UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF Bacillus STRAIN B12 AND B45 AS PUTATIVE PROBIOTICS TO IMPROVE GROWTH AND SURVIVAL OF Epinephelus fuscoguttatus (FORSSKAL, 1775) JUVENILE CHALLENGED WITH Vibrio alginolyticus

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By

NABILAH FATIN BINTI ABD RAZAK

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Masters of Science**

November 2015

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DEDICATION

To my dearest mother who never stops encouraging me and the reason for me to not give up in my dreams.

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

DEVELOPMENT OF *Bacillus* STRAIN B12 AND B45 AS PUTATIVE PROBIOTICS TO IMPROVE GROWTH AND SURVIVAL OF *Epinephelus fuscoguttatus* (FORSSKÅL, 1775) JUVENILE CHALLENGED WITH *Vibrio alginolyticus*

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November 2015

Chairman : Ina Salwany Md. Yasin, PhD
Faculty : Agriculture

Occurrences of bacterial diseases and the overexploitation to overcome it have resulted to the emergence of antibiotic resistant microorganisms. In relation to this matter, experiments were performed in this study by identifying putative probionts, screening of putative probionts against selected pathogenic strains based on their *in vitro* antagonism activities, *in vivo* safety test, feeding trial of *E. fuscoguttatus* juveniles and challenge with *V. alginolyticus.* Gramôs method had classified 44 bacterial strains as rod, gram positive cells while biochemical test (BBL Crystal^{TM} identification system) had identified 44 bacterial strains to species level. From 44 bacterial strains, 22 were non-haemolytic isolates. Agar well diffusion assay have demonstrated two bacilli strains; B12 and B45 elucidating intermediate (++; 11 mm ï 15 mm) to highest level (+++; >16 mm) of inhibition zones against *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. harveyi* and *Aeromonas hydrophila*. The broth culture assay in this study observed a complete inhibitory activity of *Bacillus*strain B12 towards *V. alginolyticus* at pre-incubation cell density of 10^5 and 10^7 cfu/mL while *Bacillus* strain B45 have displayed no inhibitory activity. 16S rRNA gene sequencing have identified *Bacillus* strain B12 as *Bacillus amyloliquafaciens* (94% similarity) and *Bacillus*strain B45 as *Bacillus subtilis* (95% similarity). Pathogenicity test of *Bacillus* strain B12 and B45 by intraperitoneal (IP) injection have revealed no sort of abnormalities or mortalities on *E. fuscoguttatus* juveniles. Supplementation of *Bacillus* strain B12 into feed of *E. fuscoguttatus* juveniles showed an efficient feed conversion ratio (FCR) of 1.17 $\pm$ 0.07, increased specific growth rate (2.66 $\pm$ 0.23) and survival (98 $\pm$ 1.73%). Application of *Bacillus* strain B12 into daily feed had significantly increased survival ($P<0.05$) to 71.65% when *E. fuscoguttatus* juveniles were challenged with *V. alginolyticus*. To summarize, experimental findings in this study showed that *Bacillus* strain B12 at cell density of 10^7 cfu/mL have the potential to be used as feed additive to improve growth and survival of *E. fuscoguttatus* juveniles.

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

PEMBANGUNAN STRAIN *Bacillus* B12 DAN B45 SEBAGAI PROBIOTIK PUTATIF BAGI PENINGKATAN PERTUMBUHAN DAN KEMANDIRIAN *Epinephelus fuscoguttatus* (FORSSKÅL, 1775) SETELAH DICABAR *Vibrio alginolyticus*

Oleh

NABILAH FATIN BINTI ABD RAZAK

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Kemunculan penyakit yang berpunca daripada jangkitan bakteria dan penyalahgunaan kaedah penyelesaian telah mengakibatkan kehadiran mikroorganisma yang rintang terhadap antibiotik. Oleh itu, kajian telah dijalankan dengan mengenalpasti spesis probiotik, eksperimen *in vitro* bagi mengenalpasti probiotik yang berpotensi melawan strain patogen yang terpilih, analisa *in vivo* keselamatan probiotik, suplementasi probiotik di dalam diet juvenil *E. fuscoguttatus* dan ujian saringan terhadap *V. alginolyticus*. Pewarnaan Gram telah mengklasifikasikan 44 strain bakteria sebagai rod dan gram positif. Ujian biokimia (Sistem identifikasi BBL Crystal™) telah mengenalpasti spesis bagi kesemua 44 strain bakteria tersebut. Dari 44 strain bakteria, 22 telah dikenalpasti tiada tindakbalas hemolisis darah. Ujian saringan awal dengan menggunakan asai telaga agar oleh strain *Bacillus* B12 dan B45 terhadap *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi* dan *A. hydrophila* mendapati diameter zon rencatan medium yang berukuran (++; 11 mm-15 mm) sehingga rencatan tertinggi (+++; > 16 mm). Rencatan penuh dapat dilihat pada kultur kaldu strain *Bacillus* B12 (10^5 cfu/mL dan 10^7 cfu/mL) terhadap *V. alginolyticus* (10^3 cfu/mL) manakala kultur kaldu strain *Bacillus* B45 terhadap *V. alginolyticus* tidak menunjukkan sebarang tindakbalas rencatan. Penjujukan gen 16S rRNA telah dapat mengenalpasti spesis strain *Bacillus* B12 sebagai *B. amyloliquafaciens* (94% keserupaan) dan strain *Bacillus* B45 sebagai *B. subtilis* (95% keserupaan). Ujian kepatogenan strain *Bacillus* B12 dan B45 dengan kaedah suntikan intraperitoneal (IP) tidak menunjukkan sebarang perlakuan abnormal atau kematian pada juvenil *E. fuscoguttatus*. Penambahan *Bacillus* B12 dalam diet juvenil menunjukkan peningkatan nisbah perubahan makanan (FCR) sebanyak 1.17$\pm$ 0.07, kadar pertumbuhan khusus (2.66 $\pm$ 0.23) dan kemandirian (98 $\pm$ 1.73%). Aplikasi strain *Bacillus* B12 ke dalam diet juvenil *E. fuscoguttatus* secara signifikan meningkatkan kadar kemandirian ($P<0.05$) sebanyak 71.65% juvenil *E. fuscoguttatus* setelah dicabar dengan *V. alginolyticus*. Kesimpulannya, kajian ini telah membuktikan bahawa strain *Bacillus* 12 pada kepekatan 10^7 cfu/mL mempunyai kebolehan untuk bertindak sebagai suplemen makanan yang berupaya meningkatkan pertumbuhan dan kemandirian juvenil *E. fuscoguttatus*.

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I certify that a Thesis Examination Committee has met on 6 November 2015 to conduct the final examination of Nabilah Fatin binti Abd Razak on her thesis entitled "Development of *Bacillus* Strain B12 and B45 as Putative Probiotics to Improve Growth and Survival of *Epinephelus fuscoguttatus* (Forsskål, 1775) Juvenile Challenged with *Vibrio alginolyticus*" 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 is to confirm that:

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LIST OF ABBREVIATIONS

CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
rpm	Revolution per minute
16S rDNA	16 subunit ribosomal deoxyribonucleic acid
16S rRNA	16 subunit ribosomal ribonucleic acid
SD	Standard deviation
SGR	Specific growth rate
sp. or spp.	Species (for singular or plural term)
ssp.	Subspecies
TCBS	Thiosulphate-citrate-bile-sucrose agar
mt	million tonne
LRFF	Life reef food fish
IDR	Indonesian Rupiah Rates
TSB	Tryptic soy broth
TSA	Tryptic soy agar
TBC	Total bacterial count
PBS	Phosphate buffered saline
etoh	ethanol
$MgCl_2$	Magnesium chloride
µM	micromolar
dNTP	deoxynucleotide
nM	nanomolar
ng	nanogram
H_20	Dihydrogen monoxide
Kb	kilobase
w/v	weight/ volume
TE	Tris-EDTA buffer
LAB	lactic acid bacteria
CRD	completely randomized design
DGA	Directorate General of Aquaculture

CHAPTER 1

INTRODUCTION

The grouper culture industry has been established for almost three decades and is one of the most value added industries in the Far East and South Eastern Asia especially Taiwan (Lee, 1995; Wong, 1995; FAO, 2000). Based on FAO (2000) data, major producers of farmed grouper are from Taiwan and Indonesia, followed by Malaysia and Thailand. According to McGilvray and Chan (2001), live reef fishes under the serranidae family convey elevated prices of up to USD70/kg wholesale in the live markets of Hong Kong and Southern China. As the aquacultural industry became more developed and the wild marine capture fisheries were not able to satisfy the cheapest protein demand of the human population, there was an immense increase of stocking density of animals raised per unit area or volume of water. Though this method could reduce cultivation cost and increase volume of fish harvest, fishes reared in high stocking density were more susceptible to stress exposure which will eventually lead to disease and high mortality. Pathogenic bacteria which usually affects the grouper species includes *Vibrio alginolyticus* (Lee, 1995), *V. carchariae* (Yii *et al*. 1997), *Pseudomonas* sp. (Nash *et al*. 1987) and *Flexibacter* sp. (Danayadol *et al*. 1996). Until a few decades ago, usages of hazardous chemicals and antibiotics have reduced due to development of standard codes of practices in the aquaculture industry and with the improvement of bio security and use of vaccines (Haya *et al*. 2005). Massive application of antimicrobials to control disease and promote growth in the aquacultural industry advocate natural emergence of bacterial resistance (World health Organization antimicrobial resistance fact sheet 194, http://www.who.int/inf-fs/en/fact194.html). Browdy (1998) indicated probiotic as one prominent technology which has evolved from the response of diseases. This idea has been supported by the Food and Agriculture Organization of the United Nations (Subasinghe, 1997) as an effort to enhance the aquatic environment.

Probiotics can be defined as dietary supplements and live microorganisms which contain potentially beneficial bacteria or yeasts. Recent definition by FAO/WHO indicated live microorganisms which when administered in adequate amounts confer health benefit on the host (Sanders, 2003). Addition of probiotic have profound effect which includes enhancement of the water quality in aquacultural conditions, benefit the host by improving its growth, inhibiting pathogenic microorganisms and improving immune responses (Verschuere *et al*. 2000; Balcazar *et al*. 2006). *Bacillus* spp. is by far the most commonly applied probiotics in the aquaculture industry. Benefits and advantages of using the *Bacillus* as probiotic includes resistant towards inhospitable environmental conditions, long extended shelf-life with established beneficial roles in the aquaculture field (Wang *et al*. 2008). Among examples from previous literature studies includes, *B. pumilus* and *B. clausii,* have been found by Sun *et al*. (2010) to exhibit resilience to artificial gastric and intestinal fluids, and enhanced growth performance and immune responses of *E. coiodes*. Liu *et al*. (2012) in his study found that *B. subtilis* E20 promotes growth, improve immunity and resistance of *E. coiodes* against *Streptococcus* sp. and iridovirus.

Species-specific isolation and thus identification of probiotic from both commercial and non-commercial local probiotic product is very crucial as a responsible quality control effort, consumer confidence in labelling of and for safety considerations (Yeung *et al*. 2002). According to Qi *et al*. (2009), the importance of knowing the exact composition of microorganisms in a probiotic product was proven when 90 microbial agent producers have gathered in Shanghai, China have signed and supported China's agreement for quality control which includes a species-specific identification of microorganisms in probiotic products. Therefore, the aim of this study was to isolate and identify microorganisms with potential as probiotic in a non-commercial local probiotic product. In general, this would be the first attempt to investigate efficiencies of *Bacillus* spp. as probiotics isolated from a non commercial local probiotic product towards survival of the *Epinephelus fuscoguttatus* when challenged with *V. alginolyticus*.

The objectives of this project were:-

1. To identify species of unknown bacterial strains by morphological, biochemical and molecular techniques (16S rRNA gene sequencing) from the marine environment
2. To determine antagonistic activities by the agar well diffusion method of *Bacillus* spp. against selected pathogenic species.
3. To determine growth performance and survival of *E. fuscoguttatus* juveniles fed diet supplemented with *Bacillus* strain B12 post challenged with *V. alginolyticus.*

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