dos yang sesuai tetapi perlindungan mungkin untuk jangka masa yang pendek.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ºC	Degree Celsius
µg	Microgram
µL	Micro litre
%	Percentage
ADJ	Adjuvant
AFA	Alcohol Formalin Acetic-acid
AHPS	Acute Hepatopancreatic Necrosis Syndrome
ANOVA	Analysis of Variance
API	Analytical Profile Index
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
BHIB	Brain Heart Infusion Broth
BW	Body Weight
CFU	Colony Forming Unit
CX	Control
DAB	Diaminobenzidine
DHC	Differential Haemocyte Count
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
DoF	Department of Fisheries
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EMS	Early Mortality Syndrome
ETC	Etcetera

EU	European Union
FAO	Food and Agriculture Organization
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
FKC	Formalin Killed Cell
g ($\times g$)	Gravitational acceleration
G	Gram
H_A	Alternative Hypothesis
H & E	Hematoxylin and Eosin
H_O	Null Hypothesis
HP	Hepatopancreas
IHC	Immunohistochemistry
Kb	Kilobase pair
kDa	Kilodalton
Kg	Kilogram
L-DOPA	L-3,4-Dihydrophenylalanine
LGH	Large Granular Haemocyte
LPS	Lipopolysaccharides
MT	Metric tonnes
Mg	Milligram
mL	Millilitre
Mm	Millimetre
mM	Millimolar
NaCl	Sodium Chloride
NaFisH	National Fish Health Research Centre

OMPs	Outer Membrane Proteins
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline Tween 20
PCR	Polymerase Chain Reactions
PG	Peptidoglycan
PPM	Part Per Million
PPT	Part Per Thousand
PO	Phenoloxidase
ProPO	Prophenoloxidase
RM	Malaysian Ringgit
ROIs	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
RPS	Relative Percentage Survival
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SE	Standard Error
SEM	Scanning Electron Microscopy
SGH	Small Granular Haemocyte
SOD	Superoxide Dismutase
Sp.	Specie
Spp.	Species
TBE	Tris Base Electrophoresis
TBS	Tris Buffer Saline
TCBS	Thiosulfate Citrate Bile salts Sucrose
THC	Total Haemocyte Count
TSB	Tryptic Soy Broth

USD	United States Dollar
USFDA	United States Food and Drug Administration
UN	United Nations
v/v	Volume/Volume
w/v	Weight/Volume
YSI	Yellow Spring Inc.

CHAPTER 1

INTRODUCTION

The growth of global production of seafood from aquaculture has been very consistent and tremendously high in the last decade, with the record of 52.5 million tonnes in 2008, which was 61.7% higher than 32.4 million tonnes in 2000. With the exclusion of aquatic plants, the value of the global harvest of aquaculture was estimated at US$98.4 billion in 2008 (FAO, 2011). The production rose to 62.7 million tonnes in 2011 at an estimated value of USD130 billion (FAO Fisheries and Aquaculture Department, 2013) and 90.43 million tonnes in 2012, and the estimate of an average global supply of food fish per person by aquaculture was 9.41 kg (FAO, 2014). The essential role of aquaculture in global hunger elimination, health promotion, poverty reduction and to some extent environmental protection cannot be over emphasized. The global production of freshwater prawns has equally gathered momentum in terms of increment in metric tons and values, with a total estimate of USD2.2 billion annually (FAO 2012). Oriental river prawn (*Macrobrachium nipponense*) was valued at USD1.13 billion with 237, 431 metric tons, giant river prawn (*M. rosenbergii*) valued at USD593.6 million with 124,713 metric tons and the rest of the species in the same genus completed the rest of the statistics (FAO, 2012). The discussion on *M. rosenbergii* would not be complete, without reference to Malaysia where it derives one of its other nomenclature (Malaysian prawn). This may be related to the discovery, of Shao Wen Ling of the FAO in the 1960s, at Penang, Malaysia, that the larval stages of *M. rosenbergii* required brackish water for development to post-larvae and survival (Wowor and Ng, 2007). This marked the beginning of modern aquaculture of this species.

Malaysia is one of the forces to reckon with, in terms of global aquaculture (FAO Fisheries and Aquaculture Department, 2013). In 2009, the total annual production of all species of freshwater prawns was 440,000 ton at the value of USD2.2 billion. Out of this total global figure, the production of farmed *M. rosenbergii* contributed 51.7%, while the oriental river prawn *M. nipponense* (exclusively reared in China) constituted 47.2% (New & Nair, 2012). However, for more than two decades, aquaculture has embraced tremendous growth in Malaysia, for instance, in 1992, the total production was estimated to be 79,699 tons of the value of RM 207.4 million, these figures surpassed that of the previous year by 23% and 25% respectively (Kechik, 1995).

Macrobrachium rosenbergii is one of the most important reared and fished crustaceans with high commercial value. For some years now, there had been a heightened interest in the culture of giant freshwater prawn (*M. rosenbergii*), due to its lower risks involvement and abundant market demands (Hameed *et al*., 2003). The value of the average unit product of both *M. rosenbergii* and all species of freshwater prawns surpassed that of various major aquaculture products such as the two-major species of marine shrimp (*Litopenaeus vannamei* and *Penaeus monodon*) and Atlantic salmon (*Salmo salar*). *M. rosenbergii* possesses unique characteristics such as large size attainment, omnivorous nature of feeding on unconventional feeds and tolerance

to changes in water quality and handling stress (El Sayed, 1997). It can thrive and grow well in both fresh and low salinity water and wide temperature range (FAO, 1975). When the penaeid shrimps culture was increasingly plagued with disease outbreaks, a global economically important cultured species, *Macrobrachium rosenbergii* gained more considerations by farmers due to the perception of its relatively less susceptibility to diseases (Pillai & Bonami, 2012). Meanwhile, the rise in demands, pronounced culture intensification and increase in world trade of this farmed species, have but come with attendant challenges such as disease outbreaks which bring about serious setbacks in management and production. Major diseases that affect the freshwater industry are of bacterial and viral origins. Apart from idiopathic diseases, others that are less common or cause serious detrimental effects than the two formers, include fungi, yeast, parasites and nutritional deficiencies have been reported (Pillai and Bonami, 2012; Bondad-Reantaso *et al.*, 2005; Eshraghi *et al.*, 2005; Michael, 2002; Karunasagar *et al.*, 1998; Bower *et al.*, 1994; Johnson, 1975). The major genera of bacteria which are incriminated in deleterious effects and economic loss in freshwater pawns are *Vibrio*, *Aeromonas*, and *Pseudomonas* (Pillai and Bonami, 2012).

However, vibriosis is one of the most important diseases reported in farmed freshwater prawns with grievous devastating effects and huge reduction or loss of production (Khuntia *et al.*, 2008; Jayaprakash *et al.*, 2006; Kennedy *et al.*, 2006; Poupard, 1976). *Vibrio alginolyticus* is one of the highly pathogenic species affecting fish and shell fish farming (Hsieh *et al.*, 2008; Jayaprakash *et al.*, 2006; Liu e*t al.*, 2004). Vibriosis causes a high rate of morbidity and mortality especially under stress and other environmental conditions. Many hatcheries and grow out ponds have been plagued by the malady and farmers have sought various means to get out of this situation, the commonest among all is the use of antibiotics.

The incessant and uncontrollable application of antibiotics in aquaculture both prophylactically and chemotherapeutically have resulted to ineffective disease combatant, many species of bacteria have developed resistance, and more virulence strains have been birthed. Subsequent drug residues also resulted from this traditional practice of disease management and eradication which pose a threat to the health of humans through consumption. Many isolates (more than 90%) of bacteria isolated from larvae and post larvae of *M. rosenbergii* showed resistance to some antibiotics including oxytetracycline, erythromycin, and furazolidone (Hameed *et al.*, 2003). Strains of *V. alginolyticus* and other vibrios were reported to carry R plasmid which is responsible for transferable drug resistance (Gomathi *et al.*, 2013). Many hatcheries and grow out ponds of farmed fish and shell fish have gone bankrupt due to outbreaks of highly antimicrobial resistant bacteria and humongous economic losses have been incurred (D. Pillai *et al.*, 2005).

In lieu of this bugging situation of antibiotic resistance, there is an urgent need for better and safer alternatives. Many studies have been carried out in the light of finding lasting solutions to this malady and to some great extents, tremendous successes have been achieved in the area of vaccination and use of probiotics. Meanwhile, researchers have explored various means to boost the non- specific immunity of crustaceans that

are of economic and health importance in order to raise protection against intending disease outbreaks through the concept of immunostimulation.

An immunostimulant in aquaculture of shellfish is said to be any substance that is used to improve immune responses and enhance disease resistance against pathogenic organisms (Smith *et al*., 2003). Various agents such as microbial components, compounds of animal and plant origins and synthetic substances have been applied as immunostimulants with varying degree of effectiveness. Components of microorganisms cell wall such as beta-glucan (Bai *et al*., 2014; Chang *et al*.,2003), lipopolysaccharides (Abbass *et al*., 2010), peptidoglycan (Purivirojkul *et al*., 2006; Itami *et al*., 1998) and outer membrane proteins (Maftuch *et al*., 2013) have been studied and reported to stimulate immune responses and enhance survival of crustaceans against infections by pathogenic organisms.

Various routes of administrations including injection, immersion and oral have been tested. Injection route is the most effective but costly, labour intensive and cause additional stress to the host species (Smith *et al*., 2003). However, the two latter are the preferred and most practicable routes (Smith *et al*., 2003).

In comparison to other components of the cell wall of bacteria, including lipopolysaccharides and peptidoglycan, little studies have been carried out on the enteral route of administration of outer membrane proteins. Hence, this study took into consideration the practicability of administration and compliance by users in investigating the effect of oral administration of outer membrane proteins (OMPs) of *Vibrio* in giant freshwater water prawns (*Macrobrachium rosenbergii*) on weight gain and protective potential against infection by *Vibrio alginolyticus*.

The objectives of this study were:

1. To determine the profiles and immunogenicity of OMPs of *Vibrio alginolyticus* using SDS-PAGE and Western blot techniques.
2. To prepare outer membrane proteins incorporated in feed and evaluate its effects on weight gain and infection by *V. alginolyticus*.
3. To examine the histopathological and immuno-histochemical reactions of prawns which have been experimentally infected with *V. alginolyticus*.

The hypotheses of this study were:

1. $\mathbf{H_O}$: OMPs of *V. alginolyticus* do not contain several minor and major polypeptides that are immunogenic
2. $\mathbf{H_A}$: OMPs of *V. alginolyticus* do contain several minors and major polypeptides that are immunogenic
3. $\mathbf{H_O}$: oral administration of OMPs does not have any effect on weight gain and infection caused by *V. alginolyticus* in freshwater prawns.

4. **H_A**: oral administration of OMPs does have any effect on weight gain and infection caused by *V. alginolyticus* in freshwater prawns.

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