UNIVERSITI PUTRA MALAYSIA

ASSESSMENT OF Enterococcus hirae STRAIN LAB3 ISOLATED FROM ASIAN SEABASS, Lates calcarifer (Bloch, 1970) AS PROBIOTIC AGAINST Vibrio harveyi INFECTION

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By

NUR FATHIAH BINTI MASDUKI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fullfilment of the Requirement for the degree Master of Science

May 2018

DEDICATION

To my beloved father and mother

En. Masduki Bin Kasbi and Pn. Siti binti Kamin

Family members:

Nur Farqhana binti Masduki

Mohd Farqhan bin Masduki

Mohamad Faiq bin Masduki

Abstract of thesis prepared to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master Science

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Chair : Murni Marlina Abd Karim, PhD
Faculty : Institute of Bioscience

Seabass farming is one of significant important contributors to the annual world aquaculture production including Malaysia. However, vibriosis has been one of the main diseases problems often cause high mortality and reducing the production. Probiotics are now become subject of interest as new alternative in preventing vibriosis in fish farming culture. This study was undertaken to discover new potential probiotics strain from the group of lactic acid bacteria (LAB) isolated from seabass (*Lates calcarifer*).

Seven potential of LABs were successfully isolated from the intestine and liver of 15 healthy seabass. In *in vitro* screening test using series of plate assays, co-culture assay and pathogenicity test on TCBS agar, one isolate was showed potential as probiotics. The potential probiont was identified as *Enterococcus hirae* (LAB3) using 16S rRNA. This strain able to grow at pH 2 to 10 with the best growth at pH 7 within 3 h incubation period and grew best at 4% of NaCl in de Man Rogosa and Sharp (MRS) broth. Antibiotic susceptibility test revealed that, this strain was resistant to: kanamycin, penincilin, gentamycin, tetracycline and streptomycin as well as able to secrete lipase enzyme. In addition, this strain was able to produce biofilm up to 30 h of incubation period.

In a preliminary *in vivo* assay using *Artemia salina*, result demonstrated a significant survival of *Artemia* treated with *E. hirae* LAB3 at 10^6 $CFUmL^{-1}$ and challenged with *V. harveyi* (70.00±3.06%) compared to the control *Artemia* with pathogen only (*V. harveyi* 13.33±1.45%). This strain was proven able to recude the number of Vibrios load in both *Artemia* and culture water. Similar results were observed using seabass larvae as a host. Larvae treated with *E. hirae* LAB3 at 10^6 $CFUmL^{-1}$ had significant high survival after challenged with the pathogen *V. harveyi* (68.33±0.88%) compared with group with pathogen only (*V. harveyi* 16.67± 3.33%).

Vibrios counts were significantly lower in larvae and culture water treated with *E. hirae* LAB3 at the end of challenged assay. In term of growth rate, larvae treated with *E. hirae* LAB3 at concentration of 10^6 $CFUmL^{-1}$ showed slightly higher but with no

significant different in term of growth rate and length increment compared with control without probiotic added. This study suggests that *E. hirae* LAB3 has potential to be one of the probiotic candidates in aquaculture industry specifically for seabass hatchery system.

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

PENILAIAN *Enterococcus hirae* STRAIN LAB3 YANG DIPENCIL DARIPADA SIAKAP, *Lates calcarifer* (Bloch, 1970) SEBAGAI PROBIOTIK TERHADAP SERANGAN *Vibrio harveyi*

Oleh

NUR FATHIAH BINTI MASDUKI

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Pertenakan ikan siakap adalah salah satu penyumbang penting kepada pengeluaran tahunan akuakultur dunia, termasuk Malaysia. Walau bagaimanapun, penyakit vibriosis menjadi salah satu masalah utama kepada kadar kematian tinggi dan mengurangkan pengeluarannya. Probiotik kini menjadi subjek perhatian sebagai alternatif baru bagi mencegah vibriosis dalam budaya penternakan ikan. Kajian ini dijalankan untuk menemukan potensi baru probiotik dari kumpulan bakteria asid laktik (LAB) yang diasingkan dari ikan siakap (*Lates calcarifer*) bagi memerangi vibriosis.

Sejumlah tujuh LAB berpotensi telah berjaya diasingkan dari usus dan hati anak ikan siakap yang sihat. Dalam ujian penyaringan *in vitro* menggunakan siri ujian plat, ujian cerakinan kultur dan ujian patogenik pada agar TCBS, satu bakteria berpotensi sebagai probiotik. Bakteria ini dikenal pasti sebagai *Enterococcus hirae* (LAB3) menggunakan 16S rRNA. E. *hirae* LAB3 dapat berkembang pada pH 2 hingga 10 dengan pertumbuhan terbaik pada pH 7 dalam tempoh masa inkubasi 3 jam dan berkembang dengan baik pada 4% daripada NaCl dalam stok Man Rogosa dan Sharp (MRS). Ujian kerentanan antibiotik mendedahkan bahawa, LAB3 ini mempunyai pertahanan terhadap: kanamicin, penincilin, gentamicin, tetracilin dan streptomicin, malah dapat merembeskan enzim lipase. Di samping itu, LAB3 ini dapat menghasilkan biofilm sehingga 30 jam selama tempoh inkubasi.

Pada prosedur awal *in vivo* menggunakan *Artemia salina*, hasil menunjukkan kelangsungan hidup *Artemia* yang dirawat dengan *E. hirae* LAB3 pada 10^6 $CFUmL^{-1}$ dan dicabar oleh *V. harveyi* (70.00± 3.06%) berbanding dengan *Artemia* terkawal dengan patogen sahaja (*V.harveyi* : 13.33± 1.45%). Terdapat pengurangan vibrios yang signifikan dalam *Artemia* dan kultur air yang dirawat dengan *E. hirae* LAB3 berbanding dengan kumpulan kawalan (*V. harveyi* sahaja). Hasil yang sama diperoleh dengan menggunakan larva ikan siakap sebagai hos. Larva yang dirawat dengan *E. hirae* LAB3 pada 10^6 $CFUmL^{-1}$ mempunyai kadar kemandirian signifikan yang tinggi selepas dicabar dengan patogen *V. harveyi* (68.33± 0.88%) berbanding dengan kumpulan dengan patogen sahaja (*V. harveyi* :16.67± 3.33%).

Angka Vibrios jauh lebih rendah pada larva dan kultur air yang dirawat dengan *E. hirae* LAB3 pada akhir penilaian yang dijalankan. Dari segi kadar pertumbuhan, larva yang dirawat dengan *E. hirae* LAB3 pada kepekatan 10^6 $CFUmL^{-1}$ menunjukkan sedikit tinggi tetapi tiada perbezaan yang signifikan dalam jangka masa pertumbuhan dan pertambahan kepanjangan berbanding dengan kawalan tanpa ditambah probiotik. Kajian ini menunjukkan bahawa *E. hirae* LAB3 berpotensi menjadi salah satu probiotik yang dicadangkan dalam industri akuakultur khususnya bagi sistem penetasan ikan siakap.

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I certify that a Thesis Examination Committee has met on 17 May 2018 to conduct the final examination of Nur Fathiah binti Masduki on her thesis entitled "Assessment of *Enterococcus hirae* Strain LAB3 Isolated from Asian Seabass, *Lates calcarifer* (Bloch, 1970) as Probiotic Against *Vibrio harveyi* Infection" 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.

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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:

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LIST OF ABBREVIATIONS

DOC	Day of culture
NaCl	Sodium chloride
TCBS	Thiosulfate citrate bile salt sucrose
TSB	Trpticase soy broth
TSA	Trypticase soy agar
MRS	Man Rogosa and Sharp
FSSW	Filtereted sterile seawater
CFU	Colony forming unit
$CFUmL^{-1}$	Colony forming unit per mililiter
rpm	Revolution per minute
µl	Microliter
h	Hour
Kb	Kilobase
bp	Basepair
16S rRNA	16 subunit ribosomal ribonucleic acid
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
sp. or spp.	Species (for singular or plural term)
SGR	Specific growth rate

CHAPTER 1

INTRODUCTION

1.1 Background of Study

One of the world's greatest challenges to be faced is how to feed more than 9.7 billion people by 2050. Gladly, in 2014, aquaculture sectors have contributed to the supply of fish for human consumption that represent one third of the world fisheries production (FAO, 2016). According to (FAO, 2015), global aquaculture production in 2014 was recorded at 73.8 million tonnes with Asian region as a whole has been produced more farmed fish than wild catch fish since 2008, with total production reached to 44.1 percent in 2014, up from 42.1 percent in 2012 and 31.1 percent in 2004.

Among 600 aquatic species that have been cultured worldwide for production, fish such as seabass (*Lates calcarifer*) seems to be the most cultured in a variety of farming systems (FAO, 2014). Moreover, fish provided more than 3.1 billion people due to their valuable nutritional properties. Considering to the global population growth, clearly, the demand for fish consumption will increase each year. Hence, the current trend in aquaculture is towards more intensive and commercialized of aquatic production.

As in aquaculture industry, presence of pathogenic microorganism in the culture system is a major concern to the industry. Vibriosis due to *Vibrio* sp. posed a threat to the most marine aquaculture production including seabass culture which causing major economic losses (Wei and Wee, 2014). This infectious disease cost the global losses of aquaculture industry in billions of dollars annually (Lafferty *et al.*, 2015).

With concerns regarding antibiotic resistance case among pathogens as well as many chemical are now being banned, environmental friendly alternatives are become subject of interest among researchers. Probiotic is one of the several alternative approaches that gaining popularity in aquaculture. Probiotic influence the composition of gut microbiota and confer beneficial health effect to their host (Nayak, 2010; Newaj-Fyzul *et al.*, 2014). Additionally, probiotics isolated from the host gut or their environment are more compatible to be commercialized due to their ability to colonize the host gut as well as prevent a threat to the surrounding ecosystem (Verschuere, 2000; Sayes *et al.*, 2018).

Desirable characteristics for the selection of probiotic include harmless to the host, should be accepted by the host and actually work *in vivo* as opposed to *in vitro* finding (Chandrakala and Soundharanayaki, 2017). Moreover, microorganism that used as probiotics in aquaculture should be safe not only for the host yet also for their environment also human (Munoz-Atienza *et al.*, 2013).

The diversity of probiotics in the host gut is correlated with their habitats and closely interacts with these microbes. For instance, probiotic of the genera *Bacillus*, *Lactobacillus* and *Enterococcus* have the potential to influence the immune system of the host (Sayes *et al.*, 2018). Recently, lactic acid bacteria (LABs) are gaining acceptance for human and animal purposes. It's known to be present in the intestine of healthy fish (Pandiyan *et al.*, 2013). In addition, *Lactobacillus* is one of the genera of LAB that most widely used in aquaculture due to their better yield in feed conversion, growth rate, weight gain (Dawood *et al.*, 2016) as well as increase the growth performance of the fish and antagonistic activity against *Vibrios* (Afrilasari and Meryandini, 2016; Gao *et al.*, 2017). Thus, it is interesting to isolate probiotic either from the wide diversity bacteria in either host or their culture environment and examine its potentially effects as potential probiotics for commercialize.

1.2 Problem Statement

Asian seabass (*Lates calcarifer*) was one of the leading species for the previous five years in Malaysia that become main cultured species comprises 37 percent over the marine total production. However, problems related to disease outbreaks have caused serious mortalities and lowered the aquaculture production (FAO, 2015). Vibriosis causes severe economical losses in shrimp, finfish and mollusk cultivation worldwide (Austin and Zhang, 2006; Defoird *et al.*, 2014).

Vibrios belong to the *Harveyi* clade is among the major pathogens of aquatic organism (Yang and Defoirdt, 2015). Asian seabass is also susceptible to *Vibrio harveyi* infections that include vasculitis, eye-lesions and luminous vibriosis (Austin and Zhang, 2006). In Malaysia, Ransangan *et al.* (2012) have stated that, *V. harveyi* infection in Asian seabass spreads rapidly among fish stocked in the same cage since in year 2008 in open cage in Sabah. In addition, *V. harveyi* also a pathogenic organism associated with luminous vibriosis that contributed mortality in penaeid shrimp farm (Wang *et al.*, 2015).

Vibriosis is highly infectious to the early stages of fish and introducing the beneficial and healthy microbial in aquatic environment in larval rearing tanks can positively influence the well-being of the fish during larviculture (Banerjee and Ray, 2017). Hence, it is interesting to develop potential probiotic bacteria isolated from the host itself for improvement of their health.

1.3 Significant of the Study

In the past few years, application of probiotics in aquaculture has been one of the major interesting research subjects due to their capability to control diseases in aquatic farms. It could be expressed by probiotic in ability to provide nutritional substances as well as enzymes, which aids in digestion process, improve, water quality and immune response and also confer resistance towards diseases (Qi *et al.*, 2009; Tuan *et al.*, 2013).

Recently, probiotic in aquaculture have been studied widely and lactic acid bacteria (LAB) have aroused as an important scientific interest in recent years (Alonso *et al.,* 2018). LAB has their own historical role as it present in the gut microbiota of freshwater fishes with beneficial effects on growth performance, nutrient digestibility and immune system modulator (Mohapatra *et al.,* 2011). Common LABs that have been used in aquaculture are *Lactobacillus lactis*, *Enterococcus* spp and *Lactococcus* spp (Lin *et al.,* 2017).

Despite being resistance to bile salts and acidity, LAB strain is indigenous population regarded as safe status adding a merit to be probiotic in aquaculture. Based on these criteria, it was possible to isolate LAB from the gut of local marine fish and develop new local probiotic especially for Asian seabass larviculture.

1.4 Objectives of Study

It is necessary to discover local strain of probiotics that can be used in Asian seabass cultures in order to increase the production of quality and healthy fish. Hence, the objectives of this research were:

1. To isolate, screen and identify lactic acid bacteria (LAB) as probiotic isolated from juvenile seabass (*Lates calcarifer*) against pathogenic *Vibrio* spp.

2. To determine the properties of selected LAB strain as potential probiont.

3. To evaluate the protective efficacy of selected potential probiotics on *Artemia salina* and Asian seabass (*Lates calcarifer*) larvae in *in vivo* challenge assay.

1.5 Hypothesis of Study

The hypothesis of the study:

Null hypothesis: Isolated LAB unable to inhibit the growth of pathogen in *in vitro* assay, did not potray any probiotic characteristics and incapable in protecting A*rtemia salina nauplii* and seabass larvae from *V. harveyi* infections.

Alternative hypothesis: Isolated LAB able to inhibit the growth of pathogen in *in vitro* assay, showed positive characteristics as probiotics as well as able to confer protection to *Artemia salina nauplii* and seabass larvae against *V. harveyi* infection.

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