
**UNIVERSITI PUTRA MALAYSIA**

***POTENTIAL PROBIOTICS FOR WHITE SHRIMP (Litopenaeus vannamei, Boone 1931) DERIVED FROM PICKLE HOMOGENATE***

**HADI ZOKAEI FAR**

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# POTENTIAL PROBIOTICS FOR WHITE SHRIMP (*Litopenaeus vannamei,* Boone 1931) DERIVED FROM PICKLE HOMOGENATE

**By**

**HADI ZOKAEI FAR**



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

**October 2012**

**DEDICATION**

To my most beloved wife, Iran,

For all her understanding, patience and supports during my study

To my lovely son, Reza,

For making everything 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 Doctor of Philosophy

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**October 2012**

**Chairman: Associate Professor Che Roos Saad, PhD**

**Faculty : Agriculture**

The present study focused on the use of candidate probiotic bacteria derived from a vegetable pickle to improve the growth performance, survival rate, culture condition and reduce the infectious disease problems in shrimp aquaculture. This study was conducted in four experiments to investigate probiotic abilities of isolated bacteria from pickle in juvenile white shrimp *L. vannamei*.

Two potential probiotic strains were isolated from vegetable pickle based on the antagonistic activity against two shrimp pathogens *Vibrio harveyi* and *Vibrio parahaemolyticus*. These probiotics were named as strain L10 (the isolate number 10 from vinegar) and strain G1 (the isolate number 1 from garlic). Both bacteria were identified by biochemical test, followed by 16s ribosomal RNA gene sequence analysis

as *Bacillus subtilis*, and characterized by PCR amplification repetitive bacterial DNA elements (rep-PCR). Subsequently, *B. subtilis*, L10 and G1 strains were tested for antibacterial activity under different physical conditions, including culture medium, salinity, pH, and temperature using the agar well diffusion assay. Among the different culture media, LB broth was the most suitable medium for antibacterial production. Both strains showed the highest level of antibacterial activity against the two pathogens at 30 ºC and 1% NaCl. Under the pH conditions strain G1 showed the greatest activity against *V. harveyi* at pH 7.3-8.0 and against *V. parahaemolyticus* at pH 6.0-8.0, whereas strain L10 showed the greatest activity against the two pathogens at pH 7.3. In addition wide ranges of tolerance to NaCl, pH and temperature were also recorded for both strains.

The potential probiotic strains were subjected for characterization of antibacterial substances and detection of antibiotic biosynthesis genes. Two strains of *B. subtilis*, L10 and G1, were thus examined against *Vibrio harveyi* for minimum inhibitory activity and temperature stability of the cell-free supernatants using well-diffusion agar assay. For minimum inhibitory activity a serial dilution of the cell-free supernatant was made at 0, 25, 50, 75% using sterile phosphate buffered saline and those supernatants were then tested for antibacterial activity. The result showed the reduction of antibacterial activity with the higher dilution factors, however, a reasonable activity (7 mm) was observed for both strains even at the highest dilution factors (75%). Temperature stability of the cell-free supernatant was tested at -20 ºC after 7 months preservation at 60, 80, 100, and 120 ºC for 20 min. Both strains showed great antibacterial activity of the cell-free supernatant preserved at -20 ºC, but not those treated at 60, 80, 100, and 120 ºC.

Additionally, in order to characterize the antibacterial substances the cell-free supernatant of both strains were treated with four different enzymes. Results showed considerable reduction of antibacterial activity for both strains, indicating the proteinaceous nature of the antibacterial substances. Extra-cellular enzyme production revealed the ability of both strains in secretion of protease and amylase in PG and SA agar. On the other hand no production of cellulase enzyme was recorded for both strains in CMC agar. Subsequently, the antibiotic biosynthesis genes were detected and identified using molecular techniques. Both strains showed similar pattern of antibiotic biosynthesis genes including *bacD*, *bacA* and *bacB* genes for production of bacilysin substances, *ppsE*/*FenB* gene for production of plipastatin/fengycin substances, *albF* and *albA* genes for secretion of subtilosin, and *srfAB* and *sfP* genes for secretion of surfactin. On the basis of the great antibacterial activity, extracellular enzyme production and possessing of antibiotic biosynthesis genes, both strains were considered for further *in vivo* studies via different application methods (feed and water) for white shrimp, *L. vannamei*.

The first *in vivo* study was carried out to evaluate the safety and effects of both probiotic candidates, *Bacillus subtilis* strains L10 and G1 through dietary administration, on the growth performance, digestive enzyme activity, immune gene expression and disease resistance of juvenile white shrimp *L. vannamei*. The safety of candidate probiotics was determined by injection a dose of $10^{10}$ CFU $ml^{-1}$ into third abodominal segment of shrimp *L. vannamei*. Both strains were harmless to shrimp as no mortality was observed after the injection for 15 days. A mixture of two probiotic strains, L10 and G1 in equal

proportions, was administered at two different doses $10^5$ (BM5) and $10^8$ (BM8) CFU $g^{-1}$ feed to shrimp for eight weeks. In comparison to untreated control group, final weight, weight gain and digestive enzyme activity were significantly greater in shrimp fed BM5 and BM8 diets. Significant differences for specific growth rate (SGR) and survival were recorded in shrimp fed BM8 diet as compared with the control; however, no significant differences were recorded for food conversion ratio (FCR) among all the experimental groups. Eight weeks after the start of the feeding period, shrimp were challenged with *Vibrio harveyi*. Statistical analysis revealed significant differences in shrimp survival between probiotic and control groups. Cumulative mortality of the control group was 63.3%, whereas cumulative mortality of the shrimp that had been given probiotics was 20.0% with BM8 and 33.3% with BM5. Subsequently, real-time PCR was employed to determine the mRNA levels of prophenoloxidase (proPO), peroxinectin (PE), lipopolysaccharide- and β-1,3-glucan- binding protein (LGBP) and serine protein (SP). The expression of all immune-related genes studied was significantly up-regulated ($P \leq 0.05$) in the shrimp fed BM5 and BM8 diets compared to the control group. These findings demonstrate that administration of *B. subtilis* strains, L10 and G1, can improve growth performance and disease resistance through an enhanced immune response in shrimp.

The second *in vivo* experiment was carried out to investigate the effect of *B. subtilis* strains L10 and G1 on the water quality, ion reduction, growth performance, digestive enzyme activity, immune response, and disease resistance of juvenile white shrimp *L. vannamei*. A mixture of two probiotic strains, L10 and G1 in equal proportion, was

administered at two different doses $10^5$ (BM5) and $10^8$ (BM8) CFU $ml^{-1}$ rearing water to shrimp for eight weeks. Both probiotic groups showed promising effects of the improvement of water quality and ion reduction. In comparison to untreated control group, final weight, weight gain, specific growth rate (SGR), and digestive enzyme activity were significantly greater in BM5 and BM8 groups. Significant differences for survival were recorded in the BM8 group as compared with the control; however, no significant differences were recorded for food conversion ratio (FCR) among all the experimental groups. Eight weeks after the start of experiment, shrimp were challenged with *Vibrio harveyi*. Statistical analysis revealed significant differences in shrimp survival between probiotic and control groups. Cumulative mortality of the control group was 80%, whereas cumulative mortality of the shrimp that had been given probiotics was 36.7% with BM8 and 50% with BM5. Subsequently, real-time PCR was employed to determine the mRNA levels of prophenoloxidase (proPO), peroxinectin (PE), lipopolysaccharide- and β-1,3-glucan- binding protein (LGBP) and serine protein (SP). The expression of all immune-related genes studied was significantly up-regulated in the BM5 and BM8 groups compared to the control group. These results suggest that administration of *B. subtilis* strains, L10 and G1, can improve growth performance, immune response and disease resistance of shrimp. In conclusion, both strains, *B. subtilis* L10 and G1, have the potential to be probiotic candidates for the improvement of shrimp culture.

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

**PROBIOTIK BERPOTENSI UNTUK UDANG PUTIH (*Litopenaeus vannamei* Boone 1931) DARI JERUK HOMOGENATE**

Oleh

**HADI ZOKAEI FAR**

**Oktober 2012**

**Pengerusi: Profesor Madya Che Roos Saad, PhD**

**Fakulti: Pertanian**

Kajian ini tertumpu kepada penggunaan bakteria calon probiotik yang berasal dari jeruk sayur-sayuran untuk meningkatkan prestasi pertumbuhan, kadar kemandirian, keadaan kultur dan mengurangkan masalah penyakit berjangkit dalam pengkulturan udang. Kajian ini telah dijalankan dalam empat eksperimen untuk menyiasat kebolehan bakteria probiotik yang dipencilkan dari jeruk bagi juvana putih udang *L. vannamei*.

Dua strain probiotik yang berpotensi telah diasingkan dari jeruk sayur berdasarkan aktiviti antagonis terhadap dua patogen udang *Vibrio harveyi* dan *Vibrio parahaemolyticus*. Probiotik ini telah dinamakan sebagai L10 (pencilan kesepuluh dari cecair cuka) dan G1(pencilan pertama dari bawang putih). Kedua-dua bakteria telah

dikenal pasti dengan ujian biokimia, diikuti oleh urutan analisis 16 ribosom RNA gen sebagai *Bacillus subtilis*, dan dicirikan oleh pengulangan amplifikasi PCR unsur DNA bakteria (wakil-PCR). Seterusnya, *B. subtilis*, L10 dan strain G1 telah diuji untuk aktiviti antibakteria di bawah keadaan yang fizikal yang berbeza, termasuk medium kultur, kemasinan, pH, dan suhu menggunakan asei susupan telaga agar. Antara media kultur yang berbeza, broth LB adalah medium yang paling sesuai untuk pengeluaran antibakteria. Kedua-dua jenis strain menunjukkan aktiviti antibakteria tahap tertinggi terhadap dua patogen pada 30 ºC dan 1% NaCl. Strain G1 menunjukkan aktiviti terbaik terhadap *V. harveyi* pada pH 7.3-8.0 dan terhadap *V. parahaemolyticus* pada pH 6.0-8.0, manakala strain L10 menunjukkan aktiviti terbaik terhadap dua patogen pada pH 7.3. Di samping julat toleransi yang luas terhadap NaCl, pH dan suhu juga telah dicatatkan bagi kedua-dua jenis strain tersebut.

Potensi bagi strain probiotik adalah tertakluk bagi pencirian bahan antibakteria dan pengesanan gen biosintesis antibiotik. Kedua strain *B. subtilis*, L10 dan G1, itu telah dikaji untuk aktiviti perencatan minimum dan kestabilan suhu supernatants sel bebas terhadap *Vibrio harveyi* menggunakan asei susupan telaga agar. Untuk aktiviti perencatan minimum pencairan bersiri supernatan sel bebas telah dibuat pada 0, 25, 50, 75% menggunakan salina steril fosfat penimbal dan kemudiannya diuji untuk aktiviti antibakteria. Hasilnya menunjukkan pengurangan aktiviti antibakteria dengan faktor pencairan yang lebih tinggi, bagaimanapun, aktiviti yang munasabah (7 mm) telah diperhatikan untuk kedua-dua jenis strain walaupun pada faktor pencairan tertinggi (75%). Kestabilan suhu supernatan sel bebas telah diuji pada -20 ºC selepas disimpan

selama 7 bulan pada suhu 60, 80, 100, dan 120 ºC selama 20 min. Sel- bebas supernatan bagi kedua jenis strain telah menunjukkan aktiviti antibakteria terbaik pada -20 ºC, tetapi tidak bagi sel-bebas yang dirawat pada 60, 80, 100, dan 120 ºC. Selain itu, untuk mencirikan bahan antibakteria, sel-bebas supernatan daripada kedua-dua jenis telah dirawat dengan empat enzim yang berbeza. Keputusan menunjukkan pengurangan besar aktiviti antibakteria untuk kedua-dua strain, menunjukkan sifat protein bahan antibakteria. Pengeluaran enzim tambahan sel mendedahkan keupayaan kedua-dua strain merembes protease dan amilase dalam agar PG dan SA. Sebaliknya tidak ada pengeluaran enzim selulase dicatatkan bagi kedua-dua strain dalam agar CMC. Seterusnya, gen biosintesis antibiotik telah dikesan dan dikenal pasti menggunakan teknik molekul. Kedua-dua strain menunjukkan corak yang sama bagi gen biosintesis antibiotik termasuk gen *bacD, bacA* dan *bacB* untuk pengeluaran bahan bacilysin, gen *ppsE/ FenB* untuk pengeluaran bahan plipastatin/fengycin, gen *albF* dan *albA* untuk rembesan subtilosin, dan gen *srfAB* dan *sfP* untuk rembesan surfactin. Bagi asas aktiviti antibakteria yang tinggi, pengeluaran enzim ekstraselular dan memiliki gen biosintesis antibiotik, kedua-dua strain telah diambil perhatian untuk kajian *in vivo* melalui kaedah yang berlainan (makanan dan air) bagi udang putih, *L. vannamei.*

Kajian *in vivo* yang pertama telah dijalankan untuk menilai keselamatan dan kesan kedua-dua calon probiotik, *Bacillus subtilis* strain L10 dan G1 melalui pemakanan, terhadap prestasi pertumbuhan, aktiviti enzim pencernaan, ekspresi gen imun dan rintangan penyakit juvana putih udang *L. vannamei*. Keselamatan calon probiotik telah ditentukan dengan suntikan dos $10^{10}$ $CFUml^{-1}$ ke dalam segmen ketiga abodomen udang

*L. vannamei*. Kedua-dua strain adalah tidak berbahaya kepada udang kerana tidak dapat kematian diperhatikan selepas suntikan selama 15 hari. Satu campuran kedua strain probiotik, L10 dan G1 dalam perkadaran yang sama, telah dilakukan pada dua dos yang berbeza $10^5$ (BM5) dan $10^8$ (BM8) CFU $g^{-1}$ makanan udang selama lapan minggu. Berbanding dengan kumpulan kawalan yang tidak dirawat, berat akhir, pertambahan berat badan dan aktiviti enzim pencernaan adalah ketara lebih tinggi dalam udang yang diberi makan diet BM5 dan BM8. Perbezaan yang signifikan ($P \leq 0.05$) bagi kadar pertumbuhan spesifik (SGR) dan kemandirian dicatatkan dalam udang yang memakan diet BM8 berbanding dengan kawalan; bagaimanapun, tiada perbezaan ketara dicatatkan bagi nisbah penukaran makanan (FCR) di kalangan semua kumpulan eksperimen. Lapan minggu selepas permulaan tempoh pemakanan, udang telah dicabar dengan *V. harveyi*. Analisis statistik menunjukkan perbezaan yang signifikan bagi kemandirian udang antara probiotik dan kumpulan kawalan. Mortaliti kumulatif kumpulan kawalan adalah 63.3%, manakala kematian terkumpul udang yang telah diberikan probiotik adalah 20.0% dengan pemberian makanan BM8 dan 33.3% dengan pemberian makanan BM5. Seterusnya, real-time PCR telah digunakan untuk menentukan tahap prophenoloxidase (proPO) mRNA, peroxinectin (PE), lipopolysaccharide dan β-1 ,3-pengikat glucan protein (LGBP) dan protein serine (SP). Ungkapan semua gen imun berkaitan dikaji adalah ketara sehingga dikawal selia ($P \leq 0.05$) dalam udang yang memakan diet BM5 dan BM8 berbanding dengan kumpulan kawalan. Penemuan ini menunjukkan bahawa melalui pemberian makanan, strain L10 dan G1 *B. subtilis*, boleh meningkatkan prestasi pertumbuhan dan rintangan penyakit melalui tindak balas imun dalam udang.

Kedua dalam eksperimen *vivo* telah dijalankan untuk mengkaji kesan *B. subtilis* strain L10 dan G1 pada kualiti air, pengurangan ion, prestasi pertumbuhan, aktiviti enzim pencernaan, tindak balas imun, dan rintangan penyakit juvana putih udang *L. vannamei*. Satu campuran dua jenis probiotik, L10 dan G1 dalam nisbah yang sama, telah ditadbir pada dua dos yang berbeza $10^5$ (BM5) dan $10^8$ (BM8) CFU $ml^{-1}$ penternakan air udang selama lapan minggu. Kedua-dua kumpulan probiotik menunjukkan kesan yang menjanjikan peningkatan kualiti air dan pengurangan ion. Berbanding dengan kumpulan kawalan yang tidak dirawat, berat akhir, pertambahan berat badan, kadar pertumbuhan spesifik (SGR), dan aktiviti enzim pencernaan adalah lebih ketara dalam BM5 dan BM8 kumpulan. Perbezaan yang signifikan untuk hidup telah direkodkan dalam kumpulan BM8 sebagai berbanding dengan kawalan; bagaimanapun, tiada perbezaan ketara dicatatkan bagi nisbah penukaran makanan (FCR) di kalangan semua kumpulan eksperimen. Lapan minggu selepas permulaan eksperimen, udang telah dicabar dengan *V. harveyi*. Analisis statistik menunjukkan perbezaan yang signifikan dalam hidup udang antara probiotik dan kumpulan kawalan. Mortaliti Kumulatif kumpulan kawalan adalah 80%, manakala kematian terkumpul udang yang telah diberikan probiotik adalah 36.7% dengan BM8 dan 50% dengan BM5. Selepas itu, real-time PCR telah digunakan untuk menentukan tahap mRNA prophenoloxidase (proPO), peroxinectin (PE), lipopolysaccharide dan β-1 ,3-mengikat glucan protein (LGBP) dan protein serine (SP). Ungkapan semua gen imun berkaitan dikaji adalah jauh dikawal selia dalam kumpulan BM5 dan BM8 berbanding dengan kumpulan kawalan. Kesimpulannya, keduadua strain B. subtilis L10 dan G1 bapotensi sebagai calon probiotik untuk penambahbaikan kultur udang.

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- My family for their love, understanding, encouragement and moral support towards this achievement.
- 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 25th October 2012 to conduct the final examination of Hadi Zokaei Far on his master thesis entitled "**POTENTIAL PROBIOTICS FOR WHITE SHRIMP (*Litopenaeus vannamei,* Boone 1931) DERIVED FROM PICKLE HOMOGENATE**" 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 Doctor of Philosophy.

Members of the Examination Committee are as follows:

**Mihdzar Abdul Kadir, PhD**
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This thesis submitted to the Senate of Universiti Putra Malaysia 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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## DECLARATION

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

**HADI ZOKAEI FAR**

Date: 25 October 2012

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| | |
|---|---|
| $(NH_4)_2SO_4$ | Ammonium Sulphate |
| ANOVA | Analysis of Variance |
| ATCC | American type culture collection |
| BLASTN | Basic Local Alignment Search Tool |
| bp | base pair |
| CFU | Colony Forming Unit |
| DDBJ | DNA Data Bank of Japan |
| $ddH_2O$ | double-distilled water |
| DNA | Deoxyribonucleic acid |
| DO | Dissolved Oxygen |
| EMBL | European Molecular Biology Laboratory |
| FAO | Food and Agriculture organization |
| FCR | Feed conversion ratio |
| GIT | Gastrointestinal tract |
| HCl | Hydrochloric acid |
| $KNO_3$ | Potassium nitrate |
| LB broth | Luria-Bertani broth |
| LGBP | Lipopolysaccharide- and β-1,3-Glucan Binding Protein |
| MB | Marine Broth |
| MHA | Muller Hinton Agar |
| MYP agar | Mannitol Egg Yolk Polymyxin Agar |

| | |
|---|---|
| NA | Nutrient Agar |
| $NaCl_2$ | Sodium Chloride |
| $NaNO_2$ | Sodium nitrite |
| NaOH | Sodium hydroxide |
| NB | Nutrient Broth |
| NSS | Normal Saline Solution |
| OD | Optical Density |
| ONPG | ortho-nitro-phenyl-β-D-galactopyranside |
| PBS | Phosphate Buffered Saline |
| P(H1) | hypothesis test ($P \leq 0.05$) |
| PCR | polymerase chain reaction |
| PE | Peroxinectin |
| PL | Post Larvae |
| proPO | Prophenoloxidase |
| Rep-PCR | Repetitive Extragenic Palindromic Sequence Polymerase Chain Reaction |
| rpm | Rotations per minute |
| rRNA | Ribosomal ribonucleic acid |
| RT-PCR | Reverse-Transcription-Polymerase Chain Reaction |
| SD | Standard Deviation |
| SGR | Specific Growth Rate |
| SP | Serine Protein |
| SPF | Specific Pathogen Free |

| | |
|---|---|
| SPSS | Statistical Package for the Social Sciences |
| TCBS agar | Thiosulfate-citrate-bile salts-sucrose agar |
| V | Voltage |

# CHAPTER 1

## INTRODUCTION

Shrimp aquaculture plays an important economic role, especially in those countries whose economy is highly dependent on the aquaculture programs. World production of shrimp, both captured and farmed, is about 6 million tonnes, owning about 60% of the world market. These values have made the shrimp as the most important internationally traded fishery commodity. Currently 40% of the world shrimp production is from aquaculture which appears to be 60% of the internationally traded shrimp (Gillett, 2008).

Promising economic achievement of the shrimp aquaculture industry began with the development of cultural system, such as intensive breeding and rearing system. Penaeid shrimp including *Penaeus japonicus, Penaeus monodon, Penaeus vannamei, and Penaeus stylirostris* have been successfully cultured using the intensive systems. Total world production of shrimp aquaculture in 1990 was recorded for 25% of total world shrimp harvest which was estimated half of all shrimp exports worldwide. Shrimp aquaculture production was steadily increasing year by year until a major production was recorded in 2004 estimating 2 million tonnes which was approximately twice the amount of the world production in 1999 (Gillett, 2008).

Among penaeid shrimp species, *Litopenaeus vannamei* has been successfully cultured worldwide. The world production of *L. vannamei* was expanded from 10% of total production to 75% in 2009. Lower production cost, rapid growth rate, tolerance to high

stocking density, tolerance to low salinity and temperature, lower protein requirement, and higher pathogen resistance have brought more international interests on *L. vannamei*'s production (FAO, 2004). Accordingly, great research attention on the development of cultural system, health management, and reproduction and breeding has been internationally considered for this valuable species.

Unfortunately the intensive production of shrimp has been accompanied with some adverse impacts, such as the outbreak of infectious diseases leading to an annual global losses of about US$ 3 billion in the shrimp aquaculture industry (Farzanfar, 2006; Vaseeharan and Ramasamy, 2003). Among the bacterial diseases, vibriosis has been reported as the most important concern (FAO, 2004). All marine crustaceans are susceptible to vibriosis during all life stages. Vibriosis may be expressed as numbers of syndromes, including appendage and cuticular vibriosis, oral and enteric vibriosis, localized vibriosis of wounds, systematic vibriosis, shell disease, and septic hepatopancreatitis (Jayasree et al., 2006). This infectious disease has been reported to be caused by different *Vibrio* species including; *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, *V. penaeicida* and *V. vulnificus* (Lightner, 1996).

In order to prevent and control these infectious diseases several strategies have been applied such antibiotics and disinfectant agents which have showed limited success in diseases management and prevention. The use of these agents thus, is not recommended and in some cases is prohibited due to the development of antibiotic resistance in pathogenic and opportunistic bacteria. In addition, the retention of antibiotic residue in shrimp aquaculture products is potentially harmful for human and animal health

(Goldburg and Naylor, 2005; Naylor and Burke, 2005). Therefore other strategies are required for prevention of diseases in shrimp aquaculture.

Among the alternative strategies, the uses of probiotics have shown promising results. In fact, it's usage is currently accepted as a key factor in order to enhance the growth and disease resistance (Verschuere L et al., 2000; Wang et al., 2008; Ziaei-Nejad et al., 2006). However, the possible mechanism of probiotic actions have not been well understood, perhaps due to methodological and ethical limitation of animal studies (Balcazar et al., 2006). Nevertheless, several studies on the effects of probiotics on aquatic animals have been conducted. Accordingly, some possible mechanisms of action have been proposed, such as competitive exclusion of pathogenic bacteria (Gomez-Gil et al., 2000; Vine et al., 2004), source of nutrient and enzymatic contribution to digestion (Verschuere L et al., 2000), direct uptake of dissolved organic material mediated by bacteria (Garriques and Arevalo, 1995; Moriarty, 1997), enhancement of the immune response (Chiu et al., 2007; Irianto and Austin, 2002), and improvement of water quality (de Souza et al., 2011; Lalloo et al., 2007; Ma et al., 2009).

In selection of new strains of probiotic bacteria it is necessary to examine and determine the probiotic abilities of the candidate bacteria. Normally, it is difficult to find all the probiotic properties in a single strain of bacterium, therefore search for the new candidate strains as probiotics in the research frameworks might be beneficial for the industry in order to lead the aquaculture to be more environmentally friendly and a sustainable industry.

Several studies have demonstrated the positive effects of probiotic on shrimp via feed application. However, due to the acidic condition of gastrointestinal tract (GIT) the candidate probiotic bacteria must not only be resistant but also able to colonize under those conditions. Therefore, according to the present statement including the mechanisms of probiotic action, this study was conducted to:

- identify and select probiotic candidates derived from pickle homogenate.
- characterize antibacterial productions and detect antibiotic biosynthesis genes in candidate probiotics.
- determine the safety of candidate probiotic on juvenile *L. vannamei.*
- investigate the *in vivo* effects of candidate probiotic using two different administrations.
- evaluate the protection of *L. vannamei* from bacterial infection by candidate probiotic.

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