UNIVERSITI PUTRA MALAYSIA

SELECTION OF POTENTIAL BACTERIAL PROBIOTICS FOR TIGER GROUPER (*Epinephelus fuscoguttatus* Forsskal) LARVICULTURE

NURHIDAYU BINTI AL-SAARI

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By

NURHIDAYU BINTI AL-SAARI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Specially dedicated to my lovely parents to whom I indebted my life.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

SELECTION OF POTENTIAL BACTERIAL PROBIOTICS FOR TIGER GROUPER (*Epinephelus fuscoguttatus* Forsskal) LARVICULTURE

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Chairman: Ina Salwany Md Yasin, PhD

Faculty: Agriculture

Rapid development in today's aquaculture industries have expanded to the level of commercial scale and has indirectly led to environmental degradation and catastrophic losses due to bacterial diseases. Thus, the application of probiotics was proposed as an environmental-friendly preventive measure. This study was carried out specifically to search for probiotic candidates for use in the larviculture of *Epinephelus fuscoguttatus*. Hypothetically, selected intestinal microfloras of *E. fuscoguttatus* are able to confer protection on larvae by exhibiting antagonistic activity against pathogenic bacteria.

A set of criteria for bacterial probiotics was used in this study, focusing on phenotypic and genotypic characterizations of the candidate strains, *in vitro* capacity to inhibit the growth of targeted competitive strains, the haemolytic activity and antibiotic susceptibility of selected candidates and virulence

assessment to the host. Initially, the intestines of healthy individual grouper which were randomly collected from three geographically distant farms in Malaysia were processed and bacterial isolation was performed. A preliminary identification and grouping method successfully identified 31 species, while 22 isolates as unidentified from a total of 123 isolated aerobes and facultative anaerobes.

The first screening process was undertaken to assess the antagonistic activity exhibited by the isolates against four common fish pathogens. Of the 123 isolates, 40 (32.5%) displayed strong antagonistic activity, mostly Gram-positive bacteria (n=32; 80%). The haemolytic nature of the short listed probionts was then assessed where only nine (22.5%) showed no breakdown of red blood cells and they were grouped into five genera based on 16S rRNA gene sequence analysis. This genotypic strategy was used to eliminate another four isolates considering their potential pathogenic nature to human and sequences similarity from which they were considered identical. Screening process was ended with the antibiotic susceptibility assay of five selected isolates which resulted in the elimination of *V. harveyi* i.e. JAQ01 and JAQ02 due to their opportunistic nature and the availability of incomprehensible evidence that these strains may potentially carry resistance genes for β-lactamase in a transferrable genetic material.

Considering the needs for an advanced study to measure the antagonism capacity of three selected candidates, bacteriocin-like inhibitory substances

(BLIS) and co-culture assays were performed. The former assay nominated *Bacillus cereus* JAQ04 as the most promising candidates with initial addition at 10^8 and/or 10^9 cfu/mL with 48 and/or 72 h pre-incubation time. Alternatively, for co-culture method, the growth of pathogenic *V. alginolyticus* can also be inhibited at 10^5 cfu/mL of all probionts tested. Both methods revealed that the highest inhibition of pathogens has always associated with the introduction of significantly higher densities of probionts.

Lastly, virulence assessment was made by determining the relationship observed between the probiont concentrations with larval survival. Conclusively, in relation to the previous antagonistic assay, 10^5 cfu/mL of *B. cereus* (JAQ04) and *Micrococcus luteus* (JAQ07) were recommended as the safest and adequate initial amount for use in field trial. Both bacteria needed at least 48 h to proliferate before being able to counteract the pathogens. Thorough studies on these bacteria including in-depth assessment on their effect *in vivo* are recommended.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMILIHAN BAKTERIA BERPOTENSI PROBIOTIK BAGI KULTUR LARVA KERAPU HARIMAU (*Epinephelus fuscoguttatus* Forsskal)

Oleh

Nurhidayu Binti Al-saari

Jun 2012

Pengerusi: Ina Salwany Md Yasin, PhD

Fakulti: Pertanian

Pembangunan pesat sektor akuakultur pada hari ini telah berkembang kepada skala komersial dan secara tidak langsung telah mengakibatkan pencemaran alam sekitar dan kerugian besar yang disebabkan oleh penyakit bakteria. Oleh itu, penggunaan probiotik telah dicadangkan sebagai langkah pencegahan yang mesra alam. Kajian ini telah dijalankan khusus untuk mencari calon-calon probiotik untuk digunakan dalam ternakan larva *Epinephelus fuscoguttatus*. Diandaikan, microflora terpilih dari usus *E. fuscoguttatus* dapat memberi perlindungan kepada larva dengan cara menentang bakteria pathogen.

Satu set kriteria pemilihan bakteria probiotik telah digunakan dalam kajian ini memberi tumpuan kepada pencirian fenotip dan genotip terikan calon, kapasiti *in vitro* untuk menghalang pertumbuhan strain sasaran, aktiviti hemolitik dan kerentanan antibiotik calon-calon yang dipilih dan penilaian kebisaan tuan

rumah. Pada mulanya, usus kerapu dari individu sihat yang dikumpulkan secara rawak dari tiga ladang berasingan di Malaysia telah diproses dan diikuti dengan pemencilan bakteria. Kaedah pengenalan dan pengkelasan awal berjaya mengenal pasti 31 spesies, manakala 22 isolat dikelaskan sebagai tidak dikenali daripada jumlah keseluruhan 123 aerobes dan anaerobes fakultatif terpencil.

Proses saringan pertama telah dijalankan untuk menilai aktiviti antagonistik yang dipamerkan oleh isolat-isolat yang dikaji terhadap empat patogen yang biasa bagi ikan. Daripada 123 isolat, 40 (32.5%) memaparkan aktiviti antagonistik yang kuat, yang mana kebanyakannya adalah bakteria Gram-positif (n = 32; 80%). Sifat hemolitik probionts yang telah disenarai pendek kemudiannya dinilai di mana hanya sembilan (22.5%) tidak menunjukkan pecahan sel darah merah dan mereka dikumpulkan kepada lima genus berdasarkan analisis jujukan gen 16S rRNA. Strategi genotip ini telah digunakan untuk menghapus kira empat lagi isolat disebabkan mereka berpotensi menjadi patogen terhadap manusia dan mempunyai persamaan jujukan gen di mana mereka dianggap serupa. Proses saringan diakhiri oleh penilaian kerentanan antibiotik oleh lima isolat terpilih yang menyebabkan penghapusan *V. harveyi* JAQ01 dan JAQ02 kerana sifat oportunis mereka dan kewujudan bukti sedia ada yang masih belum jelas yang menerangkan bahawa terikan ini mungkin membawa gen rintangan bagi β-lactamase dalam bahan genetik boleh pindah.

Melihat kepada keperluan kajian yang lebih dalam untuk mengukur kapasiti antagonisma tiga calon yang dipilih, bahan-bahan rencatan menyerupai

bacteriocin (BLIS) dan ujian kultur bersama telah dilakukan. Asai pertama telah menamakan *Bacillus cereus* JAQ04 sebagai calon yang paling berpotensi digunakan pada kepekatan 10^8 dan/atau 10^9 cfu/mL dengan 48 dan/atau 72 jam masa pengeraman. Sebagai alternatif, bagi kaedah kultur bersama, pertumbuhan patogen *V. alginolyticus* juga boleh direncatkan dengan penambahan 10^5 cfu/mL probionts yang diuji. Kedua-dua kaedah mendedahkan bahawa perencatan tertinggi patogen sentiasa dikaitkan dengan penambahan probionts pada ketumpatan yang ketara lebih tinggi.

Akhir sekali, penilaian kebisaan telah dilakukan dengan cara menentukan hubungan diantara kepekatan probiont dengan kemandirian larva. Kesimpulannya, dengan merujuk kepada penilaian antagonistik terdahulu, 10^5 cfu/mL *B. cereus* (JAQ04) dan *Micrococcus luteus* (JAQ07) adalah jumlah yang disyorkan paling selamat dan mencukupi bagi kegunaan ladang. Kedua-dua bakteria memerlukan sekurang-kurangnya 48 jam untuk berkembang sebelum dapat mengatasi patogen. Kajian yang menyeluruh ke atas bakteria ini termasuk penilaian terperinci mengenai kesan mereka secara *in vivo* adalah disyorkan.

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I also wished to thank all farms involved in specimen collection and financial support from Agro-Biotechnology Institute (ABI), Ministry of Science, Technology and Innovation (MOSTI) Malaysia

I certify that a Thesis Examination Committee has met on 12 June 2012 to conduct the final examination of Nurhidayu binti Al-saari on her thesis entitled " Selection of Potential Bacterial Probiotics for Tiger Grouper, (*Epinephelus fuscoguttatus* Forsskal) Larviculture " 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

Mohd Salleh Kamarudin
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Kamaruzaman Sijam, PhD
Associate Professor
Name of Faculty
Universiti Putra Malaysia
(Internal Examiner)

Che Roos Saad, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Najiah Musa, PhD
Associate Professor
Faculty of Fisheries and Aqua-Industry
Universiti Malaysia Terengganu
Malaysia
(External Examiner)

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Ina Salwany Md Yasin, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Hassan Mohd Daud, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Sharr Azni Harmin, PhD
Professor
Faculty of Science and Biotechnology
Universiti Selangor
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

NURHIDAYU BINTI AL-SAARI

Date: 12 June 2012

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