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EFFECTS OF PROBIOTICS ON THE GROWTH AND SURVIVAL OF WHITELEG SHRIMP (*Litopenaeus vannamei*) AND THEIR INHIBITORY ROLES AGAINST *Vibrio parahaemolyticus*

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By

HADI ZOKAEI FAR

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

April 2009

DEDICATION

To my most beloved wife, Iran,

For all her understanding, patience and support during all difficulties and for her technical help during my study

To my lovely son, Reza,

For making every thing worthwhile

To my dearest parents,

For their true love, constant trust, principle guide and encouragement since my childhood

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

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Chairman: Associate Professor Che Roos Saad, PhD

Faculty : Agriculture

A study focused on the use of putative bacteria as probiotics to reduce nutritional and disease problems in aquaculture industry was carried out. This study was conducted in two experiments to investigate the putative bacteria flora as probiotics (isolated from *Macrobrachium rosenbergii*) for enhancement of growth and survival of *L. vannamei* in duration of 2007 to 2008 at University Putra Malaysia.

In the first experiment, a feeding trial was carried out to investigate the potential probiotic properties of *Bacillus subtilis* isolated from *M. rosenbergii* on *L. vannamei*. Putative *B. subtilis* bacterium was added to commercial shrimp feed as a probiotic. Four types of diets were prepared by mixing the commercial pellet shrimp feed with; i) *B. subtilis* (T1), ii) mixture of *B. subtilis* and a commercial probiotic (T2), iii) commercial probiotic as positive control (T3), and iv) an un-supplemented feed as negative control (T4). After 60 days the shrimps fed diet mixed with *B. subtilis* showed the highest survival rate

75.5± 4.62 % and the greatest yield 190.00± 13.13 g and also there were significant differences ($P < 0.05$) for bacterial count between T1 and the other treated groups. It was found that, feed treated with *B. subtilis* appeared to enhance growth and survival rate of *L. vannamei* at concentration of 10^{10} CFU/g.

Another experiment was carried out to investigate the potential probiotic-ability of *B. subtilis* to combat with the *L. vannamei* disease problems. After 60 days of culture, shrimps were challenged by immersion method to *V. parahaemolyticus* (10^7 CFU/ml). Four treatment groups were presented in this experiment which were; i) T1- Shrimps treated with *B. subtilis* in the first experiment were challenged with *V. parahaemolyticus*, ii) T2- Shrimps treated with mixture of *B. subtilis* and commercial probiotic in the first experiment were challenged with *V. parahaemolyticus,* iii) T3- Shrimps treated with unaltered diet were challenged with *V. parahaemolyticus* as negative control group, and iv) T4- Shrimps treated with commercial probiotic diet were challenged with *V. parahaemolyticus* as positive control group. After 15 days of the challenge test, there were no significant differences in survival rate between treatment and control groups. There was no significant mortality or disease symptoms due to infection pathogen, and survival rate for all of treatment and control groups was 100%.

Another study was carried out to confirm whether *V. parahaemolyticus* is a pathogen. One hundred shrimp (a new group) with the same size and the same age of shrimps were prepared to confirm the pathogenicity of *V.*

parahaemolyticus. Survival rate after 10 days was 57% due to existing mortality from *V. parahaemolyticus*. To find the reason of non-mortality of the negative control group during the challenge test with *V. parahaemolyticus*, bacteria were isolated from digestive tract, muscles and body surface of negative control group, based on morphological observation. Forty three kinds of bacteria were isolated. From these isolated bacteria, 30% were gram positive bacteria, 30% were *Pseudomonas* spp. and 40% were Enterobacteriacea. Antagonism tests were put for isolated *Pseudomonas* spp. by the cross-streak method with three pathogens; *V. parahaemolyticus, V. alginolyticus, and V. cholerae*. Results of antagonist test for four isolated *Pseudomonas* spp. bacteria showed perfect antagonistic activity against the three pathogens.

Perhaps the reason for no observed mortality during the challenge test was due to availability of these natural microflora bacteria (*Pseudomonas* spp.) inside the body of shrimps, and perhaps they had an inhibitory role against *V. parahaemolyticus*. Interestingly, there was no Vibrionaceae bacteria found in the shrimps' bodies however a count of 5.5×10^7 CFU/ml of *Vibrio* bacteria was found from the culture water. It could be possible that, the *Pseudomonas* spp. from the control group (as a natural micro flora) and *B. subtilis* for treatment groups played the inhibitory roles against pathogen bacteria or Vibriosis, by action of competitive exclusion or adhesion site.

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

KESAN PROBIOTIK KEPADA PERTUMBUHAN DAN KADAR KEMANDIRIAN *(Litopenaeus vannamei)* DAN PENENTANGAN TERHADAP *Vibrio parahaemolyticus*

Oleh

HADI ZOKAIE FAR

April 2009

Penyelia : Professor Madya Che Roos Saad, PhD

Fakulti : Pertanian

Satu penyelidikan tentang penggunaan bakteria sebagai probiotik untuk mengurangkan masalah berkaitan pemakanan dan penyakit dalam industri akuakultur telah dijalankan. Dua eksperimen telah dijalankan untuk mengkaji keberkesanan bakteria flora (didapati dari *Macrobrachium rosenbergi)* sebagai probiotik untuk meningkatkan pertumbuhan dan kemandirian *Litopenaeus vannamei* pada tempoh 2007-2008 di Universiti Putra Malaysia.

Dalam eksperimen pertama, satu kajian pemakanan telah dilakukan untuk melihat keupayaan probiotik *Bacillus subtilis* yang diasingkan daripada *M. rosenbergii* terhadap *L. vannamei*. Bakteria *B. subtilis* telah dicampurkan ke dalam makanan udang sebagai probiotik. Empat jenis diet telah disediakan dengan mencampurkan pelet makanan udang komersial bersama i) *B. subtilis* (T1), ii) campuran *B. subtilis* dan probiotik komersial (T2), iii) probiotik komersial sebagai kawalan positif (T3), dan iv) makanan yang tiada suplemen sebagai kawalan negatif (T4). Selepas 60 hari, udang yang diberi

makanan bercampur *B. subtilis* telah menunjukkan kadar kemandirian yang paling tinggi iaitu 75.5± 4.62 %, hasil yang terbaik iaitu 190.00± 13.13 g dan juga terdapat perbezaan bererti ($P < 0.05$) untuk jumlah bakteria antara T1 dan kumpulan yang lain. Didapati juga makanan yang telah dicampur *B. subtilis* boleh meningkatkan pertumbuhan dan kemandirian *L. vannamei* pada kepekatan 10^{10} CFU/g.

Kajian seterusnya dijalankan untuk mengkaji keupayaan probiotik *B. subtilis* untuk menghadapi dan melawan penyakit terhadap *L. vannamei*. Selepas 60 hari dipelihara, udang telah didedahkan kepada *V. parahaemolyticus* (10^7 CFU/ml) mengunakan teknik rendaman. Terdapat empat rawatan yang berlainan dalam kajian ini iaitu; i) T1- udang yang diberi rawatan menggunakan *B. subtilis* dalam eksperimen pertama digunakan untuk menghadapi *V. parahaemolyticus*, ii) T2 - Udang yang diberi rawatan menggunakan campuran *B. subtilis* dan probiotik komersial dalam kajian pertama digunakan untuk menghadapi *V. parahaemolyticus,* iii) T3 - udang yang tidak diberi suplemen menghadapi *V. parahaemolyticus* sebagai kawalan negatif, dan iv) T4 - udang yang diberi rawatan menggunakan probiotik komersial dalam eksperimen pertama digunakan untuk menghadapi *V. parahaemolyticus* kawalan positif. Selepas 15 hari ujian persaingan, tiada perbezaan bererti bagi kemandirian antara kumpulan rawatan dan kawalan. Tiada kematian dan simptom penyakit kesan daripada patogen. Manakala kemandirian bagi kesemua kumpulan yang diberi rawatan dan kawalan adalah 100%.

Oleh itu, satu lagi kajian dilakukan untuk mengesahkan samada *V. parahaemolyticus* adalah satu patogen atau pun tidak. Seratus ekor udang (kumpulan baru) yang mempunyai saiz dan umur yang sama telah digunakan dalam kajian ini untuk mengesahkan kesan patogen oleh *V. parahaemolyticus*. Kadar kemandirian selepas 10 hari ialah 57% disebabkan oleh *V. parahaemolyticus*. Untuk mengetahui sebab kemandirian yang tinggi bagi rawatan kawalan positif semasa pendedahan kepada *V. Parahaemolyticus*, saringan bakteria dari usus, otot dan permukaan badan dan berasaskan pemerhatian morfologi dilakukan terhadap udang dalam rawatan kawalan ini. Sebanyak 43 jenis bakteria telah diasingkan dimana sebanyak 30% adalah merupakan gram positif, 30% merupakan *Pseudomonas* spp. dan 40% pula terdiri daripada Enterobacteriacea. Ujian antagonis telah dijalankan ke atas *Pseudomonas* sp. menggunakan teknik "cross-streak" dengan menggunakan tiga patogen; *V. parahaemolyticus, V. alginolyticus, and V. cholera*. Keputusan bagi ujian antagonis bagi empat *Pseudomonas* sp bacteria yang telah diasingkan menunjukkan terdapat aktiviti antagonis yang baik menentang 3 patogen tersebut.

Tiada kematian semasa ujian pendedahan tersebut berkemungkinan disebabkan kewujudan bakteria microflora semulajadi (*Pseudomonas* sp) di dalam badan udang, dan kemungkinan juga ia dapat menekan kewujudan *V. parahaemolyticus.* Satu lagi keputusan yang memberangsangkan adalah tidak terdapat bakteria jenis Vibrionaceae yang diasingkan dari badan udang, walaubagaimanapun bilangan *Vibrio* dari air kultur adalah 5.5×10^7 CFU/ml. Barangkali, *Pseudomonas* spp. untuk kumpulan kawalan sebagai micro flora semulajadi dan *B. subtilis* bagi kumpulan rawatan, memainkan peranan melawan patogen atau Vibriosis, melalui persaingan “exclusion” atau untuk tapak perlekatan.

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- My wife for her constant encouragement and her concerns during the study period.
- Above all, to GOD almighty for making the study possible.

I certify that a Thesis Examination Committee has met on 23th April 2009 to conduct the final examination of Hadi Zokaei Far on his master thesis entitled "Effects of Probiotics on the Growth and Survival of Whiteleg Shrimp (*Liptopenaeus vannamei*) and their Inhibitory Roles Against *Vibrio parahaemolyticus*" 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.

Members of the Examination Committee are as follows:

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Date: 18 June 2009

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master. The members of the Supervisory Committee are as follows:

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Date: 9 July 2009.

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and it is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

HADI ZOKAIE FAR

Date: 4 June 2009

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ANOVA	Analysis of variance
CFU	Colony forming unit
$CHCL_3$	Chloroform
CRD	Completely Randomized Design
DO	Dissolved oxygen
FAO	Food Agriculture Organization
FCR	Feed conversion ratio
g	Gram
ha	Hectare
L	Liter
m	Meter
min	Minute
ml	Milliliter
mm	Millimeter
MYP	Mannitol-Egg Yolk-Polymyxin
NA	Nutrient Agar
NACL	Sodium Chloride
nm	Nanometer
NSS	Normal Saline Solution
PL	Post larvae
rpm	Round per minute
SD	Standard deviation
SGR	Specific growth rate

SPF	Specific Pathogen Free
SPR	Specific Pathogen Resistance
SPSS	Statistical Package for Social Science
mt	Metric tonne
TCBS	Thiosulphate citrate bile salt agar
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
UPM	Universiti Putra Malaysia

CHAPTER 1

GENERAL INTRODUCTION

1-1 Background of the Study

1-1-1 Economic Status of Aquaculture

In 2005 world production of capture fisheries and aquaculture was about 142 million tonnes, providing an apparent per capita supply of 16.6 kg. The proportion of aquaculture production amounted to 43 percent of this total. Excluding China, annual per capita supply has shown a modest growth rate of about 0.4 percent, since 1992 as growth in supply from aquaculture more than offset the effects of static capture fishery production and a rising population (FAO, 2007).

Shrimp aquaculture expanded significantly during the 1980s and now represents a multi-billion dollar a year industry. In 2002, the global shrimp farming industry produced an estimated 1.6 million metric tons of shrimp, and production is projected to increase at a rate of 12-15% per year over the next several years (Rosenberry, 2003). Although farmed shrimp now represent about 50% of the global penaeid shrimp supply, farmers have suffered significant economic losses over the last decade, largely from viral diseases that have plagued the industry.

1-1-2 Disease Problems

The reason of low level of aquaculture production has been due to a combination of both, disease and pollution. While disease played the prominent role in declining aquaculture stocks, both factors caused massive mortality in the leading aquaculture countries (Fast and Menasveta 2000). The diseases that brought the most impact to the industry include viral infections and bacteriosis. White spot disease is a type of viral infection of shrimp, and this disease alone caused global economic losses about US$ 3,000 million per year (Hill, 2000). Disease caused devastation to developing countries whose economics are heavily dependent on aquaculture farming.

1-1-3 Probiotic Usage in Aquaculture

With the rapid population growth and human requirement to fish protein source as food and also due to lack of land and water source, the modern aquaculture system began. Researchers developed techniques for intensive breeding and rearing of various aquatic animals. By using intensive systems and recirculation water systems, requirement for exchange of rearing water was reduced from 30-100% to 5-10%. There is no serious problem for aquatic animals which are newly stocked in these systems especially when they are in the early stage of their life. When the growth rate increased and the animal population are at the high density some factors such as; how to enhance to appropriate growth? How to increase the immune system of the prawn body? and how to approach to appropriate feed conversion ratio

(FCR)? In the intensive system must be on perspective. On the other hand water management must be handled appropriately due to high density of the intensive systems. From apparent problems in the aquaculture industry, almost all owing to the use of the intensive systems, perhaps, controlling of disease outbreak, specially viral and bacterial diseases are the most important concern. Conventional approaches to control diseases include; the use of antimicrobial drugs, pesticides and disinfectants (Gomez-Gil *et al*. 2000) have had limited success in the prevention or cure of aquatic diseases. Global losses resulting from shrimp microbial diseases are around 3 billion US dollars (Farzanfar, 2006; Vaseeharan & Ramasamy, 2003). The potential negative consequences of using antibiotics in aquaculture, such as the development of drug resistant bacteria and the reduced efficiency of antibiotic resistant for human and animal diseases, have led to the use of nonpathogenic bacteria as probiotic control agents (Vaseeharan and Ramasamy, 2003) .

During the past 50 years, numerous trials were conducted with microorganisms known as probiotics in efforts to improve cultivability of food animal, and to improve human health and welfare. Appropriate probiotic applications were shown to improve intestinal microbial balance, thus leading to improved food absorption (Parker, 1974; Fuller, 1989), and reduced pathogenic problems in the gastrointestinal tract (Lloyd *et al*., 1977; Snoeyenbos *et al*., 1978; Pivnick *et al*., 1981; Cole and Fuller, 1984; Goren et al., 1984). Several probiotic species used were *Lactobacillus* spp. (Muralidhara *et al*., 1977; Pollman *et al*., 1980; Jonsson, 1986),