MIR ALLAH DAD TALPUR DOCTOR OF PHILOSOPHY 2012

ISOLATION OF THE GUT PROBIOTICS AND APPLICATION IN EARLY STAGES LARVICULTURE OF BLUE SWIMMING CRAB, *Portunus pelagicus* (LINNAEUS, 1758)

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Thesis Submitted in Fulfillment of the Requirement for the Degree of Doctor of Philosophy in the Institute of Tropical Aquaculture Universiti Malaysia Terengganu

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DEDICATION

This thesis is dedicated to:

1. *My late mother, father and young brothers, my late father in law and mother in law, my beloved wife Gul Nissa Talpur and my lovely son Mir Mhd Moazam Talpur and my smart and cute daughters, Rida Zehra Talpur and Shazma Zehra Talpur for their unforgettable love in my life.*

2. *My supervisor Assoc. Prof. Dr. Abol Munafi Ambok Bolong and his family for their support and happiness during my study in Malaysia.*

3. *Also for my respected supervisory committee members Dr. Mhd Ikhwanuddin @ Polity bin Abdullah and Dr. Muhd Danish Daniel bin Abdullahd for their support to my degree of Doctor of Philosophy in Aquaculture (Biotechnology).*

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ISOLATION OF THE GUT PROBIOTICS AND APPLICATION IN EARLY STAGES LARVICULTURE OF *Portunus pelagicus* (LINNAEUS, 1758)

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OCTOBER 2012

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Survival problems of *Portunus pelagicus* larvae rearing are encountered at early stages owing to pathogenic microbes. In rearing system pathogens enter either shed by adult female in spawning tanks or from live feed fed to larvae. To determine the pathogen prevalence in the gut of female *P. pelagicus* crab the study was conducted.

Five leading shellfish pathogens *Staphylococcus epidermidis, Vibrio harveyi*, *V. parahaemolyticus Micrococcus luteus* and *Pseudoalteromonas piscicida* were determined in the gut based on 16S rRNA gene sequencing and their pathogenicity was tested challenging to one day hatch *P. pelagicus* larvae. *V. harveyi* and *P. piscicida* resulted in 100% mortality at allowance 10^6 cfu mL^{-1} after 24 h post exposure and *V. parahaemolyticus* after 72 h post exposure.

To cope with the pathogenic problem and to enhance the larval survival of *P. pelagicus*, indigenous probiotics were isolated from the gut of female *P. pelagicus.* Five lactic acid bacteria (LAB) were isolated from the gut of female *P. pelagicus* and identified as *Lactobacillus plantarum*, *L. salivarius*, *L. rhamnosus*, *Weissella*

confusa and *W. cibaria*. They showed antagonistic properties *in vitro* towards indicator gut pathogens tested. In small scale *in vivo* and pathogen-probiotic assay, only three LAB (*L. plantarum*, *L. salivarius*, *L. rhamnosus*) showed characterstics as probiotics and enhanced the survival rate of larvae were selected for further *in vivo* trials.

LAB probionts were used at various concentrations ($1x10^6$ cfu mL^{-1}, 5×10^6 cfu mL^{-1} and $1x10^7$ cfu mL^{-1}) as water additive, bioencapsulation in live feed and bioencapsulation in live feed together addition in water in larviculture of *P. pelagicus* either as sole probiotic or in mixture of probiotics. All tested probiotics showed significantly ($p<0.05$) higher larval survival over the control. Moreover, highest specific protease and amylase activities were significantly ($p<0.05$) higher in larvae treated with probiotic allowance over the control.

Addition of probiotic significantly influenced the pH of rearing water. Enrichment of probiotics reduced the bacterial load in live feed. Furthermore, bacterial population was found lower in rearing water treated with probiotics over the non-inoculated control.

LAB probiotics showed significant effect on water quality, acted as bio-remedy and bio-control against fatal bacteria thus significantly enhanced larval survival of *P. pelagicus*.

Abstrak tesis yang dikemukan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN PROBIOTIK DAN APLIKASI DALAM PERINGKAT AWAL TERNAKAN LARVA *Portunus pelagicus* (LINNAEUS, 1758)

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Masalah dalam kemandirian hidup *Portunus pelagicus* ketika ternakan larva pada peringkat awal hidupnya adalah berpunca daripada patogen. Di dalam sistem ternakan, patogen ini berpunca daripada ketam betina daripada tangki penetasan atau daripada makanan yang diberikan kepada larva. Lima patogen utama kerang-kerangan, *Staphylococcus epidermidis, Vibrio harveyi*, *V. Parahaemolyticus, Micrococcus luteus* dan *Pseudoalteromonas piscicida* dikenalpasti di dalam melalui penjujukan gen 16S rRNA dan kesan patogeniknya diuji ke atas larva *P. pelagicus* yang baru menetas pada hari pertama. *V. harveyi* dan *P. piscicida* menunjukkan kematian 100% pada kepekatan 10^6 cfu mL^{-1} selepas pendedahan 24 jam dan *V. parahaemolyticus* selepas pendedahan 72 jam.

Untuk mengatasi masalah patogen ini dan meningkatkan kemandirian larva *P. pelagicus*, probiotik asli telah diasingkan daripada perut *P. pelagicus* betina. Lima Bacteria Asid Laktik (LAB) diasingkan dari perut *P. pelagicus* betina dan dikenalpasti sebagai *Lactobacillus plantarum*, *L. salivarius*, *L. rhamnosus*, *Weissella*

confusa and *W. cibaria.* Ia menunjukkan sifat perlawanan secara *in vitro* terhadap indikator patogen perut yang diuji. Di dalam kajian *in vivo* skala kecil dan ujian probiotik-patogen, hanya tiga LAB (*Lactobacillus plantarum*, *L. salivarius*, *L. rhamnosus*) menunjukkan ciri-ciri probiotik dan berupaya meningkatkan kadar kemandirian larva dan dipilih untuk percubaan lanjutan *in vivo*.

Probiotik LAB digunakan pada kepekatan yang berbeza ($1x10^6$ cfu mL^{-1}, 5×10^6 cfu mL^{-1} and $1x10^7$ cfu mL^{-1}) sebagai pelarut air, bio-pengkapsulan makanan hidup dan bio-pengkapsulan di dalam makanan hidup bersama-sama penambahan air ke dalam kultur larva *P. pelagicus* sama ada sebagai probiotik tunggal atau sebagai campuran probiotik. Kesemua probiotik yang diuji menunjukkan kemandirian larva yang lebih tinggi secara signifikan ($p<0.05$) berbanding kumpulan kawalan. Tambahan pula, aktiviti amilase dan protease spesifik yang tertinggi adalah lebih tinggi secara signifikannya ($p<0.05$) di dalam larva yang dirawat menggunakan kepekatan probiotik berbanding kumpulan kawalan.

Penambahan probiotik secara signifikannya mempengaruhi pH air ternakan. Pengkayaan probiotik mengurangkan jumlah sel bakeria didalam makanan hidup. Tambahan pula, populasi bakteria didapati lebih rendah didalam air ternakan yang dirawat dengan probiotik berbanding kumpulan kawalan yang tidak dirawat.

Probiotik LAB secara signifikan mempengaruhi kualiti air ternakan, bertindak sebagai kawalan dan rawatan biologi terhadap bakteria yang menyebabkan kematian dan secara tidak lansung meningkatkan kadar kemandirian larva *P. pelagicus*.