UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION AND EVALUATION OF Paenibacillus pabuli STRAIN D12 AND D14 ISOLATED FROM GUT MICROFLORA OF RED TILAPIA AS POTENTIAL PROBIOTICS FOR AQUACULTURAL USE

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By

HISHAMMUDDIN BIN HAMDAN

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By

HISHAMMUDDIN BIN HAMDAN

December 2014

Chairman: Ina Salwany binti Md Yasin, PhD

Faculty: Agriculture

Presently, the aquaculture production is facing a greater constraint due to disease outbreaks particularly in the culture of marine and freshwater fish. The application of antibiotics or chemotherapeutics agents continues to be the current method of choice for disease control. However, the evolution of antimicrobial resistance gene among pathogenic bacteria, potential health risk of antibiotic accumulation in humans and growing awareness among public and authorities concern regarding food safety issues has led to the application of probiotics, an environment-friendly and non-pathogenic beneficial bacteria in aquaculture. Therefore, the present investigation has been made to evaluate three bacterial isolates from the gastrointestinal tracts of cultured red tilapia fish as a biocontrol agent against fish pathogenic bacteria. Probionts identification procedures through phylogenetic analysis based on 16S rRNA and Internal Transcribed Spacer (ITS) gene sequencing were constructed as part of precise molecular identification. Their antagonistic ability towards common fish pathogenic bacteria was evaluated using a series of *in vitro* assessment. Successful candidates were verified *in vivo* with a bioassay using *Artemia* to confirm their safety towards these common live-feed organisms. Results derived from ITS profiles identified our probionts as *Paenibacillus pabuli* strain D12, *Paenibacillus pabuli* strain D14 and *Bacillus megaterium* strain E28. As for detection of antagonistic effect, *P. pabuli* strain D12 and *P. pabuli* strain D14 were further chosen from cross-streaking method as they exhibited antagonism ability against common fish pathogenic bacteria. Our finding from Bacteriocin Like Inhibition Substance (BLIS) assay suggests *P. pabuli* strain D12 as the most prominent probiotic since it produced maximum inhibitory capacities against target strains at 10^9 cfu/mL with 72 hours pre-incubation period of probionts. In broth co-culture assay, complete elimination of *V. alginolyticus* by *P. pabuli* strain D12 successfully occurred at 48

hours of incubation period as compared to *P. pabuli* strain D14 (72 hours). Final *in vivo* studies validate the outcomes from our *in vitro* screening test. *Artemia* survival rate is the highest when treated with *P. pabuli* strain D12 (72%) followed by *P. pabuli* strain D14 (68%) after being challenged with *V. alginolyticus*. Vibrios count in *Artemia* and in the culture water proved that our probionts suppressed the proliferation of the pathogenic strain, thus could be used as biocontrol agent in fish aquaculture industry via live-feed carrier. The present study proved the ability of *P. pabuli* strain D12 and D14 as potential probiotics in controlling pathogenic *V. alginolyticus* in *Artemia* culture system.

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

PENCIRIAN MOLEKUL DAN PENILAIAN *Paenibacillus pabuli* STRAIN D12 DAN D14 YANG DIPENCILKAN DARIPADA MIKROFLORA USUS TILAPIA MERAH SEBAGAI PROBIOTIK YANG BERPOTENSI UNTUK KEGUNAAN AKUAKULTUR

Oleh

HISHAMMUDDIN BIN HAMDAN

Disember 2014

Pengerusi: Ina Salwany binti Md Yasin, PhD

Fakulti: Pertanian

Pada ketika ini, pengeluaran akuakultur sedang mengalami cabaran besar akibat penularan penyakit terutama dalam kultur ikan air tawar dan marin. Penggunaan antibiotik atau agen kemoterapeutik terus menjadi kaedah pilihan semasa untuk mengawal penyakit. Walaubagaimanapun, evolusi gen kerentanan anti bakteria dalam kalangan bakteria patogenik, potensi risiko kesihatan akibat pengumpulan antibiotik dalam manusia dan peningkatan kesedaran dalam kalangan orang awam serta keprihatinan pihak berkuasa berkenaan isu keselamatan makanan telah membawa kepada penggunaan probiotik, sejenis bakteria berfaedah yang mesra alam dan tidak patogenik dalam akuakultur. Oleh sebab itu, penyelidikan ini telah dijalankan untuk menilai tiga bakteria yang telah dipencilkan dari usus ikan tilapia merah yang dikultur sebagai agen kawalan bio terhadap bakteria ikan yang patogenik. Prosedur mengenal pasti probiotik melalui analisis fenotip berdasarkan gen jujukan 16S rRNA dan "Internal Transcribed Spacer" (ITS) telah dibina sebagai sebahagian daripada pengenalan molekular yang tepat. Keupayaan antagonistik bakteria-bakteria ini terhadap bakteria ikan patogenik yang lazim telah dinilai menggunakan siri penilaian *in vitro.* Calon bakteria yang berjaya telah disahkan secara *in vivo* dengan kaedah bio esei menggunakan *Artemia* untuk mengenalpasti tahap keselamatan bakteria-bakteria tersebut terhadap organisma yang kebiasaannya dijadikan makanan hidup ini. Keputusan yang diperolehi daripada profil ITS telah mengenal pasti probiotik kami sebagai *Paenibacillus pabuli* strain D12, *Paenibacillus pabuli* strain D14 dan *Bacillus megaterium* strain E28. Dalam mengesan keupayaan antagonistik, *P. pabuli* strain D12 dan *P. pabuli* strain D14 telah dipilih daripada saringan awal "cross streaking method" kerana mempamerkan keupayaan antagonistik terhadap bakteria ikan patogenik yang lazim. Penemuan kami daripada esei "Bacteriocin Like Inhibition Substance (BLIS)" mencadangkan *P. pabuli* strain D12 sebagai probiotik yang paling menonjol kerana ia menghasilkan

kapasiti menyekat yang maksimum terhadap strain sasaran pada 10^9 cfu/mL dengan 72 jam tempoh pra-inkubasi probiotik. Dalam "broth co-culture" assay, penghapusan lengkap *V. alginolyticus* oleh *P. pabuli* strain D12 berjaya dilakukan dalam 48 jam tempoh inkubasi berbanding dengan *P. pabuli* strain D14 (72 jam). Kajian akhir *in vivo* mengesahkan hasil daripada ujian saringan *in vitro* kami. Kadar hidup *Artemia* adalah yang tertinggi apabila dirawat dengan *P. pabuli* strain D12 (72%) diikuti oleh *P. pabuli* strain D14 (68%) selepas didedahkan kepada *V. alginolyticus*. Kiraan *Vibrio* dalam *Artemia* dan dalam air kultur membuktikan bahawa probiotik kami menyekat pertumbuhan strain patogen, maka dengan itu boleh digunakan sebagai agen kawalan bio dalam industri akuakultur ikan melalui makanan hidup. Kajian ini membuktikan keupayaan *P. pabuli* strain D12 dan strain D14 sebagai probiotik yang berpotensi dalam mengawal patogen *V. alginolyticus* dalam sistem kultur *Artemia.*

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment 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)

Natrah Fatin Mohd Ikhsan, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Nor Fadilah Rajab, PhD
Associate Professor
School of Diagnostic and Applied Health Science
Faculty of Health Sciences
Universiti Kebangsaan Malaysia
(Member)

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BUJANG BIN KIM HUAT
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School of Graduate Studies
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Signature:_____

Name of Chairman of Supervisory Committee:

Ina Salwany Md Yasin, PhD

Signature:_____

Name of Member of Supervisory Committee:

Natrah Fatin Mohd Ikhsan, PhD

Signature:_____

Name of Member of Supervisory Committee:

Nor Fadilah Rajab, PhD

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LIST OF ABBREVIATIONS

°C	degree celcius
ANOVA	One Way Analysis of Variance
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BLIS	Bacteriocin-like Inhibition Substance
bp	base pair
cfu	colony forming units
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DoF	Department of Fisheries
EUS	Epizootic Ulcerative Syndrome
FAO	Food and Agriculture Organization
ITS	Internal Transcribed Spacer
kb	kilobase
LAB	Lactic Acid Bacteria
MAS	Motile Aeromonas Septicaemia
mM	millimolar
mt	metric tonne
NaCI	Sodium chloride
nanogram	ng
NCBI	National Centre for Biotechnology Information
NH_3-N	total ammonia
NJ	Neighbour Joining
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction

ppt	parts per thousand
rpm	Rotation per minute
rRNA	Ribosomal Ribonucleic Acid
SE	Standard Error
SPSS	Statistical Package for the Social Science
SSW	sterile sea water
TBE	Tris-Borate-EDTA
TCBS	Thiosuplhate Citrate Bile Salt Sucrose
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
UV	Ultra Violet
w/v	weight/volume
WHO	World Health Organization
µg	microgram
µL	microlitre
µM	micromolar

CHAPTER 1

INTRODUCTION

Over time, global fish aquaculture production will need to expand to cater the growing human population. The State of World Fisheries and Aquaculture Report stated that in 2012, more than half of the world's food fish consumption came from aquaculture (FAO, 2012). The rapid intensification and commercialization of aquaculture production however, facing a significant constraint due to disease outbreaks particularly in the culture of marine and freshwater fish. Among the major pathogenic bacteria associated with fish aquaculture are aeromonads and vibrios. *Aeromonas hydrophila* and *Aeromonas salmonicida* for instance have been reported from both marine and freshwater environments and identified as causative agent for fish disease (Nielsen *et al*., 2001; Aberoum & Jooyandeh, 2010; Farto *et al*., 2011) leading to surface lesions, sloughing of scales, haemorrhages, septicaemia, ulcer syndrome, motile *Aeromonas* septicaemia (MAS), furunculosis and enteritis (Austin & Austin, 1999; Shao *et al*., 2004; Zhang *et al*., 2006 and Janda & Abbott, 2010).

On the other hand, *Vibrio* species such as *V. anguillarum*, *V. alginolyticus*, *V. ordalii*, *V. salmonicida*, *V. vulnificus* (Hjeltnes & Roberts, 1993) and *V. harveyi* (Austin & Zhang, 2006; Ransangan *et al.*, 2012) have been documented as causative agent for intestinal necrosis, anaemia, septicaemia and haemorrhages in cultured aquaculture systems worldwide. These pathogens were identified globally in several fish species like salmonid, rainbow trout, turbot, burbot, carps, catfish and tilapia (Spanggaard *et al*., 2000; Al-Sunaiher *et al*., 2010; Parthasarathy & Ravi, 2011; Ransangan *et al.*, 2012; Zheng *et al*., 2012). As consequences, severe economic losses and environmental degradation were recorded in many countries.

Although the prevention of disease by vaccination is increasing, the application of antibiotics or chemotherapeutics agents continues to be the current method of choice for disease control. These practices however have been questioned, due to evolution of antimicrobial resistance gene among pathogenic bacteria as well as potential health risk of antibiotic accumulation in humans (Balcazar *et al*., 2006). Moreover, antibiotics may inhibit or kill normal beneficial microflora in the digestive tract of fish (Sugita *et al.*, 1991). Therefore, alternative methods to prevent or treat diseases are needed for a sustainable development of the aquaculture sector. Many researchers have begun to evaluate the food safety issues regarding intensive aquaculture which led to the usage of probiotics, non-pathogenic beneficial bacteria.

Many probiotic definitions has been documented since the earliest one published by Lilly and Stillwell (1965). Conclusively, the current definition that best describes probiotic role in aquaculture will be "a live microbial or cultured product feed supplements which beneficially affect the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating the immune function and improving the microbial balance" (Fuller, 1989; Verschuere *et al*.,

2000). As a promoter of health, probiotic could be beneficial through multiple ways, either as single strain or combination of several probionts.

There are several selection criteria listed for choosing probiotics including safety, function and technology (Saarela *et al.*, 2000). According to Gomez-Gil *et al*. (2000), the methods to select probiotic bacteria for use in aquaculture include: (i) collection of background information; (ii) acquisition of potential probiotic strain; (iii) assessment on the ability of potential probiotics to inhibit pathogenic strains; (iv) evaluation of the pathogenicity and the effect of potential probiotics in the host; (v) economic analysis. It is also recommended that a particular probiotic strain tend to be more effective in the host species from where it was originated (Verschuere *et al.*, 2000). Hence, the search for potential strains which suits the host biological requirements is highly encouraged (Lazado *et al*., 2010).

The present investigation has been made to evaluate three potential bacterial probionts strain namely D12, D14 and E28 which were isolated from gastrointestinal tracts of cultured tilapia fish (Khairi, 2010; Lim *et al*., 2013). According to their study, out of 135 bacterial isolates, these three strains have been proposed as potential probiotic based on; (i) their ability to inhibit fish pathogens through *in vitro* study; (ii) haemolytic profile; and (iii) safety evaluation in tilapia as fish model. The haemolytic nature of isolates showed no breakdown of red blood cell (Khairi, 2010) and proved to be safe in the host with 79% to 83% survival rate when intra-peritoneally injected in tilapia fish (Lim *et al*., 2013).

Thus, current studies which include comprehensive *in vitro* and *in vivo* evaluations were necessary to validate their true potential to become biocontrol agent against fish pathogenic bacteria. Initially, current attempts were carried out with phenotypic and molecular identification of these isolates through phylogenetic analysis of 16S rRNA gene and Internal Transcribed Region (ITS). Further investigations were assessed on *in vitro* inhibitory capacity by preliminary cross streaking method, Bacteriocin like Inhibitory Substance (BLIS) and co-culture assay against fish pathogens. Merrifield *et al.* (2010) remarked that probiotics should exhibit antagonistic ability toward one or more common pathogens. To validate the probiotic potential *in vivo*, the effects of bacterial probionts in reducing *Vibrio alginolyticus* in the *Artemia* culture were studied. *Artemia* is the most common live food organisms, and also appropriate as test organism to study the host–microbe interactions (Marques *et al*., 2004). Selected probiotic were administered into the culture water whereby *Artemia* act as the transporter targeting the host. It is necessary to confirm a probiotic for use in aquaculture should be non-pathogenic, safe to the host and the environment in which the host is living (Balcazar *et al*., 2006). At the end of the study, bacterial strains that showed positive result were expected to become potential bio control agent against pathogenic *V. alginolyticus* in the treatment for *Artemia* to be used as live food for marine hatcheries.

Therefore, the objectives of the present study were:

1. to identify and characterize three bacterial probionts by using 16S rRNA and Internal Transcribed Spacer (ITS) gene sequencing;
2. to evaluate the inhibitory capacities of potential probionts using a series of *in vitro* antagonism test;
3. to determine the ability of selected probionts in protecting *Artemia* and reducing number of *V. alginolyticus*.

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