UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION, CHARACTERIZATION AND ASSESSMENT OF *Bacillus amyloliquefaciens* AS PUTATIVE PROBIONT FOR JUVENILES BLUE SWIMMING CRAB [*Portunus pelagicus*, (Linnaeus 1758)] TO CONTROL *Vibrio harveyi* INFECTION

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By

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

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The blue swimming crab, *Portunus pelagicus* also known as flower crab which is one of the important marine crustacean in Malaysia. However, many blue crab mortalities were attributed by systemic bacterial infections, especially at larval and juvenile stages. The genus *Vibrio* mainly *Vibrio harveyi* was commonly found in blue crab. To cater this problem, the present study focused on development of local bacteria as potential probiont in controlling the growth of pathogenic *V. harveyi*. Eleven potential bacteria were successfully isolated from the hemolymph of four healthy blue swimming crab (*P. pelagicus*). All isolates were identified as *Bacillus amyloliquefaciens* by series of biochemical test using triple sugar ion test, oxidase and catalase test, followed by Internal Transcribe Spacer (ITS) gene sequence analysis. The isolates were able to inhibit the growth of *V. harveyi* in *in vitro* screening assay by using spot lawn, disc diffusion and well diffusion assay with strong antagonistic activity ranging from 7 to 16 mm. The potential probiont at 10^8 CFU mL^{-1} showed highest inhibition response towards *V. harveyi* after being co-cultured for 48h. The probionts produced three major extracellular enzymes, which were amylase, gelatinase and lipase. The potential probionts were also able to form biofilm after 24h of culture. In *in vivo* study, *B. amyloliquefaciens* L9 and *B. amyloliquefaciens* L11 were used as potential probionts in preliminary study using *Artemia* as a host. The survival rate of *Artemia* treated with probionts at concentration of 10^6 CFU ml^{-1} showed significant highest survival for *B. amyloliquefaciens* L9 and for *B. amyloliquefaciens* L11 (69 ± 0.3% and 62 ± 1.4 % respectively) after challenged with *V. harveyi.* The results of vibrios count showed that both of probionts were able to reduce the numbers of *Vibrio* in *Artemia* at the end of the challenge assay. For axenic hatched, the results showed that the number of probiotic in *Artemia* increased over time for *B. amyloliquefaciens* L9 and *B. amyloliquefaciens* L11. *Bacillus amyloliquefaciens* L11 at 10^8 CFU mL^{-1} took as early as 6h to penetrate into the *Artemia* meanwhile *B. amyloliquefaciens* L9 needed longer time to be partially penetrated into the *Artemia* (12h of incubation) with the same concentration. In *in vivo* challenged assay using blue crab juveniles, *B. amyloliquefaciens* L11 was used due to its ability in inhibited *V. harveyi* during antagonistic activity. Immersion method was

implemented using 20 crab juveniles per tanks. The treatment tanks were incubated with probiont for 24h and pathogenic *V. harveyi* was added on the next day. Results demonstrated that *B. amyloliquefaciens* L11 had significant highest survival rate 42 ± 0.5 % if compared with *V. harveyi* only (12 ± 0.8 %) at concentration of 10^6 CFU mL^{-1} after 5 days of challenged test. In addition, the results also showed that *B. amyloliquefaciens* L11 were able to reduced vibrio load, gave slightly increased in crab juvenile weight and length. Gene contents analysis study demonstrated that *B. amyloliquefaciens* L11 underwent a mutation at different base length and location. Insertion and deletion were the most abundant mutation detected from gene analysis. A 21 bases mutation as deletion occurred at two different gene loci and the longest bases insertion was 39 bases occurred at one gene loci. A number of 73 genes occurred a mutation that cause a structural variation in *B. amyloliquefaciens* L11 probiotic genome at different location of the sequences. Majority of mutation occurred in non-coding regions and intergenic regions. Antibacterial gene consist within the *B. amyloliquefaciens* L11 probiont are considered as main factors in antagonistic activity. Lipopeptid and polyketid genes are related in present of antibacterial agents. Overall, based on results of *in vitro* and *in vivo* assay, *B. amyloliquefaciens* could be considered as a promising potential probiotic with antibacterial properties.

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

PENGENALPASTIAN, PENCIRIAN DAN PENILAIAN *Bacillus amyloliquefaciens* SEBAGAI PROBIOTIK PUTATIF UNTUK JUVENIL KETAM BIRU [*Portunus pelagicus,* (Linnaeus 1758)] BAGI MENGAWAL JANGKITAN *Vibrio harveyi*

Oleh

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Ketam biru (*Portunus pelagicus*) juga dikenali sebagai ketam bunga merupakan salah satu krustasia laut yang penting di Malaysia. Walau bagaimanapun, terdapat kejadian kematian ketam biru disebabkan oleh jangkitan bakteria, terutamanya pada peringkat anak larva dan juvenil. Genus *Vibrio* terutamanya *Vibrio harveyi*, banyak ditemui pada ketam biru. Bagi mengatasi masalah ini, kajian ini menumpukan terhadap pemencilan bakteria tempatan sebagai calon probiotik dalam mengawal pertumbuhan patogen *V. harveyi*. Sebelas calon probiotik telah berjaya dipencilkan daripada hemolymph empat ekor ketam biru (*P. pelagicus*) dewasa yang sihat. Kesemua isolat ini dikenalpasti sebagai *Bacillus amyloliquefaciens* oleh beberapa siri ujian biokimia menggunakan ujian ion gula tiga kembar, ujian oksida dan pemangkin, diikuti oleh analisis jujukan gen *Internal Transcribe Spacer* (ITS). Kesemua isolat ini berupaya merencat pertumbuhan *V. harveyi* dalam *in vitro* esei menggunakan esei tompokan, esei susupan cakera dan esei susupan telaga agar dengan aktiviti antagonis kuat bermula daripada 7 hingga 16 mm. Calon probiotik pada kepekatan 10^8 CFU mL^{-1} menunjukkan perencatan tertinggi untuk tindakbalas terhadap *V. harveyi* selepas pengkulturan bersama selama 48 jam. Semua calon probiotik menghasilkan tiga enzim utama iaitu amylase, gelatinase dan lipase. Bakal probiotik ini juga mampu membentuk lapisan biofilem selepas 24 jam pengkulturan. Untuk kajian *in vivo*, *B. amyloliquefaciens* L9 dan *B. amyloliquefaciens* L11 digunakan sebagai calon probiotik dengan menggunakan *Artemia* sebagai perumah untuk kajian awal. Kadar kemandirian *Artemia* yang dirawat dengan probiotik pada kepekatan 10^6 CFU ml^{-1} menunjukkan kemandirian tertinggi untuk *B. amyloliquefaciens* L9 dan *B. amyloliquefaciens* L11 (69 ± 0.3 % and 62 ± 1.4 %) selepas ditentang dengan *V. harveyi.* Keputusan pengiraan *Vibrio* menunjukkan kedua-dua calon probiotik mampu mengurangkan bilangan *Vibrio* pada *Artemia* di akhir esei penentangan. Bagi pembiakan Axenik, keputusan menunjukkan jumlah bilangan probiotik dalam *Artemia* meningkat mengikut masa bagi *B. amyloliquefaciens* L9 dan *B. amyloliquefaciens* L11. Bagi *B. amyloliquefaciens* L11 pada kepekatan 10^8 CFU mL^{-1}, masa seawal 6 jam diperlukan untuk menembusi badan *Artemia* sementara *B. amyloliquefaciens* L9 memerlukan masa lebih panjang untuk memasuki ke dalam *Artemia* (12 jam

pengeraman). Untuk esei penentangan secara *in vivo* menggunakan ketam biru juvenil, *B. amyloliquefaciens* L11 dipilih untuk digunakan berdasarkan keupayaannya dalam merencat pertumbuhan *V. harveyi* ketika aktiviti antagonis. Kaedah perendaman telah dilaksanakan dengan menggunakan 20 ketam juvenil bagi setiap tangki. Tangki untuk rawatan dieram dengan probiotik selama 24 jam dan pathogen *V. harveyi* dimasukkan pada hari berikutnya. Keputusan menunjukkan calon probiotik *B. amyloliquefaciens* L11 mempunyai kadar kemandirian yang tinggi iaitu 42 ± 0.5 % berbanding dengan *V. harveyi* sahaja (12 ± 0.8 %) pada kepekatan 10^6 CFU mL^{-1} selepas 5 hari ujian penentangan dijalankan. Tambahan daripada itu, keputusan juga menunjukkan bahawa *B. amyloliquefaciens* L11 berupaya mengurangkan jumlah *Vibrio*, memberi peningkatan dalam berat dan panjang ketam biru juvenile. Dalam analisis kandungan gen menunjukkan probiotik *B. amyloliquefaciens* L11 mengalami mutasi pada lokasi dan panjang bes yang berbeza. Mutasi kemasukan dan pembuangan adalah yang paling banyak dikesan daripada analisa gen. Mutasi pembuangan sebanyak 21 bes berlaku pada dua tempat gen yang berbeza dan 39 bes terpanjang untuk mutasi kemasukan berlaku pada satu tempat. Sebanyak 73 gen berlakunya mutasi yang menyebabkan kepelbagaian struktur pada probiotik *B. amyloliquefaciens* L11 genom pada lokasi berbeza. Kebanyakan mutasi berlaku pada kawasan tidak mengkod dan antara dua kawasan. Gen penentang bakteria terkandung dalam probiotik *B. amyloliquefaciens* L11 dianggap sebagai faktor utama dalam aktiviti penentangan. Gen lipopeptida dan poliketida adalah berhubung rapat dengan kewujudan agen penentang bakteria. Secara keseluruhannya, berdasarkan keputusan esei *in vitro* dan *in vivo*, *B. amyloliquefaciens* adalah dianggap sebagai calon probiotik yang digemari dengan sifat antibakteria.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABREVIATIONS

NaCl	Sodium Chloride
CFU	Colony forming unit
CFU mL^{-1}	Colony forming unit per milliliter
µL	Microliter
µm	Micrometer
mL	Milliliter
mg	Miligram
ºC	Degree centigrade
%	Percent
TSA	Tryptic soy agar
TCBS	Thosulfate citrate bile salt sucrose
TSB	Tryptic Soy Broth
rpm	Rotation per minute
FSSW	Filtered sterile seawater
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
$Na_2S_2O_3$	Sodium Thiosulfate
DMSO	Dimethyl sulfoxide
H_2O_2	Hydrogen peroxide
HCl	Acid hydrochloric
w/v	Weight per volume
OD	Optical density

CHAPTER 1

INTRODUCTION

1.1 Background of study

Aquaculture which comprised of aquatic organisms including fish, mollusks, crustaceans and other non-fish animals including aquatic plants are among most important food supply for human consumption (FAO, 2016). World aquaculture industry has been expand intensively for the past 20 years ago to meet world food demand by 2020 which worth around US$10 billion (Ashokkumar and Mayavu, 2014). The scenario is hope to fill the value of seafood protein sources since wild fisheries were declined as an effect of overexploited which could be seen based on fish landing per capita (Barman *et al.,* 2011; FAO, 2016). The byproduct of fisheries production in cosmetic (collagen) and fishmeal become one of the high demands for fish supply (FAO, 2016).

In addition, Malaysia's future total fish production boosted by the aquaculture sector that has a very good growth potential. It is undeniable that the fisheries sector comprising of fisheries and aquaculture plays an important role in the national economy in terms of employment, income, foreign exchange and especially food production (Tan, 1998). Malaysia has the highest fish consumption with an estimated average per capita consumption of 49 kg per capita in year 2000. This further increased to 53 kg per capita in the year 2005 (Othman, 2006) and small rise up to 53.1 kg per capita in the year 2011 (Yusoff, 2015). In addition, 56.6 kg per capita are recorded in year 2014 (Othman *et al.,* 2017) and estimated to increase to 61.1 kg in year 2020. High value fish such grouper, snapper and shrimp are exported to foreign country with good income contribution to the country (Yusoff, 2015).

In Asian country, crustaceans' aquaculture such shrimp, mussel and crab have been developed for a few decades. Mud crab being a popular species especially in India which molted crabs are place in isolated tank for a short period until fattening of soft-shelled reach marketable size (Kathirvel *et al*., 2004). This method is one of aquaculture practice which contribute to increase the vibrant of seafood industry and fulfill the demands.

The blue swimming crab, *Portunus pelagicus* (Linnaeus, 1758) also known as flower crab is one of the species that being cultured in Malaysia. Crab fisheries is estimated to grow and expand in years (Ikhwanuddin *et al.,* 2012). The cultured of female blue crab in hatchery as a broodstocks are important to maintain larvae and juveniles supply since over capture of adults crab in wild environment (Abol- Munafi *et al.,* 2017). However, according to Talpur *et al.* (2012), low survival and high mortality rate of crab larval occur in hatchery. It can be a bottleneck to this crab culture industry along with other factors contributed to this scenario such as bacterial infection, water quality, temperature

and management of the system (hygiene of the tanks) (Hewitt, 2008). It was also detected that the spread of disease that caused mobility and mortality (Wu *et al.,* 2014) were started from contaminated food which transfer to larvae culture during feeding (Nicolas *et al.,* 1989; Keskin *et al.,* 1994).

With development of this industry, disease become a major problem as a constrain contributions. Lighther *et al*. (1992), stated that uncontrolled development of cultured species led to the outbreaks of infectious diseases either from microorganisms such as bacteria, parasites and virus. The bacteria such as *Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda, E. ictaluri, Vibrio harveyi,* and *V. anguillarum* are among the most predominant pathogens in the aquaculture system. Bacterial disease such as vibriosis is cause by *Vibrio* sp. which infect crustaceans and fishes (Ashokkumar and Mayavu, 2014).

According to Otta *et al.* (2001), *Vibrio* sp. is an aquatic bacterium that widely distributed in freshwater, estuarine and marine environment. These bacteria may lead to an increase of cultured mortality rate up to 100% (Karunasaga *et al*., 1994). The infection of these bacteria was started with penetration to the host tissue and follow by deployment to other part of the host system such cellulatic enzyme production (Chatterjee and Haldar, 2012).

Other than that, haemolysin activity of the *Vibrio* sp. is also contributed to the problem. According to Martin-Laurent *et al.* (2001) the pathogen colonization on the host body is a complex process and common symptoms of disease could be seen including necrosis, anemia, and haemorrhagic. During growth out stage, blue crab larval and juvenile will lost their appetites and no body growth is notice as early symptom. It will continue with the colour changes of hepatopancrease to dark (Jithendran *et al.,* 2010).

To overcome this problem, farmers used inappropriate antibiotics or other disinfectants intensively in aquaculture such as chloramphenicol, tetracycline and oxolinic acid (Towers, 2014). Usually these antibiotics are added to cultured organism as prophylactic or growth enhancement (Marshall and Levy, 2011) and continuously used to ensure the production. Some of these products are not suitable for aquaculture farm due to the concerns relating to bioaccumulation and carcinogenicity effects to people who consuming the fish. These unethical practice also driven to the emergence of antibiotic resistant bacteria in the aquaculture (Huang *et al.,* 2015). As a consequences, the earthen culture ponds become an antibiotic resistance genes reservoirs (Tomova *et al.,* 2015). Moreover, these antibiotic resistance genes can easily be spread via horizontal gene transfer to susceptible bacteria.

In addition, these antibiotic resistant bacteria in the environment combining with human bacterial pathogens which can be transferred back to animal and human through food chain become a public health hazards concerns and leading to hardness during infectious diseases treatment (Tomova *et al.,*2015). Some of the antibiotics residual still remain in the cultured organisms after processing to customer (Pham *et al.,* 2015). In addition,

European Country has banned the application of antibiotic as growth promoter in their agriculture and aquaculture product (Done *et al.,* 2015).

Due this problem, an alternative method were introduced such probiotic which is used in aquaculture sector to control the pathogenic bacteria and for demand of environmental friendly substance. The delivery of these probiotic to the target host could be done by mixing with pharmaceutical formula and also through food consumption (Govender *et al.,* 2014). The used of *Lactobacillus plantarum* as a probiotic for blue crab larviculture gave positive impact on decreasing of *Vibrio* sp. growth (Talpur *et al.,* 2012). The antagonistic activity properties in probiotic are consider as main factor in contribution to its effect.

1.2 Problem statement

The development of the aquaculture along with a disease infection is a dilemma problem to the culture system. *Vibrio harveyi* is usually found in crab culture and other crustaceans which passed from infected broodstock to larvae through feaces route (Talpur *et al.,* 2011). High mortality during crab culture period cause by *Vibrio* infection lead to vibriosis is the major losses in economic to certain countries. Since that, studies have been done to overcome the problems and probiotic is used as one of the best alternative method (Wang *et al.,* 2008).

In Malaysia, blue crab culture is still in research progress and has a great potential to be cultured by farmers or aquaculture industry players. The cannibalism among blue crab larvae and juvenile during growth out process in hatcheries is one of the constraint in culturing system as apart from disease problem. The high mortalities of larvae and juvenile by bacterial infection is also giving more stress to the industry. Furthermore, the study on potential bacteria as a probiotic against *Vibrio* sp. in blue crab is still lacking.

1.3 Justification of the study

a. To establish environmental friendly potential probiont for *P. pelagicus* juveniles against *Vibrio harveyi* infection in Malaysia. This approach could improve the reliability and sustainability of crab hatchery systems through the use of probiotic bacteria as an alternative to disease control. It is also to characterize the properties and test their ability as the potential probiotic to the blue crab juvenile before it could be used as a probiotic.

b. To investigate the gene contents in the isolated probiotic bacteria. The structural variation, deletion and insertion of the gene were revelaed using single nucleotide polymorphism method.

1.4 Objectives of the study

Therefore, the objectives of this study were:

1. to isolate, screen and identify local bacteria as potential probionts against pathogenic *Vibrio harveyi* from different organs of crab such muscle, hemolymph, ovary, testis, gill, carapace and hepatopancreas.

2. to evaluate the characteristics of the potential probionts in biofilm formation ability, extracellular enzyme production, spore forming, biochemical properties and hemolytic activity.

3. to determine the ability of potential probionts in protecting *Artemia fransiscana* nauplii and blue crab (*Portunus pelagisus*) juveniles against *V. harveyi* infection in *in vivo* challenge assay.

4. to analyze gene contents of the probiont that involve in antibacterial activities and detection of antibiotic biosynthesis genes.

1.5 Hypothesis of the study

The hypothesis of the study:

Null hypothesis: Isolated local bacteria do not have probiotic properties and not able to confer protection towards blue crab juveniles against *Vibrio harveyi* infection.

Alternative hypothesis: Isolated local bacteria show probiotic properties and able to confer protection towards blue crab juveniles against *Vibrio harveyi* infection.

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Noor Azrin Binti Abdul Rahman was born in Alor Setar, Kedah, Malaysia on Feb 28, 1981. She received her early primary education at Sekolah Rendah Kebangsaan Suka Menanti, Alor Setar, Kedah. After completed her primary level, she continued for secondary education at Sekolah Menengah Sultanah Asma, Alor Setar, Kedah. In year 2003, she enrolled her study at University Putra Malaysia, Serdang as a graduate with a degree of Bachelor of Science in Biotechnology. In 2004 she was admitted as a Master Degree Candidate at the Aquatic Animal Health Unit, Faculty of Veterinary Medicine, UPM under Graduate Research Assistant (GRA). Previously, she was working as a young lecturer from April 2006 until February 2015 at University Teknologi MARA Cawangan Sabah. She was actively involved in research activities and consultation work for private company.

Currently, she is doing her PhD project majoring in Microbial Biotechnology at Aquaculture Department, Universiti Putra Malaysia under supervision of Dr. Murni Marlina Abd Karim, co-supervised with Associate Prof. Dr Yuzine Esa and Associate Prof. Dr Ina Salwany Md Yasin. Her study was involved in "Identification, Characterization and Assessment of *Bacillus amyloliquefaciens* as Putative Probiont for Juveniles Blue Swimming Crab (*Portunus pelagicus,* Linnaeus 1758) To Control *Vibrio harveyi* Infection". She was awarded with MyBrain scholarship from Ministry of Higher Education to support her study.

LIST OF PUBLICATION

Journal of Publication

Azrin N.A.R, Yuzine E, Ina-Salwany M.Y, and Murni Karim. (2019). The Efficacy of Potential Probiont *Bacillus amyloliquefaciens* Strain L11 in Protecting *Artemia* Nauplii and Blue Crab Juveniles Against *Vibrio harveyi* Infection. Journal of Pure and Aplied Microbiology, Vol. 13(2)

Azrin N.A.R, Yuzine E, Ina-Salwany M.Y, Suzana R, Fathiah Masduki, Murni Karim. (2017). Characterization of Potential Probionts from Blue Swimming Crab *Portunus pelagicus* and Its Antagonistic Activity Against *Vibrio harveyi*. *International Journal of Bioscience* (IJB), Vol. 11, No. 4, p. 292-303

Azrin N.A.R, Yuzine E, Ina-Salwany M.Y, and Murni Karim. (2019). Antibacterial Genes Variation in *Bacillus amyloliquefaciens* Strain L11 as Potential Probiont. (In progress).

Seminar

Oral presentation

Noor Azrin Abdul Rahman, Ina Salwany Md Yasin, Yuzine Esa and Murni Marlina Abd Karim. (2017). Isolation and Assessment of Potential Probiont Isolated from Blue Swimming Crab (*Portunus pelagicus*) Haemolymph against *Vibrio harveyi.* International Conference on Advances in Fish Health 2017. Auditorium, Office of the Deputy Vice Chancellor (Research and Innovation), UPM, Serdang, Selangor.

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