



UNIVERSITI PUTRA MALAYSIA

PERFORMANCE OF PROBIOTIC *Bacillus subtilis* G1 AS A DIETARY SUPPLEMENT FOR *Hemibagrus nemurus Valenciennes* FINGERLINGS

FARHANA AHMAD AFFANDI

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By

FARHANA AHMAD AFFANDI

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

October 2014

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

PERFORMANCE OF PROBIOTIC *Bacillus subtilis* G1 AS A DIETARY SUPPLEMENT FOR *Hemibagrus nemurus* Valenciennes FINGERLINGS

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October 2014

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A study was carried out to investigate the probiotic activity of *Bacillus subtilis* G1 isolated from fermented pickles on *Hemibagrus nemurus* fingerlings at Universiti Putra Malaysia. *Hemibagrus nemurus* is a highly price fish that is commercially cultured in Southeast Asia for its excellent taste. However, *H. nemurus* is a slow-growing fish that take almost a year to reach marketable price. Large-scales aquaculture of this fish has exposed the fish to a stressful condition such as diseases. *Aeromonas hydrophila* is known to be the famous bacterial infections in Bagridae catfishes. Growth hormones and antibiotics have been used for a long time in aquaculture to increase production and to prevent diseases. However, these substances have caused many problems. Therefore, probiotics are used as an alternative method for sustainable aquaculture.

This probiotic was mixed in feed at doses of 0 (C, control), 10^9 (T1), 10^7 (T2) and 10^5 (T3) cfu g⁻¹ and administrated to *H. nemurus* fingerlings for nine weeks. Results showed that *H. nemurus* fed with diet containing 10^7 cfu g⁻¹ of *B. subtilis* G1 had significantly higher weight gain, total length and specific growth rate with $248.69 \pm 3.31\%$, 13.65 ± 0.09 cm and $1.98 \pm 0.09\%$ respectively, and better protein efficiency ratio and food conversion ratio with 19.71 ± 0.33 and 1.68 ± 0.03 respectively, than those fed with the control, 10^5 , and 10^9 cfu g⁻¹ diets. Total protein gain of the body fish fed probiotic diets also increases with the increasing weight gain. This probiotic was also found to improve the water quality by lowering the NH³-N concentration. The haematological parameters such red blood cells, haemoglobin, haematocrit and mean corpuscular haemoglobin concentration, in the fish fed probiotic diets was higher compared to the control.

Inhibitory activity of the probiotic *B. subtilis* G1 against fish pathogens such as *Aeromonas hydrophila* and *Streptococcus agalactiae* was evaluated by well diffusion agar method. Inhibition zone measured showed strong inhibitory activity against *A. hydrophila* and *S. agalactiae* with 16.13 ± 0.91 mm and 17.5 ± 1.84 mm respectively. Later, *H. nemurus* of all groups were fed with their respective diets for three weeks and then were challenged with 10^6 cfu ml⁻¹ of *A. hydrophila* (0.1 ml) by intraperitoneal injection. After 14 days, the cumulative mortality of *H. nemurus* were

significantly lower in the group T1 with $30 \pm 5.8\%$ as compared to the T2 ($36.7 \pm 3.3\%$), T3 ($46.7 \pm 3.3\%$) and C ($56.7 \pm 3.3\%$) groups. These findings proved that supplementation of *B. subtilis* G1 in the diet at 10^9 cfu g⁻¹ can improve growth and disease resistance in *H. nemurus* fingerlings.



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

PRESTASI PROBIOTIK *Bacillus subtilis* G1 SEBAGAI PENAMBAHAN DIET UNTUK JEJARI BAUNG (*Hemibagrus nemurus Valenciennes*)

Oleh

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Satu kajian telah dijalankan untuk mengkaji aktiviti probiotik *Bacillus subtilis* G1 yang diasingkan dari fermentasi jeruk ke atas jejari Baung di Universiti Putra Malaysia. Ikan Baung merupakan ikan mahal yang diternak secara komersil di Asia Tenggara kerana rasanya yang lazat. Namun, Baung adalah ikan yang lambat membesar yang mengambil masa hampir setahun untuk mencapai saiz pasaran. Kepesatan penternakan ikan Baung ini telah mendedahkan ikan kepada penyakit. Penyakit dari bakteria *Aeromonas hydrophila* terkenal sebagai patogen yang menyerang ikan dari famili Bagridae. Pelbagai hormon tumbesaran dan antibiotik telah digunakan sekian lamanya untuk melawan penyakit di dalam akuakultur. Namun, bahan-bahan ini telah menyebabkan pelbagai masalah. Oleh itu, probiotik digunakan sebagai satu cara terbaik untuk akuakultur yang mampan.

Probiotik dicampurkan ke dalam makanan ikan dengan dos 0 (C), 10^9 (T1), 10^7 (T2) dan 10^5 (T3) sel g^{-1} dan diberi makan kepada jejari Baung selama sembilan minggu. Keputusan telah menunjukkan bahawa Baung yang diberi diet yang mengandungi 10^7 sel g^{-1} *B. subtilis* G1 mempunyai peratus pertambahan berat, panjang dan kadar pertumbuhan spesifik yang nyata sekali lebih tinggi dengan nilai masing-masing $248.69 \pm 3.31\%$, 13.65 ± 0.09 sm dan $1.98 \pm 0.09\%$, serta mempunyai nisbah kecekapan protein dan nisbah penukaran makanan yang baik dengan nilai masing-masing 19.71 ± 0.33 dan 1.68 ± 0.03 , berbanding dengan diet kawalan, 10^5 , dan 10^9 sel g^{-1} . Jumlah protein terkumpul dalam Baung yang diberi diet berprobiotik juga bertambah dengan pertambahan berat badannya. Probiotik tersebut juga didapati mampu meningkatkan kualiti air dengan merendahkan kepekatan NH^3-N . Parameter hematologi seperti sel darah merah, hemoglobin, hematokrit dan kepekatan min korpuskular hemoglobin Baung yang memakan diet berprobiotik turut bertambah berbanding dengan kawalan.

Aktiviti perencatan probiotik *B. subtilis* G1 terhadap patogen *Aeromonas hydrophila* dan *Streptococcus agalactiae* dinilai melalui ujian resapan gel agar. Zon perencatan yang terbentuk menunjukkan aktiviti perencatan yang kuat terhadap *A. hydrophila* dan *S. agalactiae* dengan masing-masing 16.13 ± 0.91 mm dan 17.5 ± 1.84 mm.

Jejari Baung dari semua kumpulan kemudiannya diberi diet masing-masing selama tiga minggu dan kemudian diuji dengan 10^6 sel ml^{-1} *A. hydrophila* (0.1 ml) melalui suntikan intraperitoneal. Selepas 14 hari, kematian terkumpul Baung nyata sekali lebih rendah dalam kumpulan T1 dengan $30 \pm 5.8\%$ berbanding dengan kumpulan T2 ($36.7 \pm 3.3\%$), T3 ($46.7 \pm 3.3\%$) dan C ($56.7 \pm 3.3\%$). Penemuan ini membuktikan bahawa penambahan *B. subtilis* G1 pada dos 10^9 sel g^{-1} dalam diet ikan mampu meningkatkan pertumbuhan dan ketahanan penyakit dalam jejari ikan Baung.



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Finally, I dedicate this thesis to my family, especially my mom and dad, for the faith and support that have given me the inspiration and patience necessary to see this journey through. For that, I am immeasurably grateful.

I certify that a Thesis Examination Committee has met on 31 October 2014 to conduct the final examination of Farhana Ahmad Affandi on her thesis entitled "Performance of probiotic *Bacillus subtilis* G1 as a dietary supplement for *Hemibagrus nemurus* Valenciennes fingerlings" 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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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
cfu	Colony forming unit
CP	Charoen Pokphand
DM	Dry-matter
DO	Dissolved oxygen
DoF	Department of Fisheries
dpi	Days of post-infection
EDTA	Ethylene-diamine-tetra-acetic acid
FAO	Food Agriculture Organization
FCR	Feed conversion ratio
g	Gram
h	Hour
Hb	Haemoglobin
HCl	Hydrochloric acid
Hct	Haematocrit
K ₂ SO ₄	Potassium sulfate
L	Liter
m	Meter
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
min	Minute
ml	Milliliter
mm	Millimeter
n	Number of samples
NaOH	Sodium hydroxide

NH ₃ -N	Ammonia-Nitrogen
nm	Nanometer
NSS	Normal saline solution
PER	Protein efficiency ratio
ppm	Part per million
ppt	Part per thousand
RBC	Red blood cell
rpm	Round per minute
SE	Standard error
SGR	Specific growth rate
SPSS	Statistical Package for Social Science
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UPM	Universiti Putra Malaysia
WBC	White blood cell
wkpf	Weeks of post-feeding

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of Study

Hemibagrus nemurus is commonly known as the Asian redbtail catfish or locally known as Baung (Rainboth, 1996). This catfish is widely cultured in floating net cage along the river rather than in a pond. *Hemibagrus nemurus* is a highly price aquarium fish but is commercially cultured as live food fish trade for its high nutrient content and good taste. *Hemibagrus nemurus* is a slow-growing fish that can take almost a year to reach marketable price as compared to other catfishes, under similar culture condition.

Aquaculture is widely practiced in many countries and has become an important source of income. Unfortunately, the large-scales of aquaculture practices have exposed the cultured fish and shellfish to stressful conditions, such as diseases and environmental changes that results to economic losses. Consequently, the fish production is maximised through application of commercial diets, addition of antibiotics, growth promoters, and several other additives. These drawbacks can be overcome by the use of probiotics or beneficial bacteria in aquaculture to control diseases as well as increase growth to reach marketable size in a shorter culture period. Probiotics have directly become an alternative way to antibiotic treatment, winning itself as being more environmental friendly for a sustainable aquaculture (Cruz *et al.*, 2012; Mohapatra *et al.*, 2012).

According to Soccol *et al.* (2010), probiotics are microorganisms that are capable of living successfully in the human and animal gut, and producing beneficial physiological effects by assisting in the establishment of intestinal microbial population. This will then have an antagonistic effect towards harmful bacteria, thus being advantageous towards the host. The global market of probiotic ingredients, foods and supplements for humans and animals use in 2008 was approximately US\$15,900 million and is estimated to increase up to US\$19,600 million in 2013.

As evidence above, probiotics beneficially affect the host by producing inhibitory compounds, competing for adhesion site, nutrient and energy source, as source of nutrients and enzymatic contribution to digestion, enhancing immune response, improving water quality, interacting with phytoplankton, and showing antiviral activity (Cruz *et al.*, 2012; Mohapatra *et al.*, 2012; Chiu *et al.*, 2010; Sun *et al.*, 2010; Son *et al.*, 2009; Sahu *et al.*, 2008; Verschuere *et al.*, 2000).

A fast and simple method of culturing, storing and administrating are more preferred by the aquaculturist. Currently, there are several commercial probiotic products that contain one or more live microorganisms that are marketed in powder or liquid forms. Probiotics are applied to cultured species as a feed additive that is either directly apply to water culture, or mixed with feed and administrated orally.

1.2 Problem Statement

Problems in aquaculture are associated with diseases, floods, predation, chemical poisoning, theft, and other miscellaneous causes. However, disease problems have become the most significant factor to aquaculture losses (De *et al.*, 2014; FAO, 2012; Bostock *et al.*, 2010; Meyer, 1991). In recent years, disease outbreaks have affected farms and hatcheries in several countries in Asia, South America and Africa, resulting in a huge loss of production. In 2010, China suffered losses of 1.7 million tonnes of aquaculture production caused by natural disasters, diseases and pollution. In 2011, Mozambique suffered outbreak of disease on their marine shrimp farming (FAO, 2012). Unfavorable conditions such as overcrowding, temperature fluctuations, inadequate dissolved oxygen (Wedemeyer *et al.*, 1976 as cited in Meyer, 1991), and poor culture practices such mishandling, uncontrolled feed usage and untreated waste management (Subasinghe, 2005), may alter the water culture quality thus contributing to an outbreak of diseases.

Growth and survival of catfish fry to fingerlings vary greatly depending on the condition of the culture tank, stocking densities, food abundance and the incidence of infectious diseases. About 45% of inventory losses on catfish fingerling farms are due to infectious diseases. Of the overall losses, 60% resulted from bacterial infections, 30% from parasitic infestation, 9% from fungal infections, and 1% are viral infections (Anonymous, 2010; Al-Dohail *et al.*, 2009). Pathogens from the genus *Aeromonas* were commonly found to infect the freshwater fishes in Malaysia and of these pathogens were *Aeromonas hydrophila* (69.6%), *Aeromonas caviae* (8.7%) and *Aeromonas sobria* (21.7%) (Anonymous, 2004).

Controls on many serious infectious diseases have been done through chemical disinfectants, antibiotics and vaccines. However, the use of chemical disinfectants kills not just the bacteria which caused problems to the aquatic species, but also most of the beneficial bacteria in the water column (Sahu *et al.*, 2008). Meanwhile, misuse of antibiotics and the use of unlicensed antibiotics could cause an outburst of antibiotic resistant bacteria (Hashim, 2008; Subasinghe, 2005). Taufik and Bastiawan (2003) have studied the susceptibility of *A. hydrophila* isolated from Baung to antibiotics such tetracycline, chloramphenicol and nalidixic acid. The results have showed that from 10 isolates of *A. hydrophila* tested, five isolates were resistant, two isolates were intermediate and three isolates were sensitive to

tetracycline and chloramphenicol. While one isolate was resistant, six isolates were intermediate and two isolates were sensitive to nalidixic acid. From the above findings, that majority of *A. hydrophila* strains were resistance to antibiotics due to the development of bacterial antibiotic resistance.

Vaccines are also well-known to control both bacterial and viral diseases. Unfortunately, vaccination is an expensive, time consuming and complicated process (Taylor, 2012). Moreover, killed vaccines must be inoculated via injection, thus ineffective for commercial application; and live vaccines are known to have possible reversion to virulence (Trust, 1986). Therefore, the use of probiotic bacteria has been suggested to become an alternative method to prevent and control various diseases in aquaculture while improving growth and survival (Chiu *et al.*, 2010; Sun *et al.*, 2010; Son *et al.*, 2009). Moreover, probiotics can be applied at larval and fry stages, where vaccination cannot (Mohapatra *et al.*, 2012).

1.3 Objectives

Bacillus subtilis strain G1 used in this study was isolated by Zokaeifar *et al.* (2012a) from fermented pickles. It has shown to improve the growth of juvenile marine shrimp (*Litopenaeus vannamei*) and increases the survivability of the shrimp from vibriosis. Therefore, this study was conducted on freshwater species against freshwater pathogens. The aims of this study were;

- a) To investigate the effect of *Bacillus subtilis* G1 on the growth performance and body composition of *H. nemurus* fingerlings
- b) To investigate the effect of *Bacillus subtilis* G1 on disease resistance towards *A. hydrophila* infection on *H. nemurus* fingerlings.

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