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Comparison of bacterial population in intestines of black tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) during the normal farming conditions and after exposure to *Vibrio harveyi*

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เดือน ปี ที่เสร็จโครงการ

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Abstract

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Project Title: Comparison of bacterial population in intestines of black tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) during the normal farming conditions and after exposure to *Vibrio harveyi*

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Project Period: 2 years (June 2011 to 2013)

Thailand is a leading exporter of the black tiger shrimp in the world. Although the global demand is increasing annually, the black tiger shrimp production in Thailand has been falling due to complications in domestication such as poor reproductive maturation and disease outbreak. Despite a better market price of the black tiger shrimp, many farmers have changed to cultivate the Pacific white shrimp, which have far less farming difficulties. To revive the black tiger shrimp production, this project aimed to examine microbial structures between the two shrimp under different growth stages and to determine approaches to enhance disease resistance in the black tiger shrimp. Here, we compared the bacteria profiles in intestines of the black tiger shrimp and the Pacific white shrimp from different growth stages. The bacterial profile analysis by denaturing gradient gel electrophoresis revealed that dominant bacteria in both shrimp species were highly similar. However, the DGGE profile of 20-day-old post-larvae was distinct from those of juvenile stages in both shrimp species. This observation suggested that growth stages had more influences on bacterial population in intestines than shrimp species, when both shrimp species were reared under the same diet and environmental conditions. We further compared intestinal bacterial profiles of the black tiger shrimp and the Pacific white shrimp under no pathogen challenge control and under pathogenic *Vibrio harveyi* challenge. The DGGE profiles showed that the presence of *V. harveyi* altered the intestinal bacterial patterns in comparison to the control. This implies that bacterial balance in shrimp intestines was disrupted in the presence of *V. harveyi*. Furthermore, bacteria were isolated from shrimp intestines and screened for antagonistic activity against *V. harveyi*. The bacteria isolates will be further tested for feasibility application in shrimp farming to protect against colonization of harmful bacteria in a subsequent project. Besides beneficial bacteria approach, we also investigated on effects of bacterial lipopolysaccharide (LPS) as feed supplement to improve the immunity of the black tiger shrimp. The up-regulation of the immune gene levels in intestines and higher level of resistance to *V. harveyi* of the shrimp fed with LPS provide the evidence for potential application of LPS as an immunostimulant in shrimp farming. The findings of this work will ultimately lead to alternative disease prevention approaches such as beneficial bacteria as biocontrol agents and immunostimulants to modulate host immune system to increase shrimp survival.

Keywords: Black tiger shrimp, Pacific white shrimp, Gut microbiota, Lipopolysaccharides, DGGE

บทคัดย่อ

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ประเทศไทยเป็นหนึ่งในผู้ผลิตและส่งออกกุ้งกุลาดำรายใหญ่ของโลก แม้ว่าความต้องการทางตลาดของกุ้งชนิดนี้จะเพิ่มขึ้นในแต่ละปี แต่การผลิตกุ้งกุลาดำในประเทศไทยนั้นกลับมีปริมาณที่ลดลงเนื่องจากประสบปัญหาในการเพาะเลี้ยง เช่น ปัญหาเรื่องการไม่เจริญพันธุ์ตลอดจนสภาวะการไวต่อการติดเชื้อโรคได้ง่าย และแม้ว่ากุ้งกุลาดำจะมีมูลค่าทางการตลาดที่สูงกว่ากุ้งขาว แต่ทางกลุ่มเกษตรกรผู้เลี้ยงกุ้งจำนวนมากได้เปลี่ยนมาเพาะเลี้ยงกุ้งขาวซึ่งเป็นกุ้งที่มีปัญหาในการเพาะเลี้ยงน้อยกว่ากุ้งกุลาดำ โครงการนี้มีวัตถุประสงค์ที่จะศึกษาแนวทางในการเพิ่มอัตราการรอดของกุ้งกุลาดำโดยมุ่งเน้นที่การสร้างความสมดุลของแบคทีเรียในลำไส้ โดยงานวิจัยนี้ได้ศึกษาและเปรียบเทียบประชากรแบคทีเรียในลำไส้กุ้งกุลาดำและกุ้งขาวที่ระยะวัยอ่อนจนถึงระยะวัยรุ่นที่เลี้ยงภายใต้สภาวะแวดล้อมเดียวกันโดยเทคนิค denaturing gradient gel electrophoresis เมื่อเราเทียบกับโปรไฟล์ของแบคทีเรียในลำไส้ของกุ้งกุลาดำกับกุ้งขาวพบว่ากลุ่มแบคทีเรียหลักที่พบในลำไส้นั้นคล้ายคลึงกัน แต่เมื่อเทียบโปรไฟล์ของแบคทีเรียในลำไส้ของกุ้งในแต่ละระยะการเจริญเติบโตพบว่า แบคทีเรียในลำไส้กุ้งทั้งสองสายพันธุ์ที่ระยะวัยอ่อนนั้นแตกต่างจากโปรไฟล์ของแบคทีเรียในลำไส้กุ้งที่อายุหนึ่งถึงสามเดือน ข้อสังเกตนี้แสดงให้เห็นว่าเมื่อกุ้งทั้งสองสายพันธุ์ถูกเลี้ยงภายใต้อาหารและสภาพแวดล้อมเดียวกัน ช่วงระยะการเจริญเติบโตมีอิทธิพลต่อประชากรของแบคทีเรียในลำไส้มากกว่าชนิดสายพันธุ์กุ้ง และเมื่อเราเทียบรูปแบบของแบคทีเรียในลำไส้ของกุ้งกุลาดำและกุ้งขาวภายใต้สภาวะที่มีเชื้อก่อโรค *Vibrio harveyi* กับสภาวะที่ไม่มีเชื้อก่อโรค พบว่ารูปแบบของแบคทีเรียในลำไส้ของกุ้งทั้งสองสายพันธุ์มีการเปลี่ยนแปลงเมื่อมีเชื้อก่อโรค *V. harveyi* แสดงให้เห็นว่าสมดุลของแบคทีเรียในลำไส้นั้น

เปลี่ยนไปในสภาวะที่มีเชื้อก่อโรค นอกจากนี้เรายังคัดเลือกกลุ่มเชื้อแบคทีเรียที่มีความสามารถในการยับยั้งการเจริญเติบโตของเชื้อก่อโรค *V. harveyi* เพื่อนำไปศึกษาประยุกต์ใช้เป็นอาหารเสริมในการเลี้ยงกุ้งเพื่อลดอัตราการติดเชื้อในบ่อเลี้ยงในอนาคต และเราได้ทดสอบศักยภาพของ lipopolysaccharide (LPS) ที่สกัดจากแบคทีเรียต่อการเพิ่มภูมิคุ้มกันในกุ้งกุลาดำ ในกลุ่มกุ้งกุลาดำที่ได้รับอาหารเสริมด้วย LPS นั้นมีการเพิ่มระดับการแสดงออกของยีนที่เกี่ยวข้องกับระบบภูมิคุ้มกันในลำไส้กุ้งกุลาดำ และมีความต้านทานต่อเชื้อโรค*V. harveyi* ได้มากกว่ากุ้งที่ไม่ได้รับอาหารเสริม LPS และสามารถนำ LPS ไปประยุกต์ใช้เพื่อกระตุ้นภูมิคุ้มกันในการเพาะเลี้ยงกุ้งกุลาดำได้ และผลจากงานวิจัยนี้ สามารถนำไปต่อยอดองค์ความรู้สู่การป้องกันโรคในการเพาะเลี้ยงสัตว์น้ำ โดยวิธีทางเลือกที่ไม่ใช้สารเคมี เช่น การคัดเลือกแบคทีเรียที่มีประโยชน์เป็นสารควบคุมทางชีวภาพและการใช้สารจากธรรมชาติมากระตุ้นภูมิคุ้มกัน และเพิ่มอัตราการรอดในการเพาะเลี้ยงกุ้งเศรษฐกิจได้ต่อไปในอนาคต

คำหลัก: กุ้งกุลาดำ, กุ้งขาว, แบคทีเรียในลำไส้, Lipopolysaccharide, DGGE

Chapter 1

Introduction

Thailand is one of the world's leading shrimp farming and exporting countries. As one of the main producers to worldwide markets, the Thai shrimp industry brings in the net annual income of US$ 2 billion (Tanticharoen et al., 2009). In the past decades, the black tiger shrimp (*Penaeus monodon*), native shrimp species in Southeast Asia, was used to be a major shrimp species exported from Thailand. However, during the last 10-year period, the black tiger shrimp farming has encountered disease outbreak problems such as yellow head and white spot viruses that severely affect shrimp production. Concurrently, the Pacific white shrimp (*Litopenaeus vannamei*), a native species to the Pacific coast, has been introduced to Thailand and its farming has rapidly been expanding due to a faster growth rate and less susceptibility to diseases than the black tiger shrimp. Thus, many Thai shrimp industries have switched to cultivate the Pacific white shrimp (Wyban, 2007).

Despite difficulties in domestication, the black tiger shrimp remains as a premium grade shrimp with higher market demands and higher prices than the Pacific white shrimp. Therefore, numerous research programs have been focusing on different approaches to revive the black tiger shrimp farming industry. To address the disease outbreak, one approach is to monitor microbial balance in both environments and inside host shrimp, especially shrimp intestines. The animal intestine is a dynamic ecosystem consisting of various microorganisms, host cells, and nutrients. Therefore, the tissue surface on shrimp intestines directly contacts to external environments, which can be easily susceptible to various pathogens. In addition to host shrimp immune system, intestinal surface is lined with commensal microorganisms, which have been reported to influence host immunity, nutrient absorption and barrier against pathogen colonization (Hooper et al., 2002; Li et al., 2008). In the germfree mouse, its mucosal layer in intestine is poorly developed and a germfree mouse is highly susceptible to intestinal infection (Hooper et al., 2001). The introduction of microorganisms to intestines of the germfree zebrafish induces expression of genes involving in nutrient absorption, mucosal modification and immunity (Rawls et al., 2004). Moreover, the gut bacteria of ruminants and termites help breaking down ingested polysaccharides to monosaccharides to short chain fatty acids as nutrients for their hosts (Brune and Friedrich, 2000). The intestinal bacteria modulate host gene expression to generate suitable environmental conditions for themselves and successful colonization prevents growth of other competitive bacteria, especially pathogens (Xu and Gordon, 2003).

Due to the importance of microbial communities in intestines to their hosts, the first part of this project aimed to characterize intestinal bacteria in the black tiger shrimp under different growth stages in order to examine whether different genetics of the host shrimp influence bacteria population in their intestines. The bacteria population in intestines of the black tiger shrimp and the Pacific white shrimp were compared using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA fragments. Furthermore, the bacterial populations in intestines were compared in shrimp under normal condition and a pathogen challenge. Both shrimp species were exposed to the shrimp pathogen, *Vibrio harveyi*, which causes vibriosis, resulting in high mortality rates in shrimp farming

(Jiravanichpaisal et al., 1994; Vandenberghe et al., 1999). The pathogenic *V. harveyi* can enter host shrimp via an oral route (Aguirre-Guzmán et al., 2010; Austin and Zhang, 2006). In order to successfully colonize and infect the host shrimp, *V. harveyi* needs to compete against commensal bacteria population in shrimp intestines. In consequence, the bacterial balance in shrimp intestines might be altered. We determined if the *V. harveyi* infection would influence on major bacteria in the intestine of the black tiger shrimp and the Pacific white shrimp.

In the second part of the project, we aimed to explore other means to improve shrimp survival under a pathogen exposure. The application of natural immune stimulants in aquaculture has been shown to increase survival of aquatic species upon a presence of pathogens (Nya and Austin, 2010; Smith et al., 2003; Yogeeswaran et al., 2012). Lipopolysaccharides (LPS) have been increasingly investigated as a potential immunostimulant in aquaculture (Lorenzon et al., 1999; Smith et al., 2003). LPS is a cell wall component of Gram-negative bacteria, in which it triggers host immune responses such as macrophages and antimicrobial peptide systems (Paulsen et al., 2003). LPS injection in salmon stimulates lysozyme activity in head, kidney and intestine (Paulsen et al., 2003). When a diet was supplemented with LPS, it increases the survival rate in rainbow trout under pathogenic bacteria challenge (Nya and Austin, 2010) and the Kuruma prawn (Takahashi et al., 2000) under exposure to shrimp virus. However, the effect of LPS when given as dietary supplement on *P. monodon* survival under a pathogen challenge has never been determined. Here, we evaluated the feasibility of an LPS application in enhancing disease resistance by oral administration. LPS was given as a supplement in *P. monodon* feeds for 10 days, and shrimp growth rates, percent weight gains, total hemocyte/granulocyte counts and survival rates were assessed. To determine efficacy of disease protection, shrimp survival rates were evaluated under the *V. harveyi* exposure after the 10-day LPS feeding trial. Moreover, to determine the molecular effect of LPS as an immune stimulator, the transcript levels of several immune-related genes such as antimicrobial peptides encoding genes *(anti-lipopolysaccharide factor isoform 3, penaeidin3a* and *crustin), C-type lectin* and *mucin-like peritrophic membrane* were compared in *P. monodon* intestines after feeding with LPS supplemented diet to non-LPS diet.

Research Objectives

The primary goal of this project was to compare the bacteria population in the black and white shrimp intestines in normal condition and under *V. harveyi* infection. The secondary goal of the project was to determine feasibility to stimulate immunity to increase shrimp survival under a pathogen exposure. The bacteria were isolated from shrimp intestines and determined for their potential application to increase shrimp protection against *V. harveyi*. The lipopolysaccharide, a cell wall component of Gram-negative bacteria was also evaluated for their efficacy to improve shrimp survival.

Specific Aims

Aim 1: To determine and compare bacteria population in intestines of the black tiger shrimp and the Pacific white shrimp. The post-larval stages of the Black tiger shrimp and the Pacific white shrimp were maintained under the same facility with the same conditions to minimized environmental variation. The intestinal bacterial profiles were compared using denaturing gradient gel electrophoresis (DGGE).

Aim 2: To determine bacteria population dynamics in intestines of the black tiger shrimp and the Pacific white shrimp after an oral infection by *Vibrio harveyi*. Both black tiger shrimp and Pacific white shrimp were exposed to the vibriosis pathogen, *V. harveyi*. The susceptibility to *V. harveyi* infection was compared between the two shrimp species. The bacteria population in intestines was characterized by DGGE. The diversity and relative ratio of bacteria were compared between non-pathogen and under *V. harveyi* infection conditions in intestines of both black tiger shrimp and Pacific white shrimp.

Aim 3: To isolate bacteria from shrimp intestine and screen for potential protection against *V. harveyi*. The bacteria were isolated on media agar and screened for antagonistic activity against *V. harveyi*. The 16S rRNA from bacterial candidates were sequenced and compared to a public database for bacteria species identification.

Aim 4: To determine feasibility of lipopolysaccharides to increase the black tiger shrimp survival under *V. harveyi* challenge. The lipopolysaccharides (LPS) were fed to the black tiger shrimp for 10-day period. Shrimp were challenged to pathogen and survival rates were compared between the groups fed with LPS- and non-LPS diets.

Chapter 2

Materials and Methods

2.1 Experimental animals and diets

To minimize variation in rearing environments, the black tiger shrimp and the Pacific white shrimp from hatcheries were reared under the same facility managed by Shrimp Biotechnology Business Unit (SBBU) (Fig. 2.1). The SBBU facility provided a re-circulating system for experimental aquaria with a regular water quality control. Shrimp were tested to be free of specific pathogens (Taura Syndrome Virus, White Spot Syndrome Virus, Yellow Head Virus, and Infectious Hypodermal and Hematopoietic Necrosis Virus) using IQ2000 PCR test kits (Genereach Biotechnology Corporation, Taiwan). Shrimp were acclimatized in a 2,000 L tank and maintained at SBBU, Thailand. They were fed with commercial feed pellets (Starfeed, CPF, Thailand).

The post larval stage of the black tiger shrimp and the Pacific white shrimp were raised until 20-day old post-larval and 1-month-old, 2-month-old, and 3-month-old juvenile stages, in which intestines were collected for subsequent experiments.

Water quality was assessed every three days for temperature, pH, and dissolved oxygen and weekly for ammonia-nitrogen, nitrite-nitrogen, and alkalinity levels.

Figure 2.1. Shrimp rearing tanks at Shrimp Biotechnology Business Unit (SBBU) Facility, BIOTEC

2.2 Intestine sample preparation

The entire intestines were aseptically removed from 20-day old post-larvae, 1-, 2- and 3- month-old juveniles and preserved in a commercial formaldehyde-free preservative buffer. For each replicate, fifty intestine samples were pooled for post larval stage whereas five intestines were pooled for juvenile stages to allow enough tissue mass for efficient DNA extraction. In a parallel experiment, water were collected for DNA extraction as and used as controls for all experiments. The genomic DNA samples were extracted from the intestine sample using QIAamp DNA extraction kit (Qiagen).

2.3PCR amplification of 16S rRNA genes and denaturing gradient gel electrophoresis (DGGE)

PCR-DGGE profiles of intestine samples from four shrimp stages were performed according as previously described with some modifications (Chaiyapechara et al., 2011; Muyzer, 1999; Muyzer et al., 1993). Briefly, genomic DNA sample (final concentration of 1ng/μl) from each library was used as a template for PCR reaction with primer pair 338F-GC clamps and 517R (Table 2.1). The PCR cycle parameters were 3 min initial denaturation at 94°C, 30 cycles of 30 sec at 94°C, 1 min at 55°C and 1.5 min at 72°C, and 10 min final extension at 72°C. The presence of a 200-bp fragment was confirmed on a 1.5% agarose gel electrophoresis. PCR-DGGE products were purified by Illustra GFX PCR DNA and gel purification kit according to supplier's manual (GE Healthcare, USA). Each PCR-DGGE product (600 ng) was loaded directly onto an 8% polyacrylamide gel with 40% to 60% denaturant vertical gradient. The in-house standard ladders were loaded as a control of DGGE analysis. The electrophoresis was performed at 80 V 60°C for 14 h using a DCode DGGE Electrophoresis System (Bio-Rad Laboratories, Inc., USA). After electrophoresis, the gels were stained using SYBR gold (Invitrogen, USA) and visualized under UV light gel doc system (Bio-Rad Laboratories, Inc.). All gel analyses were performed using InfoQuest™ Software (Bio-Rad Laboratories, Inc.). DGGE bands were excised and cloned to pGEM-T according to supplier's instruction (Promega). Plasmid was extracted and submitted for sequencing.

2.4 Sequencing and phylogenetic analysis

The sequence data were BLAST against GenBank database at National Center of Biotechnology Information (NCBI)(Altschul et al., 1990) . The phylogenetic tree were be constructed by PHYLIP software (http://evolution.genetics.washington.edu/phylip.html).

2.5. *V. harveyi* exposure experiments

A group of 1-month-old black tiger shrimp juveniles (N= 400) from SBBU were divided into two water tanks where one group (N=200) was inoculated with *V. harveyi* 1526 (Rengpipat et al., 2003) and another group (N= 200) remained untreated as a control of the experiment (Fig. 2.2). The same treatment condition was done with a group of Pacific white shrimp juveniles. The percent of survival were determined and the intestine samples were individually collected from 10 juvenile shrimp at 6, 12, 24, 48, 72 and 96 hours after inoculation.

The survival curve of juvenile shrimp after exposure to *V. harveyi* were plotted and compared between the black tiger shrimp and the Pacific white shrimp.

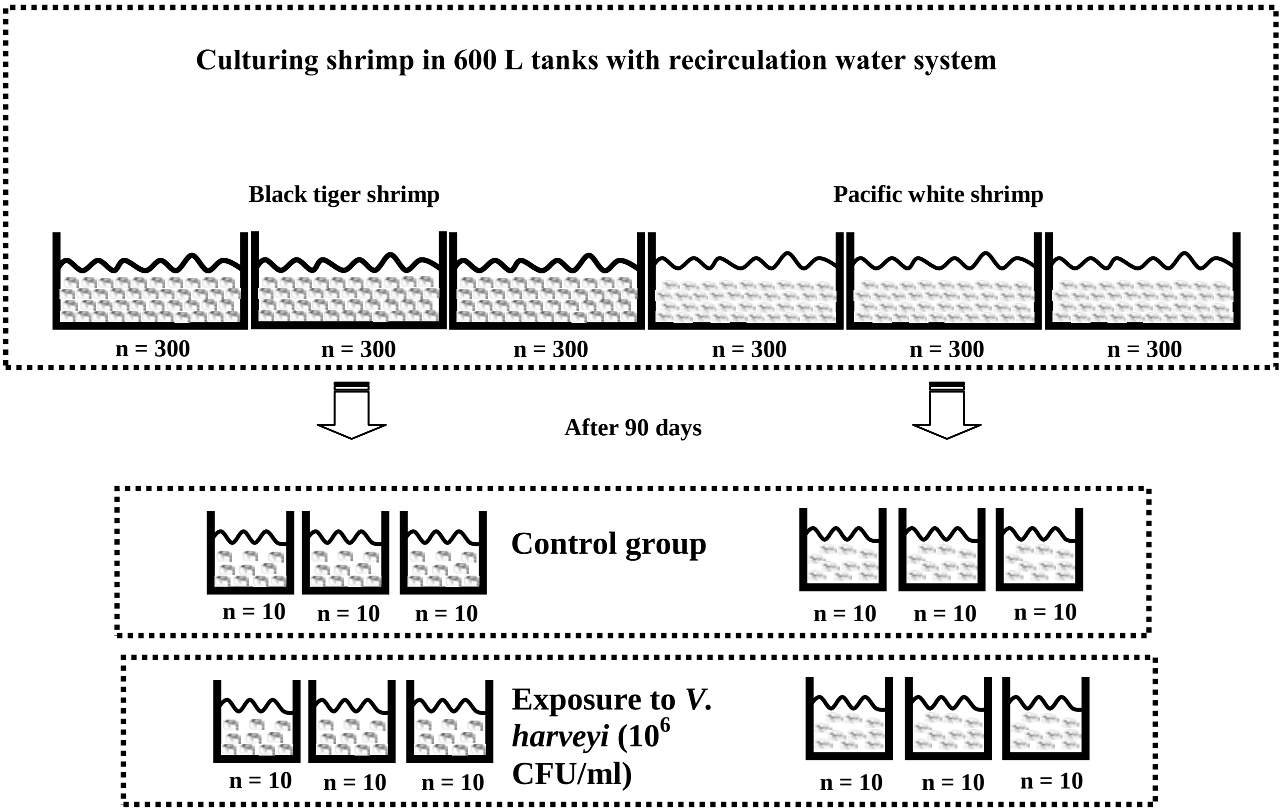


Figure 2.2. Overview of *V. harveyi* challenge of 3-month-old juveniles of the black tiger shrimp and the Pacific white shrimp

2.6 Search for potential bacterial candidates for pathogen inhibition activity

The juvenile shrimp intestines were aseptically dissected out and carefully ground in sterile phosphate saline buffer (PBS) and spread onto TCBS (Oxoid, England) agar and Luria-Bertani agar. The plates were incubated at 30°C overnight. The antagonistic activity was screened by agar overlay technique, in which *V. harveyi* 1526 was inoculated in top agar layer. The bacteria isolates from shrimp intestines were then streaked onto the solidified agar overlaid plates and incubated overnight at 30°C. The bacterial isolates with inhibition zone were collected. The potential bacterial candidates were subsequently tested in triplicates to ensure consistency of antagonistic activity against *V. harveyi*. The 16S rRNA sequences of bacterial candidates were determined to identify the unknown bacteria.

2.7 The LPS feeding trials of black tiger shrimp juveniles

The black tiger shrimp juveniles (*P. monodon*) with a weight range of 1-2 g (approximately 2-month-old shrimp) were obtained from a commercial farm in Pathum Thani province, Thailand. Shrimp were tested to be free of specific pathogens (Taura Syndrome Virus, White Spot Syndrome Virus, Yellow Head Virus, and Infectious Hypodermal and Hematopoietic Necrosis Virus) using IQ2000 PCR test kits (Genereach Biotechnology Corporation, Taiwan). Shrimp were acclimatized in a 2,000 L tank and maintained at Shrimp Biotechnology Business Unit (SBBU), BIOTEC, Thailand. They were fed with commercial feed pellets (Starfeed, CPF, Thailand). Water quality was assessed every three days for temperature, pH, and dissolved oxygen and weekly for ammonia-nitrogen, nitrite-nitrogen, and alkalinity levels.

Once the weight of juvenile shrimp reached 2-4 g, the shrimp were transferred to a 220 L rearing tanks (a total of 28 tanks) with a recirculation water system (Fig. 2.3).Shrimp were randomly divided into three groups (n =200 each), where they were fed with commercial shrimp feed pellets twice a day (Group 1), lipopolysaccharide (LPS)-coated feed pellets for once a day in the morning and normal feed pellets in the evening (Group 2) or LPS-coated feed pellets twice a day (Group 3). The commercial shrimp diet (Starfeed)contained protein $\geq$ 38%. The purified LPS supplement was extracted from Escherichia coli O26:B6 (Sigma Chemical, USA). LPS was prepared in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) with a concentration of 4 µg LPS/g shrimp body weight/meal. Shrimp feed were prepared every 3 days by coating commercial feed pellet with PBS for the commercial pellet control (Group 1), and coating with LPS for experimental diet (Group 2 and Group3), then dried at 20°C for 1 hr. Diets were stored in plastic bags at 4°C until used. Shrimp were fed twice daily at 08:00 and 18:00 hr. Daily feeding rate was 5-6% of total body weight. Each experimental diet was done in four replicates with 30 shrimp per replicate for a 10-day period. For further analysis of immune gene expression, intestine samples were aseptically collected from each diet group (n = 10 each). The intestine tissue samples were kept frozen at -80 $^{\circ}$C until further used.

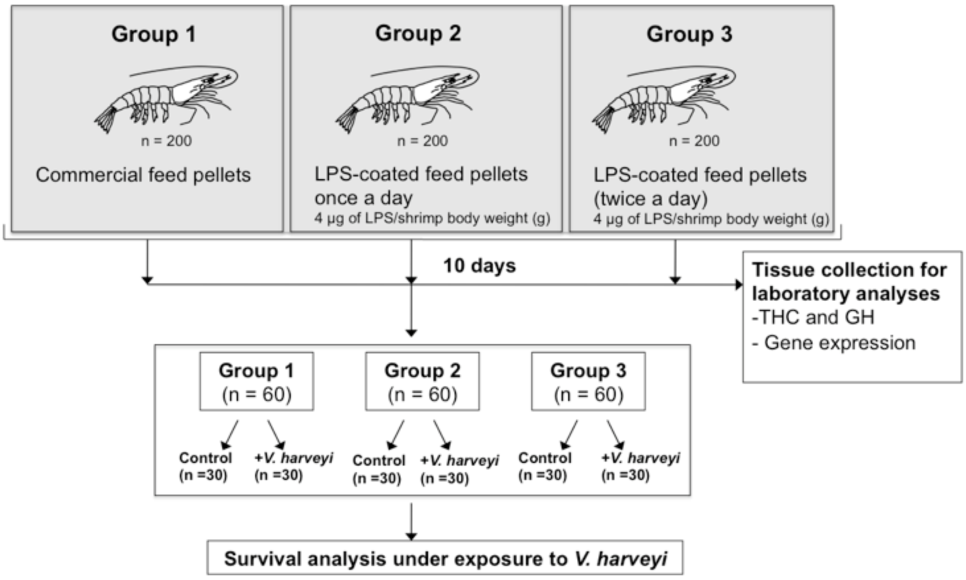


Figure 2.3.Overview of experimental design to examine effects of LPS in *Penaeus monodon*.

2.8 Laboratory analysis for LPS feeding trial

Shrimp growth performance were evaluated daily on days 0 and 10 and calculated a percent weight gain, specific growth rate, average daily growth, feed conversion ratio, and survival rates the end of the feeding trial. After 10 days of feeding trial, sub-samples of shrimp in each experimental diet were used for determining total hemocyte (THC) and granulocyte (GH) counts, which were done according to a previously published method (Sritunyalucksana et al., 2005). THC and GH counts were determined from hemolymph collected from shrimp in each experimental diet (n =12). A volume of hemolymph (100 µl) were drawn from a ventral sinus of the first abdominal segment by using a syringe containing with 100 µl of fixative agents (10% formalin in 0.45 M NaCl), mixed immediately and incubated at room temperature at least for 10 min before transferring to a 1.5 ml microcentrifuge tube. A 20 μl aliquot of each sample was transferred into a second tube containing with 20 μl of 1.2% Rose Bengal Solution (Sigma-Aldrich, USA) in 50% ethanol. The staining step was incubated at room temperature for 20 min before counting THC by hemacytometer or smeared on a microscope slide for GH counts. For GH count, a smeared slide was dried and counterstained with hematoxylin solution (Sigma-Aldrich, USA) for 10 min, rinsed with tap water for 10 min followed by dehydration with 95% ethanol (10 dips) and 100% ethanol (10 dips). A slide was rinsed in xylene (Sigma-Aldrich, USA) twice for 5 min before mounting with Permount (Fisher Scientific, USA). A proportion of GH per 200 of total hemocytes was recorded for calculating the total number of GH.

2.9 Comparison of shrimp survival rates under *Vibrio harveyi* exposure between shrimp fed with LPS and non-LPS diets

After 10 days, the black tiger shrimp from each diet group (n = 60) were transferred to a 70 L glass aquarium and acclimated for three days. Juvenile shrimp were equally divided into two groups: a *V. harveyi* 1526 exposure group and a control group without the bacterial exposure (n= 30 each). In this experiment, the LD_{50} of *V. harveyi* infection in *P. monodon* was determined to be 8.9×10^7 CFU/ml, which was subsequently used in pathogen exposure experiment. To mimic natural bacterial infection, shrimp were immersed in a final bacteria concentration of 8.9×10^7 CFU/ml (Jiravanichpaisal et al., 2007). Shrimp were monitored for seven days for survival comparison. Their survival rates under pathogen exposure were determined from triplicate experiments (n = 10 for each replicate).

2.10 Gene expression analysis by relative quantitative real-time PCR

To determine transcript levels of host immune genes, intestine samples (n = 5) were collected and ground using a mortar in liquid nitrogen for RNA extraction. RNA extraction was performed using TriReagent® (Molecular Research Center, USA) according to supplier's instruction. The RNA pellets were resuspended in 50 µl of RNase-free water and treated with DNaseI (0.5 unit/µg, Promega) for 30 minutes at 37 ºC to remove DNA contamination. The DNA-free RNA was purified by phenol: chloroform extraction and precipitated with 1/10 volume of sodium acetate (3M) and 1 volume of isopropanol. RNA concentration was determined using NanoDrop UV-Vis Spectrophotometer (ND-8000). Treated RNA samples were further used as templates in PCR reactions with the primer pair specific for the housekeeping gene ef1α (Table 1) to verify for an absence of DNA contamination. A Improm-II™ Reverse Transcription System (Promega, USA) was used to synthesize the first strand cDNA using total RNA (1.5 µg) as a template in a total reaction volume of 20 µl. Aliquots of 0.5 µl of cDNA preparation were used for real-time PCR analysis. Relative abundance of ALF3, crustin, pen3a, C-lectin and mucine-like PM transcripts was determined by relative quantitative real-time PCR (qRT-PCR). The reactions were carried out in CFX96 System (BIORAD, USA) using IQ™SYBR® Green Supermix (BIORAD). The target genes and primer sequences were listed in Table 2.2. The housekeeping gene *ef1α* was used as an internal control for both shrimp species (Table 2.2). All reactions were carried out according to the supplier's instructions. The cycling parameters used were as follows: an initial denaturation at 95 ºC for 3 min, 40 cycles at 95 ºC for 30 sec, 57 ºC for 20 sec and 72 ºC for 30 sec. The fluorescent signal intensities were recorded at the end of each cycle. Melting curve analysis was performed from 55 ºC to 95 ºC with continuous fluorescence reading every 0.5 ºC increment to validate specificity of PCR amplification. The relative fold of induction were calculated by the $\Delta\Delta$ct method (Livak and Schmittgen, 2001).

2.11 Statistical analysis

Statistical analysis was performed using an analysis of variance (ANOVA) and Duncan's New multiple range tests were used to determine whether significant differences existed between population means. All statistical analyses were conducted using SPSS of Windows version 15.0 (Landau and Everitt, 2004).

Table 2.1 Oligonucleotide used in denaturing gradient gel electrophoresis.

Name	*Sequence 5’ to 3’
338F	ACTCCTACGGGAGGCAGCAG
338GC-F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCA
518R	5′ ATTACCGCGGCTGCTGG 3′

*The underlined sequence is GC-clamp region for DGGE analysis.

Table 2.2 Oligonucleotide used in gene expression analysis by real-time PCR.

Shrimp	**Gene**	**Sequence 5' to 3'**	**Size (bp)**	**Accession No. or EST libraries**	**Reference**
P. monodon	*ALF3* (Antilipopolysaccharide factor isoform 3)	F: TCTCATCTCTCAACAGGAGGCCAA R: GGTAGAGCTTCCATTGCCAACTGC	103	EF523562	(Soonthornchai et al., 2010)
	crustin	F: AGTTCCTGGAGTTGGAGGTGGATT R: ACCTCGTTCTGCAGTAATTGCACTC	119	BI784446	
	c-lectin (C-type lectin)	F: AGTGCTGGACGAGTGCTTCTATCT R: TTGAGAGCATAGACGTTCCTGGGT	117	DQ078266	
	mucine-like PM (Mucine-like peritrophin)	F: ACTGGAAACCGAAGGATGTTCCCT R: TTGTTGCAGTCCTTGTGTGGCTTG	123	IN-N-S01-0247-LF	
	ef1α (Elongation factor 1α)	F: AGGCGTACTGGTAAGGAACTGGAA R: AGAGGAGCATACTGTTGGAAGGTCTC	123	DQ021452	
	pen3a (Penaeidin 3a)	F: CCCAGCAGGTCCTCGTGTATT R: ACCAACCACACACAGACCCATAC	94	FJ686016.1	This study
	Lyz (Lyzozyme)	F: GGAATGACATCAAGAACTGGGTGTGC R: TCTGGAAGATACCGTAGTCTGTGCT	108	EU095851	
	Toll1 (Toll1 protein)	F: CCTCCTGCTCTGCTGACGAATAC R: ACACTGGTGAGTCCGTTGTCC	143	GU014556.1	
L. vannamei	*pen3a* (Penaeidin3a)	F: CGTGGTCTGCCTGGTCTTCTT R: CAAAGGTCTCACGAAGGGTGGT	112	Y14926.1	
	crus (Crustin)	F: CCACAACCTGTTCCAACGACTACAA R: AAACCTGCGATCCGAAGAATGAGG	111	AF430076	
	ALF1 (Antilipopolysaccharide factor isoform 1)	F: AGGACCTCATCCCTTCGCTAGT R: GGACACCACATCCTGCCATTGA	145	EW713395.1	
	ef1α (Elongation factor 1α)	F: CGTCGCTTCCGACTCGAAGAA R: TGGCAATCAAGCACAGGTGAGTAG	97	GU136229.1	
	Lyz (Lyzozyme)	F: GGAGTTCGAGTCGTCCTTCAACA R: GTAGTCGCTTCCGCACCAGTA	109	AY170126	
	Toll1 (Toll1 protein)	F: CGAGAGCGAGTTGGACGAGAAG R: CCTGTGGGTGTGGCATGATGTA	113	DQ923424.1	

Chapter 3

Results and Discussion

3.1. Stocking of experimental animals and rearing conditions

The post larval stage of the black tiger shrimp (*Penaeus monodon*) and the Pacific white shrimp (*Litopenaeus vannamei*) were raised until they reached 20-day-old post-larval, 1-, 2-, and 3-month-old juvenile stages (PL20, J1, J2, and J3, respectively).

Water temperature, pH, and DO were monitored every two-day interval (Table 3.1). The levels of ammonia-nitrogen, nitrite-nitrogen, and alkalinity were measured weekly. To ensure consistency of rearing conditions, the shrimp culture water was monitored and adjusted to have pH ranges from 7.5-8.5, dissolved oxygen > 5 mg/L, temperature between 27-31 °C, salinity level at 15 ppt, ammonia-nitrogen < 0.03 mg/L, nitrite-nitrogen ratio < 1 mg/L, and alkalinity range of 80-150 mg$CaCO_3$/L.

The weights of both shrimp species were recorded on day 0, 30, and 60 and 90 of the rearing period, corresponding to PL20, J1, J2, and J3 stages (Table 3.2). The average weight was obtained from weighing approximately 180 shrimp per species, which was accounted for 20% of each shrimp population. Interestingly, the average body weights of *P. monodon* and *L. vannamei* were significantly different under PL20, J1 and J2 stages ($p < 0.05$). Once both shrimp populations reached J3 stage, there were no significant differences in the body weight of shrimp (Table 3.2).

Table 3.1. The water quality of the culture system in each week (Mean ± SD).

Week	**Water quality**						
	Dissolved oxygen (mg/L)	**Temperature (°C)**	**pH**	**Salinity (ppt)**	**Alkalinity (mg $CaCO_3$/L)**	**Nitrite-Nitrogen (mg N/L)**	**Ammonia-Nitrogen (mg N/L)**
0	5.81±0.08	28.17±0.14	7.71±0.03	15	87.17±1.17	0.2072±0.1083	0.0002±0.0001
1	5.74±0.13	27.85±0.13	7.56±0.08	15	85.83±0.75	0.3827±0.1083	0.0013±0.0008
2	5.44±0.18	28.12±0.65	7.55±0.08	15	81.67±1.86	0.2219±0.0421	0.0020±0.0008
3	5.94±0.28	27.73±0.54	7.58±0.15	15	85.83±1.33	0.3828±0.2377	0.0012±0.0004
4	5.64±0.29	28.13±0.13	7.50±0.04	15	84.17±1.47	0.3326±0.0421	0.0022±0.0010
5	5.61±0.25	17.62±0.44	7.59±0.05	15	81.67±1.03	0.3329±0.0257	0.0018±0.0003
6	5.54±0.32	27.45±0.24	7.40±0.06	15	82.17±3.06	0.0839±0.1596	0.0011±0.0007
7	6.52±0.74	28.48±0.63	7.34±0.20	15	84.83±1.17	0.0821±0.0373	0.0026±0.0023
8	5.52±0.28	29.03±0.29	7.10±0.18	15	81.67±1.21	0.1171±0.0195	0.0063±0.0013
9	5.64±0.28	28.32±0.57	7.28±0.18	15	82.50±3.54	0.1589±0.0064	0.0012±0.0012
10	5.36±0.20	29.68±0.33	7.60±0.06	15	104.00±1.41	0.0696±0.0069	0.0050±0.0005
11	5.75±0.23	29.32±0.58	7.65±0.14	15	115.00±0.00	0.0673±0.0194	0.0018±0.0004
12	5.60±0.15	29.28±0.38	7.49±0.14	15	96.00±1.41	0.0659±0.0000	0.0024±0.0001
13	5.43±0.10	29.12±0.50	7.56±0.02	15	101.00±1.41	0.0767±0.0163	0.0025±0.0002
14	5.25±0.17	28.88±0.23	7.60±0.05	15	99.00±1.41	0.0539±0.0007	0.0009±0.0012

Table 3.2. The body weight (grams) of shrimp during the 90-day rearing period (Mean ± standard deviation).

Shrimp Stage*	**Average body weight (grams)**			
	Day 0	**Day 30**	**Day 60**	**Day 90**
	PL20	J1	J2	J3
Black tiger shrimp (*P. monodon*)	0.0051±0.0013 ^{b}	0.10±0.09 ^{b}	0.76±0.36 ^{b}	3.85±1.97 ^{a}
Pacific white shrimp*(L. vannamei)*	0.0125±0.0035 ^{a}	0.46±0.19 ^{a}	1.89±0.69 ^{a}	3.89±1.31 ^{a}

*PL20 denotes 20-day old post-larval stage, whereas J1, J2 and J3 denote 1-, 2- and 3- month old juvenile stages, respectively.

3.2 Comparison of bacterial profiles in *P. monodon* and *L. vannamei* under different growth stages

The intestinal bacterial profiles of *P. monodon* (the black tiger shrimp) and *L. vannamei* (the Pacific white shrimp) were compared using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA sequences (Fig. 3.1). Cluster analysis of DGGE profiles was performed in intestines from four different growth stages; 20-day-old post-larval, 1-, 2-, and 3-month-old juvenile stages (PL20, J1, J2, and J3, respectively). Moreover, bacterial population in pond water from each growth stages was also analyzed as the control of bacteria in external environments. The cluster analysis showed two major clusters, in which the first cluster contained intestinal bacterial profiles from *P. monodon* and *L. vannamei* larvae and bacteria in pond water and the intestinal bacterial profiles from juvenile stages of both shrimp species formed another cluster (Fig. 3.1). This observation suggested that shrimp growth stages have more influences on bacterial population in intestines than shrimp species, when both shrimp were reared under the same diet and environmental conditions.

In both shrimp species, the intestinal bacterial population in post-larvae was distinct from those found associated to three juvenile stages. The difference in bacterial composition in post-larva and juveniles might also be due to different feed conditions. In this study, bacterial populations were investigated in shrimp rearing under commercial production, where post-larval shrimp were fed with live *Artemia* nauplii (brine shrimp) as nutritional supplement in addition to commercial feed pellets until they reach juvenile stage. Once shrimp reached juvenile stages, their diet is solely based on commercial pellets (Chen, 1993; Wilkenfeld et al., 1984). The gut layer in Penaeid shrimp is ectodermal origin, which regularly shreds off during the molting period (Chang, 1995). Therefore, bacteria from ingested food influence re-colonization of the shrimp gut's microbial flora after shredding. The shifting from the live feed in post-larval shrimp to commercial pellets in juvenile shrimp might explain the difference in diversity of bacterial composition in shrimp rearing ponds.

One other factor that influences intestinal bacterial diversity is the selective pressure from the host such as resistance to host immune system (Ley et al., 2006). The shrimp digestive system is fully developed in the post-larval stage (Ribeiro, 2010). However, the immune system of post-larval shrimp is not as developed as in juvenile shrimp (Jiravanichpaisal et al., 2007; Soonthornchai et al., 2010). For example, penaedin, a shrimp antibacterial peptide, is expressed weakly in post-larval shrimp in comparison to juvenile shrimp (Jiravanichpaisal et al., 2007). This work revealed that bacterial profile found in post-larvae intestine was similar to bacterial profile in rearing condition. This could be explained by the lower immune level in the post-larval stage resulted in less harsh condition for bacteria to reside within (Jiravanichpaisal et al., 2007; Ley et al., 2006), resulting in different intestinal bacterial diversity from the harsher conditions in the juveniles. In contrast, the more developed immune system in juvenile shrimp imposes a higher selective pressure for those bacteria that can resist the host immune system, resulting in different bacterial pattern in their intestines from rearing condition.

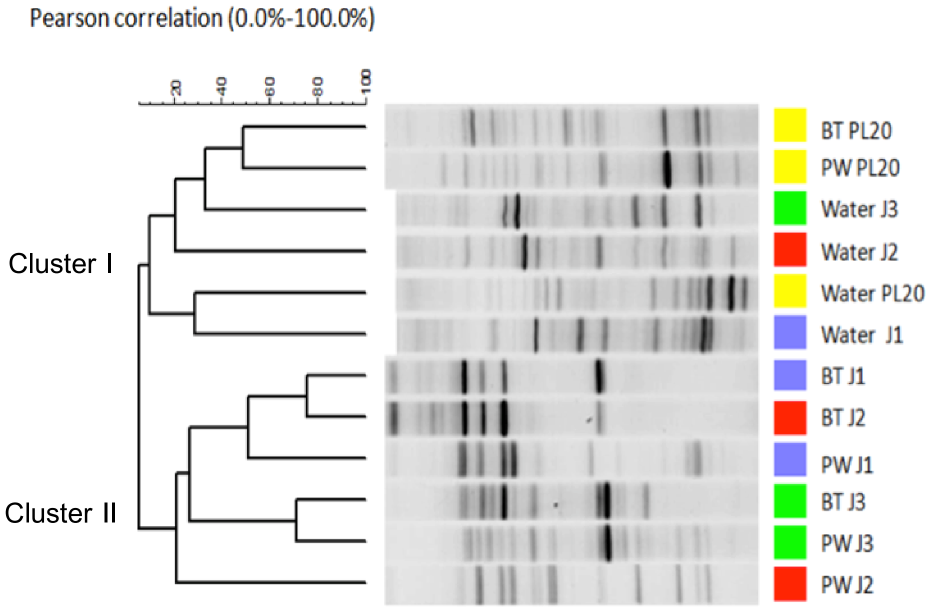


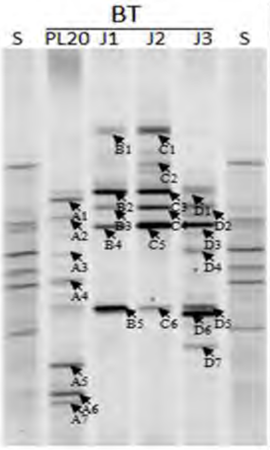
Figure 3.1. Dendogram analysis of DGGE profiles of predominant bacterial population in intestines of *Penaeus monodon* (BT, Black tiger shrimp) and *Litopenaeus vannamei* (PW, Pacific white shrimp) from 20-day-old post-larvae (PL20), 1-month-old, 2-month-old and 3-month-old juveniles (J1, J2, and J3, respectively). Bacterial profiles were also analyzed in pond water collected from each growth stage (Water PL20, Water J1, Water J2, and Water J3). Pearson correlation indicates similarity level, where 100% is the highest similarity.

3.3 Intestinal bacterial communities in *P. monodon* and *L. vannamei* under different growth stages

To identify bacteria associated to shrimp intestines, the selected DGGE bands were sequenced (Fig. 3.2). Sequencing of major DGGE bands showed that *Pseudoalteromonas*, *Tenacibaculum*, *Ruegeria*, *Muricauda*, *Shewanella*, *Maritibacter*, and *Thalassobius* were associated in PL20, all were belong to phylum *Proteobacteria* except for *Tenacibaculum* and *Muricauda* were from phylum *Bacteroidetes* (Fig. 3.2A). On the other hand*, Vibrio* and *Pseudoalteromonas* (belong to phylum *Proteobacteria*) were found across of J1, J2 and J3 of *P. monodon* (Fig. 3.2A).In *L. vannamei*, DGGE bands of PL20 were similar to *Ruegeria*, *Rhodobacter*, *Shewanella*, *Bowmanella*, and *Albidovulum*, in which all were members of *Proteobacteria* (Fig. 3.2B). *Vibrio* spp. were shared across all intestines of *L. vannamei* juveniles, which were similar to observation in *P. monodon*. Meanwhile, DGGE bands of bacteria detected in water were similar to *Paracoccus*, *Donghicola*, *Roseovarius*, *Pelagibaca*, *Legionella*, *Meiothermus* and *Photobacterium* (Fig. 3.2C). Most of bacterial detected in water were not found associated in shrimp intestine, except for *Photobacterium*. This observation suggested that there was a level of host gut selective pressure for intestinal associated bacteria. The phylogenetic tree was constructed to visualize relationship of bacterial species detected in DGGE analyses of different samples (Fig. 3.2D), where the bacterial detected in shrimp intestines were closely related species.

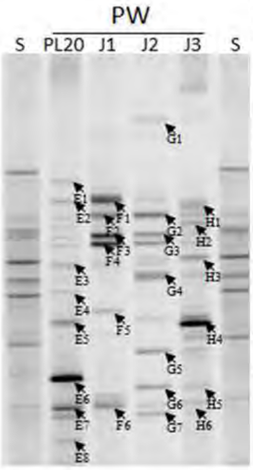
The dominant bacteria detected in intestines of both shrimp species were mainly belong in phylum *Proteobacteria*. This was consistent with the previous report of bacteria associated in intestines of *P. monodon* from commercial farms (Chaiyapechara et al., 2011). It has been reported that the most common bacterial genera in aquatic animal intestines are *Vibrio*, *Pseudomonas*, *Flavobacterium*, *Micrococus* and *Aeromonas*(Harris, 1993), in which *Vibrio* is reported as dominant genus in penaeid shrimp (Rungrassamee et al., 2013). Most of bacteria in this genus have been isolated as free-living or commensal in marine animal tracts, while some have been reported as pathogens. In shrimp aquaculture, *Vibrio harveyi* is the major bacterial pathogen, including that of the black tiger shrimp, and its infection results in high mortality rates in the larval and juvenile stages (Jiravanichpaisal et al., 1994; Lavilla-Pitogo et al., 1998; Soto-Rodriguez et al., 2012). Infection with *V. harveyi* is lethal, especially during the early larval stages in black tiger shrimp farms (Jiravanichpaisal et al., 1994). Currently, *V. harveyi* is considered an opportunistic pathogen that causes infection under certain conditions, such as high nutrients in water and high animal density in rearing ponds (Kraxberger-Beatty et al., 1990; Vaseeharan and Ramasamy, 2003). The recent outbreak of early mortality syndrome (EMS) has also been linked to *V. parahaemolyticus*, however its pathogenicity and roles are still under controversial (FAO, 2013). Despite the pathogenicity of several *Vibrio* species, there is some evidence suggesting beneficial roles of other *Vibrio* species to the aquatic animal host (Austin et al., 1995; Oxley et al., 2002). Although the bacteria population was characterized solely based on the DGGE analysis, an in-depth analysis of bacterial community structures in *P. monodon* and *L. vannamei* is currently underway to obtain a more comprehensive view of bacterial member associated in shrimp intestines.

A



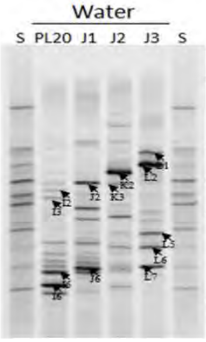
Sample	DGGE band	Phylum	Bacterial classification	GenBank accession	Identity (%)	Query length (bp)
BT-PL20	A1	Proteobacteria	*Pseudoalteromonas luteoviolacea*	NR_026221.1	100	196
	A2	Bacteroidetes	*Tenacibaculum aestuarii*	NR_043713.1	99	192
	A3	Proteobacteria	*Ruegeria* sp.	NR_042675.1	96	171
	A4	Bacteroidetes	*Muricauda ruestringensis*	NR_074562.1	99	192
	A5	Proteobacteria	*Shewanella amazonensis*	NR_074842.1	97	197
	A6	Proteobacteria	*Maritimibacter alkaliphilus*	NR_044015.1	98	173
	A7	Proteobacteria	*Thalassobius aestuarii*	NR_042903.1	99	172
BT-J1	B1	Proteobacteria	*Bowmanella pacifica*	NR_044500.1	97	197
	B2	Proteobacteria	*Glaciecola* sp.	NR_044430.1	96	197
	B3	Proteobacteria	*V. coralliilyticus*	NR_028014.1	97	197
	B4	Proteobacteria	*V. sinaloensis*	NR_043858.1	97	199
	B5	Proteobacteria	*Pseudoalteromonas* sp.	NR_074761.1	94	197
BT-J2	C1	Proteobacteria	*Oxalicibacterium flavum*	NR_037133.1	98	197
	C2	Proteobacteria	*V. pectenicida*	NR_029344.1	97	197
	C3	Proteobacteria	*Vibrio* sp.	NR_074990.1	92	197
	C4	Proteobacteria	*V. coralliilyticus*	NR_028014.1	97	197
	C5	Proteobacteria	*Vibrio sp.*	NR_043858.1	96	198
	C6	Proteobacteria	*Pseudoalteromonas sp.*	NR_074761.1	93	197
BT-J3	D1	Proteobacteria	*Vibrio sp.*	NR_074990.1	91	197
	D2	Proteobacteria	*Vibrio sp.*	NR_028014.1	95	198
	D3	Proteobacteria	*V. sinaloensis*	NR_043858.1	97	199
	D4	Proteobacteria	*P. spongiae*	NR_043172.1	97	197
	D5	Proteobacteria	*Pseudoalteromonas sp.*	NR_026218.1	92	197
	D6	Proteobacteria	*Thalassomonas sp.*	NR_043649.1	96	197
	D7	Proteobacteria	*S. haliotis*	NR_044134.1	98	197

B



Sample	DGGE band	Phylum	Bacterial classification	GenBank accession	Identity (%)	Query length (bp)
PW-PL20	E1	Proteobacteria	*Ruegeria pomeroyi*	NR_074150.1	99	172
	E2	Proteobacteria	*Rhodobacter maris*	NR_042629.1	100	172
	E3	Proteobacteria	*Ruegeria* sp.	NR_042675.1	95	171
	E4	Proteobacteria	*Bowmanella* sp.	NR_044500.1	96	197
	E5	Proteobacteria	*Albidovulum inexpectatum*	NR_025222.1	97	172
	E6	Proteobacteria	*Shewanella. amazonensis*	NR_074842.1	97	197
	E7	Proteobacteria	*S. amazonensis*	NR_074842.1	97	197
	E8	Proteobacteria	*S. amazonensis*	NR_074842.1	97	197
PW-J1	F1	Proteobacteria	*Vibrio sp.*	NR_074990.1	93	197
	F2	Proteobacteria	*V. coralliilyticus*	NR_028014.1	97	197
	F3	Proteobacteria	*V. sinaloensis*	NR_043858.1	97	199
	F4	Bacteroidetes	*Ulvibacter antarcticus*	NR_044279.1	97	192
	F5	Proteobacteria	*Halochromatium* sp.	NR_042529.1	91	196
	F6	Proteobacteria	*Shewanella* sp.	NR_044418.1	90	197
PW-J2	G1	Proteobacteria	*Vibrio* sp.	NR_029344.1	96	197
	G2	Proteobacteria	*R. vinaykumarii*	NR_042572.1	100	172
	G3	Bacteroidetes	*Actibacter* sp.	NR_044349.1	93	192
	G4	Bacteroidetes	*Actibacter* sp.	NR_044349.1	93	192
	G5	Proteobacteria	*R. maris*	NR_042629.1	100	172
	G6	Proteobacteria	*Vibrio* sp.	NR_025476.1	96	197
	G7	Bacteroidetes	*Actibacter* sp.	NR_044349.1	93	192
PW-J3	H1	Bacteroidetes	*Actibacter* sp.	NR_044349.1	92	192
	H2	Proteobacteria	*Photobacterium damselae*	NR_040831.1	99	197
	H3	Bacteroidetes	*Actibacter* sp.	NR_044349.1	93	192
	H4	Proteobacteria	*Pseudoalteromonas* sp.	NR_074761.1	94	197
	H5	Proteobacteria	*S. amazonensis*	NR_074842.1	99	197
	H6	Proteobacteria	*P. ulvae*	NR_025032.1	98	197

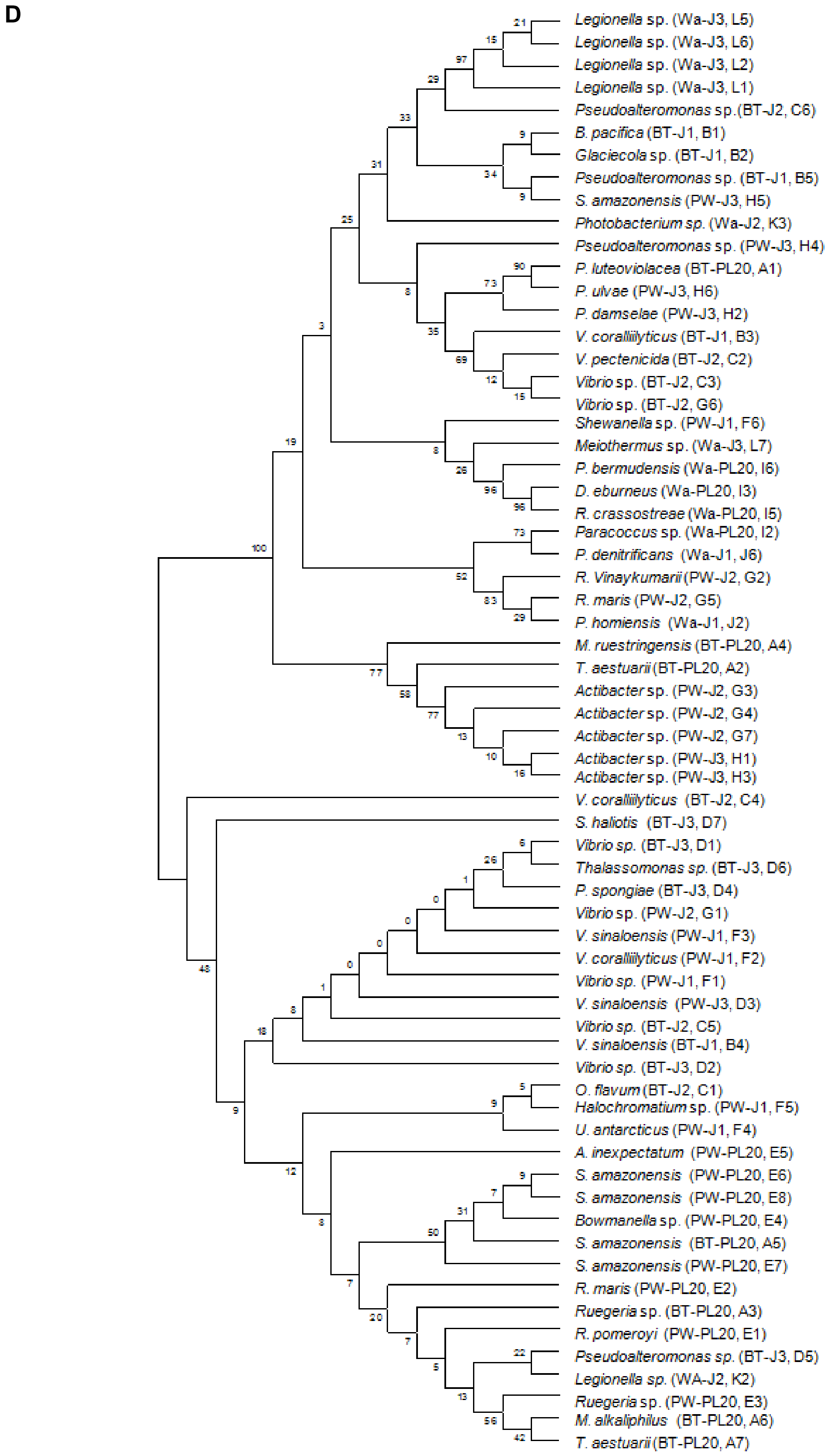
C



Sample	DGGE band	Phylum	Bacterial classification	GenBank accession	Identity (%)	Query length (bp)
Wa-PL20	I2	Proteobacteria	*Paracoccus sp.*	NR_043733.1	95	172
	I3	Proteobacteria	*Donghicola eburneus*	NR_043928.1	100	172
	I5	Proteobacteria	*Roseovarius crassostreae*	NR_041731.1	99	172
	I6	Proteobacteria	*Pelagibaca bermudensis*	NR_043611.1	99	172
Wa-J1	J2	Proteobacteria	*P. homiensis*	NR_043733.1	99	172
	J6	Proteobacteria	*P. denitrificans*	NR_074152.1	97	172
Wa-J2	K2	Proteobacteria	*Legionella sp.*	NR_044965.1	94	198
	K3	Proteobacteria	*Photobacterium sp.*	NR_043188.1	93	197
Wa-J3	L1	Proteobacteria	*Legionella sp.*	NR_044965.1	95	198
	L2	Proteobacteria	*Legionella sp.*	NR_044965.1	94	198
	L5	Proteobacteria	*Legionella sp.*	NR_044966.1	94	198
	L6	Proteobacteria	*Legionella sp.*	NR_044953.1	93	198
	L7	*Deinococcus/Thermus*	*Meiothermus sp.*	NR_042366.1	90	170

Figure 3.2 Bacterial similarity of DGGE band sequences from intestines of *Penaeus monodon* (BT, panel A) and *Litopenaeus vannamei* (PW, panel B) intestines and water (panel C) and Neighbor-joining phylogenetic tree (panel D). DGGE sequences were blast to ribosomal rRNA database. PL denotes post-larvae, where J1, J2, and J3 indicate 1-month-old, 2-month-old and 3-month-old juveniles shrimp, respectively.

Fig. 3.2 –continued-



3.4 Survival of *P. monodon* and *L. vannamei* under *V. harveyi* exposure

To determine *V. harveyi* concentration for bacterial challenge, the 3-month-old juvenile stage of *P. monodon* (n= 8) and *L. vannamei* (n= 8) were randomly selected for *V. harveyi* immersion trials. The group of shrimp was immersed into four water tanks (10 L) containing 10^5, 10^6 and 10^7 CFU/ml of *V. harveyi* and no *V. harveyi* as a control of the trial experiment. Shrimp survival was monitored daily for 7 days (Fig. 3.3). The exposure to *V. harveyi* at 10^5 CFU/ml showed no differences in survival rates in *P. monodon* and *L. vannamei* in comparison to the experimental control (Fig. 3.3A and B). Both Black tiger shrimp and Pacific white shrimp survival rates were affected when exposed to the higher concentration of *V. harveyi* at 10^6 CFU/ml and severely affected when exposed to 10^7 CFU/ml. However, the exposure to *V. harveyi* at 10^7 CFU/ml also affected water quality in the tank. Thus, a *V. harveyi* concentration of 10^6 CFU/ml was used for the immersion experiment for both shrimp populations in all subsequent experiment trials.

To compare survival rates between *P. monodon* and *L. vannamei* under the pathogen challenge, both shrimp species (n= 60 for each species) were transferred to experimental tanks and acclimated for 5 days before the bacterial challenge. The shrimp from each species were divided into 2 groups: one group was immersed to a tank containing *V. harveyi* (~ 4.43×10^5 CFU/ml) and the other to a tank without pathogen as a control group. The survival rates were monitored for a 7-day period (Fig. 3.4). The exposure to *V. harveyi* had an effect on shrimp survival for both species. *L. vannamei* showed a higher tolerance level to *V. harveyi* than *P. monodon*, suggesting that *P. monodon* shrimp were more sensitive to the pathogen. The finding was consistent with the previous report that *L. vannamei* have higher degree of disease resistance than *P. monodon*(FAO, 2004). However, it still remains unclear on what contributes to higher disease tolerance in *L. vannamei* than *P. monodon*.

To confirm for bacterial infection inside host shrimp, the transcript levels of shrimp antimicrobial peptides (*penaeidin*, *crustin*, and *antilipolysaccharide factor*), *lysozyme*, and *toll receptor* were determined in shrimp intestines of *P. monodon* (Fig. 3.5) and *L. vannamei* (Fig. 3.6). The transcripts of these genes have previously been shown to be inducible during the exposure to *V. harveyi*(Soonthornchai et al., 2010). In *P. monodon*, the transcript levels of *penaeidin*, *crustin* and *antilipolysaccharidefactor* were significantly up-regulated in the first 6hours after the pathogen exposure (Fig. 3.5A to C). Similarly, the transcripts of *lysozyme* and *toll1* were significantly induced in the first 6hours and their levels remained induced during the 72-hour period of the pathogen challenge (Fig. 3.5D and E). Surprisingly, the induction levels of these genes were not significantly induced in intestines of *L. vannamei* (Fig 3.6 A to E), suggesting that there was no activation of immune response to the pathogen exposure. *V. harveyi* 1526 was originally isolated from infected *P. monodon*(Rengpipat et al., 2003), hence, it might be possible that this *V. harveyi* isolates might not be pathogenic to *L. vannamei*. This might contribute to the higher survival rate observed in *L. vannamei* than *P. monodon*.

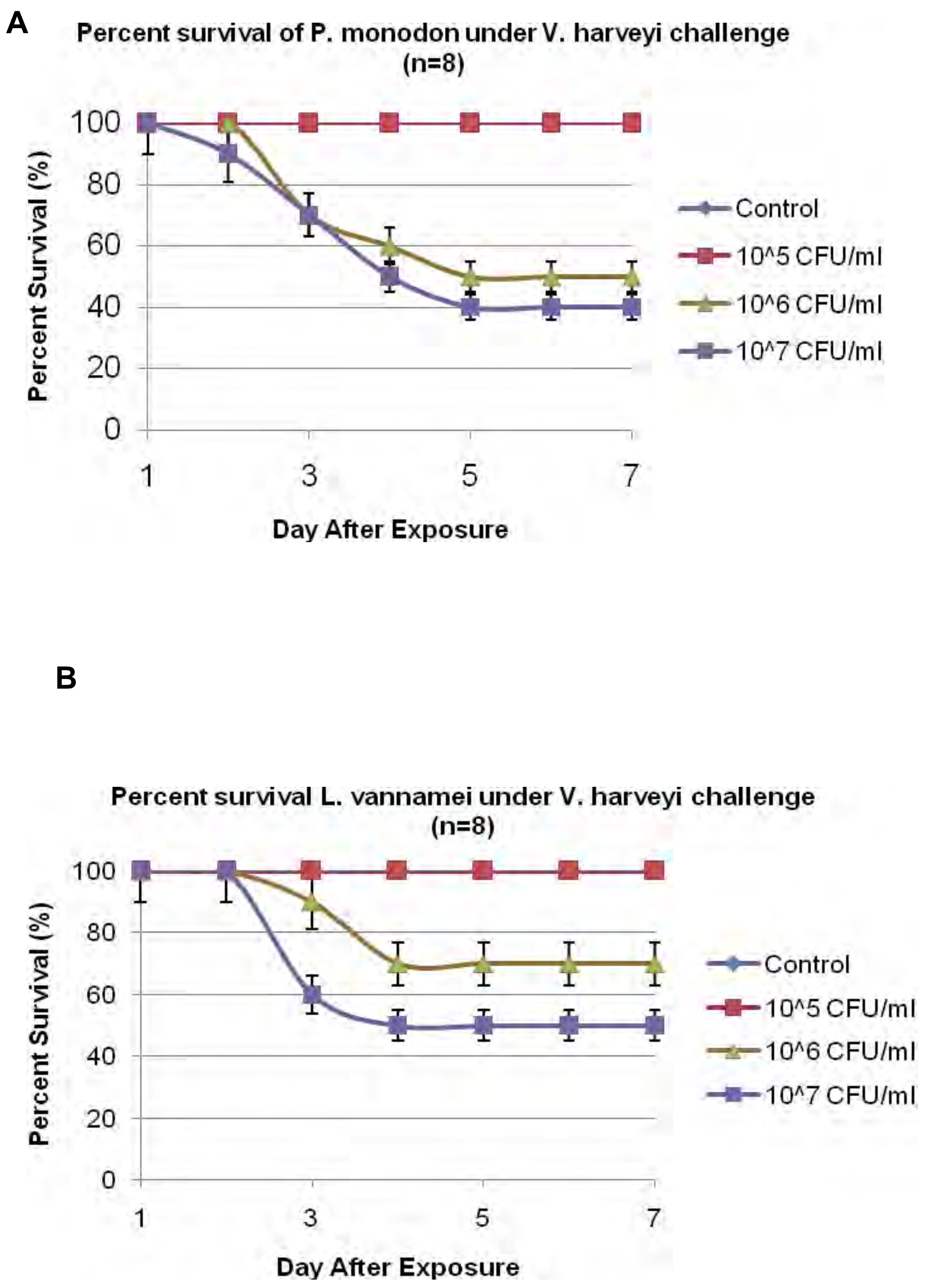


Figure 3.3. Survival rates of *Penaeus monodon* (Black tiger shrimp) (**A**) and *Litopenaeus vannamei* (Pacific white shrimp) (**B**) under exposure to different *V. harveyi* concentrations for 7 days. Error bars represent standard deviation calculated from triplicate trials with 10 shrimp per replicate for each species.

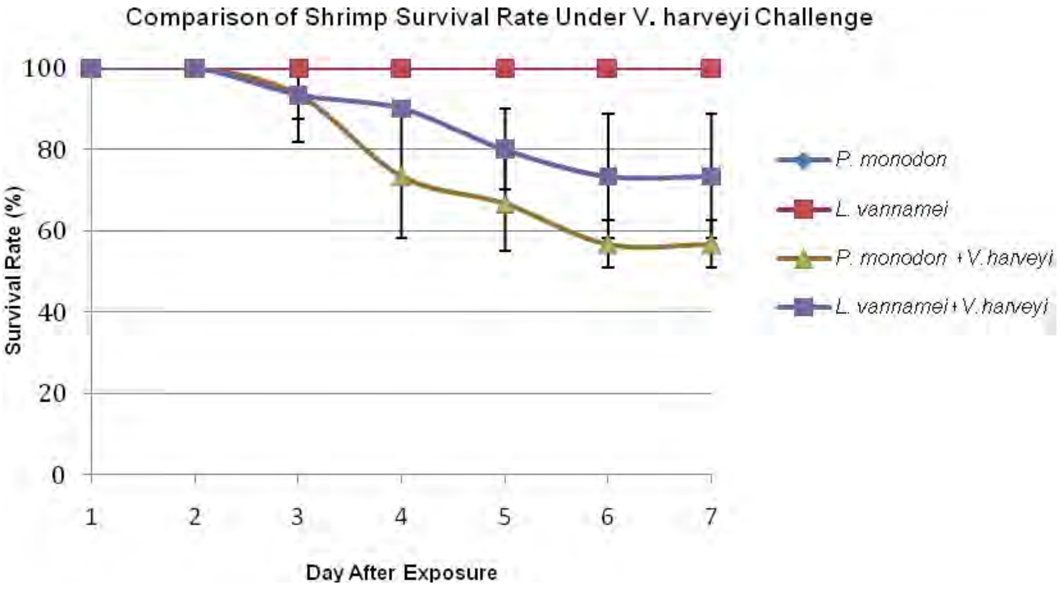


Figure 3.4. The survival rates of 3-month old juveniles of *Penaeus monodon* (Black tiger shrimp) and *Litopenaeus vannamei* (Pacific white shrimp) when exposed to *V. harveyi*. Error bars represent standard deviation calculated from triplicate trials with 10 shrimp per replicate for each species.

3.5 Monitoring of intestinal bacterial communities during the exposure to pathogenic *V. harveyi*

To determine if bacterial community was affected by the presence of pathogen, bacterial profiles were determined in intestine samples from *P. monodon* and *L. vannamei* at 6, 12, 24, 48 and 72 hours after the *V. harveyi* exposure. The DGGE profiles of *V. harveyi* challenge group were then compared the control group with no *V. harveyi* (Fig. 3.7). In *P. monodon*, DGGE profile of a time-course *V. harveyi* challenge group was clustered together, and in a distinct cluster from the control group (Fig. 3.7A). The DGGE profiles of the pathogen challenge group also showed higher bacterial pattern variations with 20 to 50% similarity range. In contrast, the DGGE profiles showed higher bacterial similarity of~80% in the control group (Fig. 3.7A). The DGGE profiles of intestinal bacteria in *L. vannamei* also showed similar observation with *P. monodon*, where the bacterial profiles of the *V. harveyi* challenge group were clustered together (Fig. 3.7B). These findings provide the evidence that the presence of *V. harveyi* altered the intestinal bacteria. The changes in bacterial profiles after *V. harveyi* challenge could be either directly through microbial interaction or indirectly through the host intestinal response to the pathogen. The disruption of intestinal bacterial balance by the pathogen has been reported as a temporary effect, which is likely due to the host immune response to control bacterial invasion (Barman et al., 2008). The effect of pathogen to bacterial community will be further determined to see if it is transient or permanent in shrimp intestine.

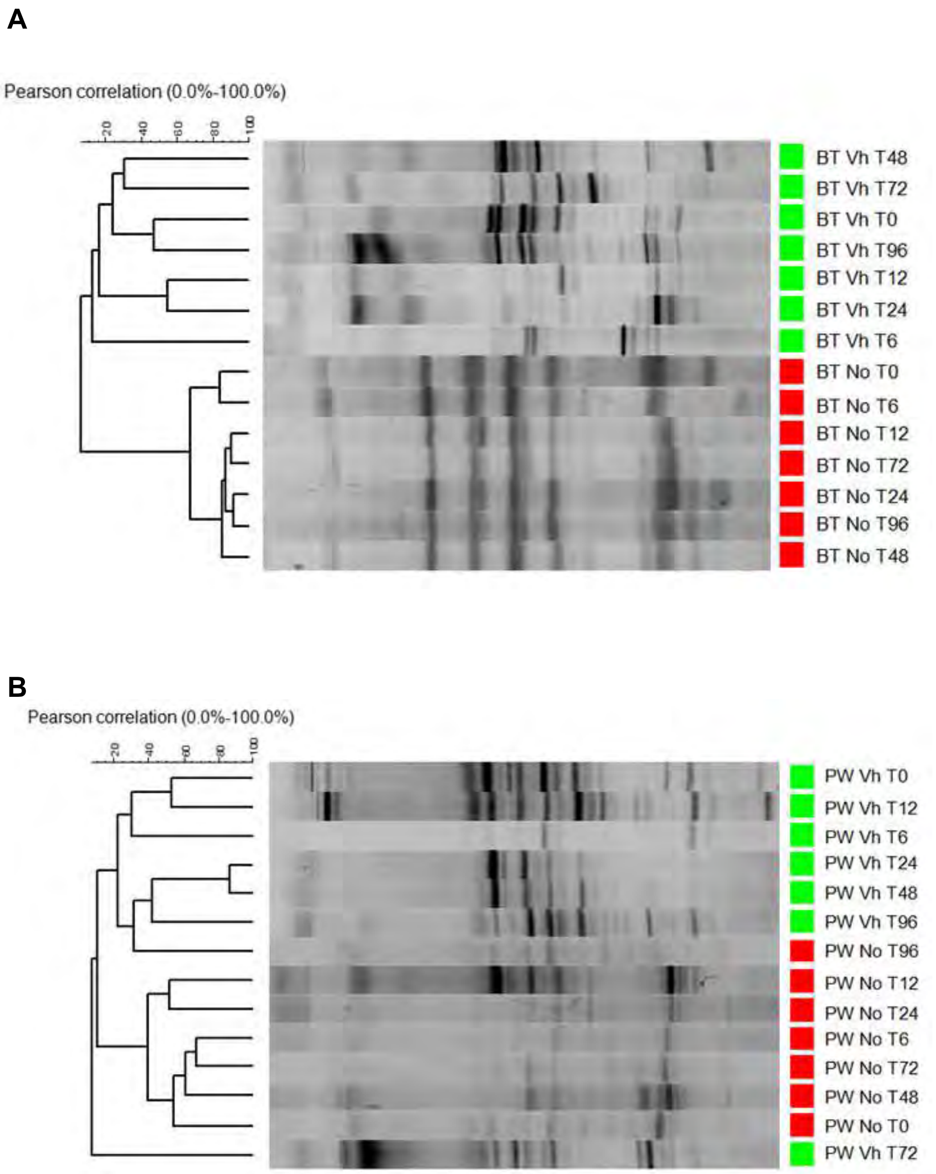


Figure 3.7. Dendogram analysis of DGGE profiles of predominant bacterial population in intestines of *Penaeus monodon* (BT, Black tiger shrimp) and *Litopenaeus vannamei* (PW, Pacific white shrimp) under *Vibrio harveyi* challenge. The 3-month-old juveniles were exposed to *V. harveyi* (Vh) and pooled intestine samples (N=5) were collected from 0, 6, 12, 24, 48 and 72 hour after the exposure (T0, T6, T12, T24, T48, and T72, respectively). The no *V. harveyi* control group (No) was also conducted in parallel. Pearson correlation indicates similarity level, where 100% is the highest similarity.

3.6 Screening of bacteria isolated from shrimp intestines for antagonistic activity against *V. harveyi*

Potential candidates of beneficial bacterial were screened from the black tiger shrimp intestines of wild-caught broodstock and 3-month-old domesticated juvenile and from the water from the rearing tank. Total of 344 bacterial were screened and the first pathogen (*Vibrio harveyi*) inhibition assay resulted in 18 promising candidates. However, only five candidates gave consistent inhibition activity after repeated testing. Among the five isolates, two isolates (VK101 and VK116) exhibited strong antagonistic activities against *V. harveyi* (Fig. 3.8). The 16S rRNA gene fragments of VK101 and VK116were sequenced and revealed high similarity to *Shewanella haliotis* and *Vibrio brasiliensis*, respectively (Fig. 3.9). The antagonistic activity of our bacteria isolates to other *Vibrio* species or pathogens will be further determined. If promising, these bacterial candidates will be tested for their beneficial roles in shrimp ponds in future research.

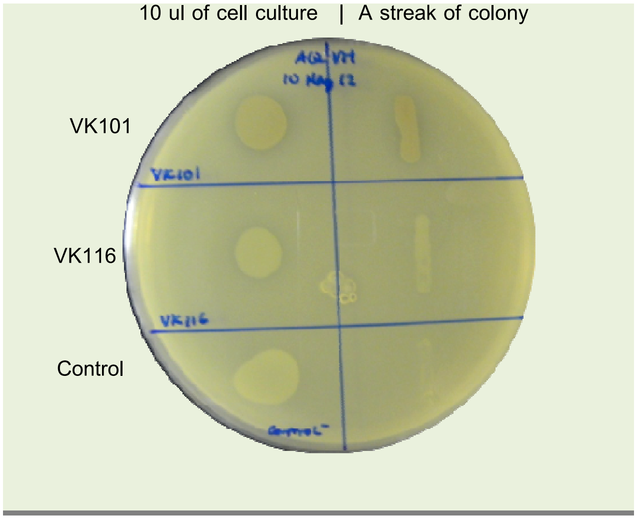


Figure 3.8. The inhibition zone observed around two bacterial isolates (VK101 and VK116) when grown on agar-overlay with *V. harveyi* 1526 (arrow). The molten agar containing *V. harveyi* 1526 (10^6 CFU/ml) was poured to LB agar. A droplet of 10 ul from VK101 or VK116 culture was placed on top (left panel) or a single isolate colony of each strain was streaked on the plate (right panel). The plate was incubated overnight at 30°C. The control was a droplet or a cell streak of *V. harveyi* 1526.

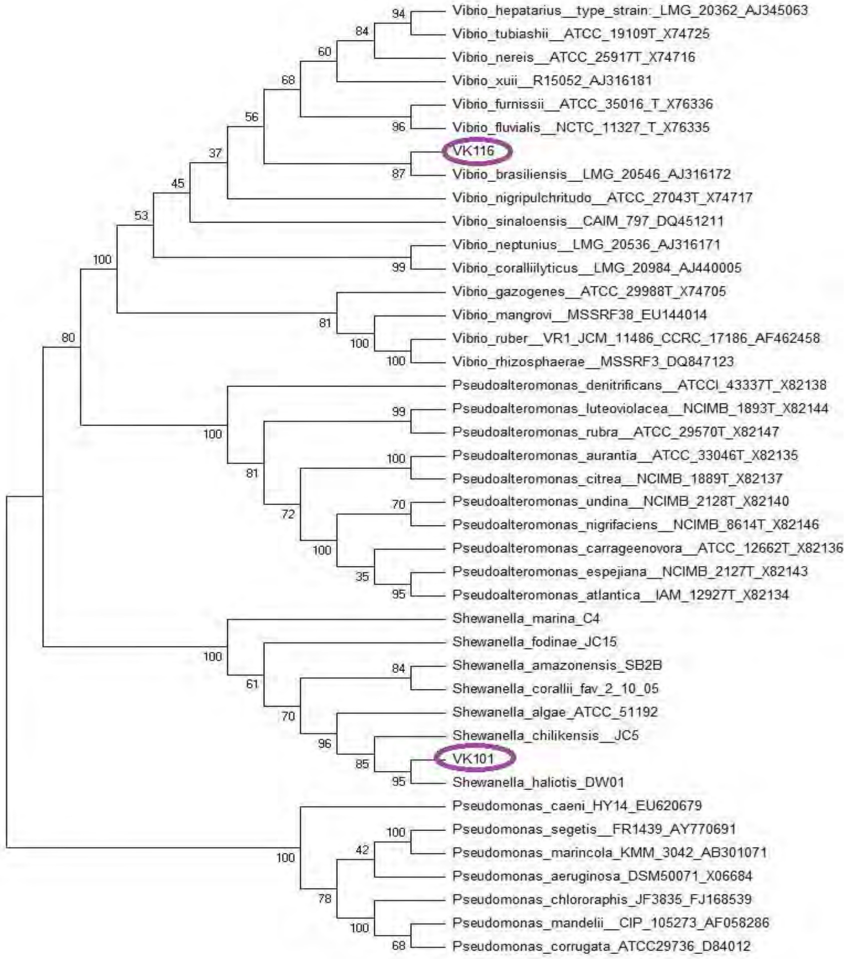


Figure 3.9. The phylogenetic tree of 16S rRNA sequences from VK101 and VK116.

3.7 Evaluation of lipopolysaccharide application for disease protection in *P. monodon* farming

The potential application of lipopolysaccharide (LPS) in shrimp feed to increase disease resistance was explored in this part. The 3-month-old *P. monodon* juveniles were divided into three groups fed with commercial feed pellets (Group 1), LPS-coated feed pellets given once a day (Group 2) and LPS-coated feed pellets given twice a day (Group 3) (Fig. 2.3). To determine the effects of LPS on growth performance of the black tiger shrimp, average shrimp weights, specific growth rate and average daily growth in the three groups were measured during the 10-day feeding trial and no significant differences were found among the three groups (Table 3.3). These similar growth indicators suggested that LPS as feed supplement has no negative effect on shrimp growth performance in comparison to the control diet.

Table 3.3. Effect of diets on the body weight (grams), percent weight gain (%), specific growth rate (%/day), and average daily growth (g/day) of the black tiger shrimp in the 10-day feeding period (Mean ± SD).

Group	**Diets**	**Average body weight (grams)**		**Percent weight gain (%)**	**Specific growth rate (%/day)**	**Average daily growth (g/day)**
		(day) 0	**10**			
1	**Control diet**	2.87±0.48^{a}	4.18±0.86^{a}	45.68±6.07^{a}	3.76±0.41^{a}	0.13±0.01^{a}
2	**LPS once a day**	2.87±0.47^{a}	4.20±0.87^{a}	46.43±0.79^{a}	3.81±0.05^{a}	0.13±0.00^{a}
3	**LPS twice a day**	2.87±0.47^{a}	4.16±0.83^{a}	44.71±2.97^{a}	3.69±0.21^{a}	0.13±0.01^{a}

Remark: Same superscript indicates no statistically significant difference among the three groups in each measurement at the same time point ($p>0.05$).

To further assess an effect of LPS on the shrimp survival under pathogen exposure, the black tiger shrimp juveniles from each diet group were challenged with *V. harveyi* and the immersion approach was employed for pathogen exposure to mimic natural bacterial entry route (Soonthornchai et al., 2010). Shrimp from each diet group were divided into two subgroups: one immersed in a water tank with *V. harveyi* (~10^7 CFU/ml) and the other without *V. harveyi* (Fig. 2.3). Unsurprisingly, shrimp mortality was only observed in the group exposed to *V. harveyi* (Fig. 3.10). When exposed to *V. harveyi*, shrimp fed with LPS supplement diets (Group 2 and Group 3) had higher percent survival rates than those fed with commercial pellets (Group 1) throughout the 7-day time course and significantly higher on Day 4 ($p < 0.05$), where Group 1 had 75% survival rate and Group 2 and Group 3 had 97% and 90%, respectively. The survival rates of the three groups remained unchanged after Day 7 (data not shown). During the *V. harveyi* challenge, the survival rates between Group 2 (LPS-coated feed pellet fed once a day) and Group 3(LPS-coated feed pellet fed twice a day) were not significantly different during the *V. harveyi* challenge. Hence, the frequency of LPS feeding did not significantly impact shrimp survival rate under pathogen challenge and this suggests that feeding LPS once a day would suffice to enhance shrimp disease resistance. The *V. harveyi* challenge experiment revealed that *P. monodon* juveniles fed with LPS supplementary diet showed increased resistance to *V. harveyi*. This observation was consistent with the previous study on LPS containing diet shows increase in disease resistance in Kuruma shrimp (*Penaeus japonicus*) (Takahashi et al., 2000).

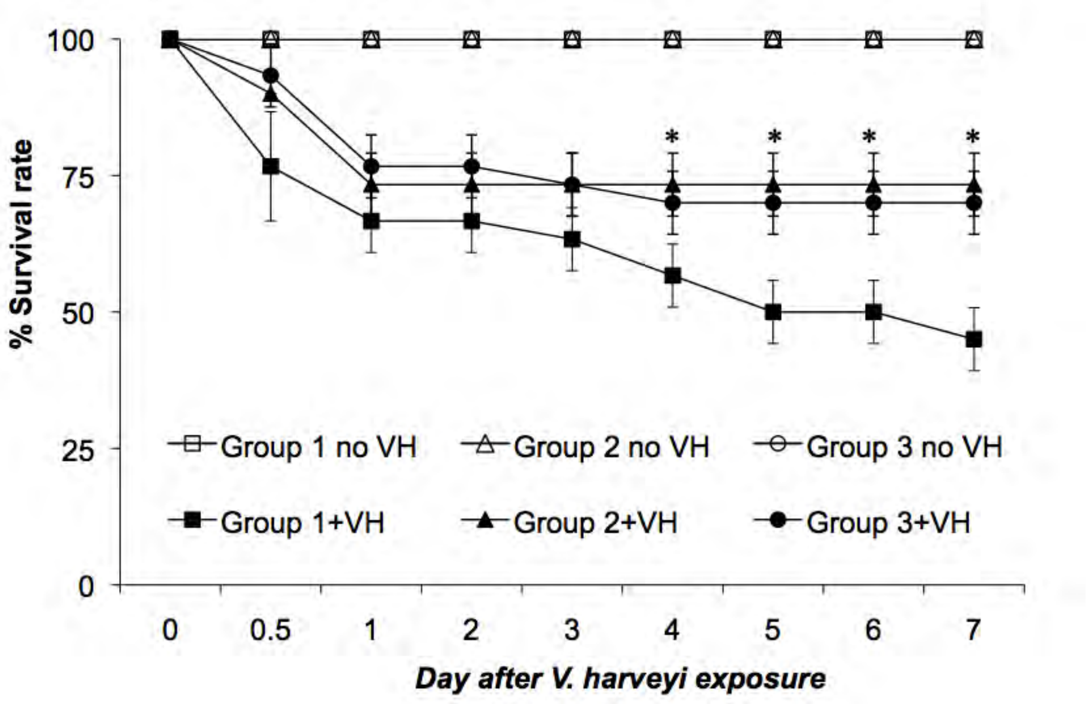


Figure 3.10. Percent survival of *Penaeus monodon* under the *V. harveyi* 1526 (VH*)* exposure. Group 1 represents shrimp fed with commercial feed pellets, Group 2 represents shrimp fed with LPS-coated feed pellets for once a day, and Group 3 represents shrimp fed with LPS-coated feed pellets for twice a day. The open symbols were the control groups without *V. harveyi* exposure, whereas the closed symbols were the groups of shrimp with *V. harveyi* exposure. Percent survival is an average of triplicate experiments (n = 10 per replicate in each group). Error bars represent standard deviations from triplicate trials. Asterisk indicates significant difference between groups fed with LPS and non-LPS containing diets ($p < 0.05$).

To assess direct effect of LPS on the shrimp immune system, the total hemocyte (THC) and granulocyte (GC) counts were determined after the feeding trials (Table 3.4). Hemocytes are one of cellular immune responses in *P. monodon* (Jiravanichpaisal et al., 2006), hence total hemocyte count is often used as an immunological indicator for shrimp immune level and health status. Total hemocyte counts have been reported to decrease in infected shrimp in comparison to the healthy population (Chang et al., 2008). Although the THC and GC counts in shrimp fed with LPS were slightly lowered than Group 1 (control diet), the THC and GC counts from all three diet groups were in the similar range of 10^6 cells/ml with no significant difference among the three groups. Therefore, the given dosage of LPS via oral administration in this study did not directly alter total hemocyte and granulocyte counts. The LPS-supplemented diet might contribute to enhancement of intestinal immunity without damaging intestinal epithelia, thus THC and GC counts were not significantly affected. In contrast, when LPS was given to shrimp via injection, the total hemocyte counts decreased, suggesting for cytotoxicity effects to the animals (Lorenzon et al., 1999). The intravenous administration of LPS also causes toxicity in mammals, but the oral administration of high dosage of LPS does not induce cell toxicity in mammals, birds or fish (Inagawa et al., 2011). The oral administration of LPS might be advantageous in minimizing damages to tissues and hemocytes in comparison to the direct LPS injection approach.

Table 3.4. Effect of diets on the total hemocyte count (THC, cells/ml) and granulocyte count (GH, cells/ml) of the black tiger shrimp after the 10-day feeding period (Mean ± SD).

Group	Diets	THC (cells/ml)	GH (cells/ml)
1	**Control diet**	$5.21 \pm 3.01 \times 10^{6\ a}$	$1.61 \pm 1.08 \times 10^{6\ a}$
2	**LPS once a day**	$3.68 \pm 3.08 \times 10^{6\ a}$	$1.22 \pm 1.34 \times 10^{6\ a}$
3	**LPS twice a day**	$3.99 \pm 1.97 \times 10^{6\ a}$	$1.20 \pm 0.71 \times 10^{6\ a}$

Remark: Same superscript indicates no statistically significant difference among the three groups in each measurement ($p>0.05$)

To further assess molecular effects of LPS supplement on host immune system, transcript levels of genes encoding the antimicrobial peptides (*ALF3*, *crustin* and *pen3a)*, C-type Lectin *(c-lectin)*, and mucine-like peritrophic membrane (*mucin-like PM)* were determined in the black tiger shrimp intestine. These genes of interest were previously shown to be induced in digestive tract of the black tiger shrimp as part of host defense mechanisms against *V. harveyi*(Soonthornchai et al., 2010). Therefore, relative expression levels of these aforementioned transcripts in intestines of the black tiger shrimp on Day 10 after fed with the LPS supplement diets (Groups 2 and 3) to those fed with the non-LPS as a control diet were determined (Fig. 3.11). The*ALF3* expression level was significantly induced by 2.5-fold when given LPS supplement diet twice daily ($p < 0.05$), while LPS did not seem to affect the transcript level of *pen3a*, whose gene product also belongs in antimicrobial peptide class. This suggested that the up-regulation of *ALF3* was specific to LPS treatment. Anti-lipopolysaccharide factor isoform 3 (*ALF3*) is one of anti-lipopolysaccharide factors (ALFs) found in *P. monodon*. The ALFs are small proteins that bind to LPS and exhibit strong antimicrobial activity against Gram-negative bacteria, but some have reported activities against Gram-positive bacteria and fungi as well (de la Vega et al., 2008; Somboonwiwat et al., 2005). Therefore, the induction of *ALF3* by LPS-supplemented diet might contribute to disease resistance under the *V. harveyi* challenge. On the other hand, the transcript level of *crustin* was slightly decreased by 0.75- and 0.60-fold in the group fed with LPS once and twice daily, respectively. Thus, the down-regulation of *crustin* by LPS in *P. monodon* was consistent with the previous work on LPS injection to the Pacific white shrimp that reported a decreasing pattern of the transcript level of *crustin* after 24 hour of injection (Okumura, 2007). This suggested that LPS-supplemented diet resulted in a negative regulation of *crustin*, however, the mechanism still remains unclear. In crustaceans, crustin proteins are part of antimicrobial peptide family, and they play important role in immune response to infection of various bacterial pathogens and viruses (Antony et al., 2011; Donpudsa et al., 2010; Shockey et al., 2009; Vargas-Albores et al., 2004). Crustin proteins might also have other alternate roles that were interfered by the presence of LPS.

The transcript level of *C-lectin* was significantly increased by 2.3-fold ($p < 0.05$) in shrimp fed with LPS-supplemented diet once a day, and lowered to1.8-fold induction in shrimp fed LPS-supplemented diet twice per day. The C-type lectin, encoded by *C-lectin*, is member of lectin family proteins. C-type lectin has been reported to bind to carbohydrate in calcium-dependent manner (Weis et al., 1998), and it plays important role in pathogen recognition by detecting conserved pathogen cell wall components such as LPS for Gram negative bacteria and further induces immune response to invaders (Medzhitov and Janeway, 2002; Wang and Wang, 2013). Lectins show strong bacterial-agglutination and opsonic activity, which facilitate phagocytosis (Luo et al., 2006). In the Chinese white shrimp, C-type Lectin has been demonstrated *in vivo* to mediate in *V. anguillarum* clearance (Wang et al., 2009). Thus, the induction of *C-lectin* could provide a local immune response and protection in *P. monodon* intestine against bacterial pathogen invasion.

Strikingly, the *mucin-like PM* showed the highest induction among the LPS – inducible immune-related genes in *P. monodon* intestines. The *mucin-like PM* transcripts were significantly increased by 23.2-fold ($p < 0.05$) when LPS-supplemented diet was given once a day, and further induced to 88-fold when LPS was administered twice a day. The *mucin-like PM* encodes for a peritrophic membrane, which lines in intestines of many invertebrates including crustaceans to protect against microorganisms and parasites invasion (Levy et al., 2011; Martin et al., 2006; Wang et al., 2012). In digestive tract of Penaeid shrimp, their mid-gut section does not line with cuticle; consequently, this site can be prone for pathogen invasion (Lovett and Felder, 1990; Soonthornchai et al., 2010). The up-regulation of *mucin-like PM* transcript by LPS treatment might increase pathogen protection by enhancement of the peritrophic membrane, which is the first defensive barrier in this gut section to prevent pathogen adhesion.

In this part, we provide the evidence of pathogen resistance in the black tiger shrimp when feeding with LPS-supplemented diet. While the LPS containing diet did not affect on growth rate, it resulted in significantly higher survival rates when exposed to *V.*

harveyi than the normal diet without LPS. Moreover, the LPS supplement showed induction of some crucial immune-related transcripts such as *ALF3*, *C-lectin* and *mucin-like PM* in shrimp digestive tracts. These findings suggest that LPS as an immunostimulant was able to activate the immune system at a molecular level in digestive tract of the host and enhanced disease resistance in the black tiger shrimp. LPS supplement is therefore a promising candidate to increase disease resistance in black tiger shrimp farming. Further investigation on optimal dosages and formulation for the LPS supplement for this purpose should be conducted for future application.

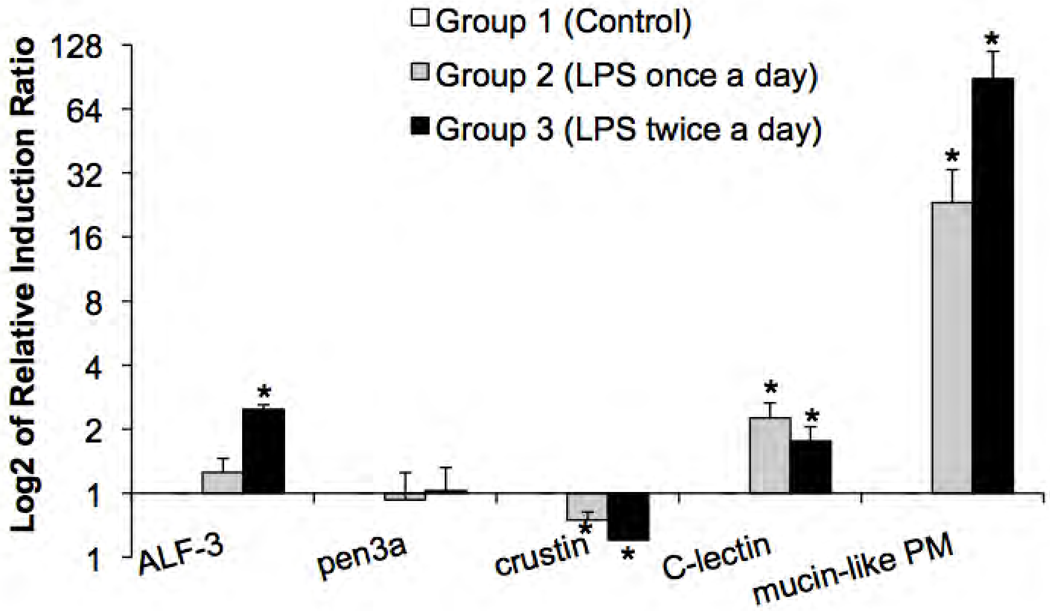


Figure 3.11. Relative gene expression analysis of immune-related genes in *P. monodon* digestive tracts after a 10-day of LPS feeding trials (Group 2 and Group 3). The fold changes of transcript levels were determined by real-time PCR in relative to the control diet (Group 1). Error bar is a standard deviation, which was calculated from five replicates for each sample. Asterisk indicates significant difference in fold-changes between groups fed with LPS and non-LPS containing diets ($p < 0.05$).

Chapter 4

Conclusions

Attempts for prevention and control of diseases in aquaculture result in a prophylactic application of antibiotics, which can lead to a high risk of antibiotic resistances transferred to animals and human pathogens (Tendencia and de la Pena, 2001). The increasing concerns on the food safety issues from contamination of these antibiotics and chemicals in aquaculture products force the industries to seek other alternatives. Based on other animal models, the intestinal bacteria communities have been known to play crucial roles in host immunity development and protective barriers against pathogens. In this project, we characterized intestinal bacterial population in intestine of the black tiger shrimp and Pacific white shrimp to generate fundamental knowledge in attempt to set a foundation for future development of non-chemical disease preventive approaches in shrimp industry.

This project embarked on comparison of intestinal bacterial communities in the black tiger shrimp and the Pacific white shrimp under different growth stages from 20-day-old post-larvae to 1-month-old, 2-month-old and 3-month-old juveniles. The bacterial profile analysis by DGGE revealed similar dominant bacteria in both shrimp species. However, the bacterial profile of 20-day-old post-larvae was distinct from those of juvenile stages. This observation suggested that the shrimp growth stages have higher influences on bacterial population in intestines than the shrimp species, when both shrimp were reared under the same diet and environmental conditions. To determine if bacterial balance was disrupted by a presence of bacterial pathogen, we further compared intestinal bacterial profiles of the black tiger shrimp and the Pacific white shrimp under normal (non-pathogen) condition and under a presence of *Vibrio harveyi* condition. The DGGE profiles showed that the presence of *V. harveyi* altered the intestinal bacterial patterns in comparison to the non-pathogen control. The changes in bacterial profiles after *V. harveyi* challenge could be either directly through microbial interaction or indirectly through the host intestinal response to the pathogen. Hence, this observation also provided the evidence that microbial gut balance is one of natural defense mechanisms to protect the host from invading pathogens. Furthermore, we have isolated potential bacteria from shrimp intestines that might be used in shrimp farming to protect against colonization of harmful bacteria. We hypothesized that the application of endogenously bacteria isolates from shrimp might be advantageous for successful colonization in intestines. The feasibility and efficacy trials of probiotic bacteria to increase disease protection in aquaculture are currently underway.

Besides characterizing beneficial bacteria approach, we also investigated onan effect of bacterial lipopolysaccharide (LPS) as feed supplement to stimulate immunity level of the black tiger shrimp. The up-regulation of the immune gene levels in intestines and higher degree of resistance to *V. harveyi* of the shrimp fed with LPS provide the evidence for potential application of LPS as an immunostimulant in shrimp farming.

The findings of this work will ultimately lead to alternative disease prevention approaches such as beneficial bacteria as biocontrol agents and lipopolysaccharides (LPS) as an immunostimulant to modulate host immune system to increase shrimp survival. The use of probiotic bacteria and/or natural immunostimulants to enhance shrimp health as disease prevention will also lessen the usage of antibiotics and chemicals in aquaculture and lead to a better and more sustainable shrimp farming.

Chapter 5

References

Aguirre-Guzmán, G., Sánchez-Martínez, J.G., Pérez-Castañeda, R., Palacios-Monzón, A., Trujillo-Rodríguez, T., and De La Cruz-Hernández, N.I., 2010. Pathogenicity and Infection Route of *Vibrio parahaemolyticus* in American White Shrimp, *Litopenaeus vannamei*. *Journal of the World Aquaculture Society* 41:464-470.

Altschul, S., Gish, W., Miller, W., Myers, E., and Lipman, D., 1990. Basic local alignment search tool. *J Mol Biol* 215:403 - 410.

Antony, S.P., Singh, I.S.B., Sudheer, N.S., Vrinda, S., Priyaja, P., and Philip, R., 2011. Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, *Penaeus monodon*, and its expression profile in response to various immunostimulants and challenge with WSSV. *Immunobiology* 216:184-194.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I., and Griffith, D.R.W., 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii*. *J Fish Dis* 18:93-96.

Austin, B. and Zhang, X.H., 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Lett Appl Microbiol* 43:119-124.

Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., and Salzman, N., 2008. Enteric Salmonellosis Disrupts the Microbial Ecology of the Murine Gastrointestinal Tract. *Infection and Immunity* 76:907-915.

Brune, A. and Friedrich, M., 2000. Microecology of the termite gut: structure and function on a microscale. *Curr Opin Microbiol* 3:263-269.

Chaiyapechara, S., Rungrassamee, W., Suriyachay, I., Kuncharin, Y., Klanchui, A., Karoonuthaisiri, N., and Jiravanichpaisal, P., 2011. Bacterial Community Associated with the Intestinal Tract of *P. monodon* in Commercial Farms. *Microb Ecol* 63:938-953.

Chang, C.-C., Yeh, M.-S., Lin, H.-K., and Cheng, W., 2008. The effect of *Vibrio alginolyticus* infection on caspase-3 expression and activity in white shrimp *Litopenaeus vannamei*. *Fish & Shellfish Immunology* 25:672-678.

Chang, E.S., 1995. Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. *J Exp Mar Biol Ecol* 193:1-14.

Chen, H., 1993. Recent Advances in Nutrition of *Penaeus monodon*. *J World Aquacult Soc* 24:231-240.

de la Vega, E., O'Leary, N.A., Shockey, J.E., Robalino, J., Payne, C., Browdy, C.L., Warr, G.W., and Gross, P.S., 2008. Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. *Mol Immunol* 45:1916-1925.

Donpudsa, S., Rimphanitchayakit, V., Tassanakajon, A., Soderhall, I., and Soderhall, K., 2010. Characterization of two crustin antimicrobial peptides from the freshwater crayfish *Pacifastacus leniusculus*. *J Invertebr Pathol* 104:234-238.

FAO, 2004. Introductions and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the Pacific. RAPI Publication.

FAO, 2013. Culprit behind massive shrimp die-offs in Asia unmasked, Rome.

Harris, J.M., 1993. The presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis. *Microbial Ecol* 25:195-231.

Hooper, L.V., Midtvedt, T., and Gordon, J.I., 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr* 22:283-307.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I., 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291:881-884.

Inagawa, H., Kohchi, C., and Soma, G., 2011. Oral administration of lipopolysaccharides for the prevention of various diseases: benefit and usefulness. *Anticancer Res* 31:2431-2436.

Jiravanichpaisal, P., Lee, B.L., and Soderhall, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* 211:213-236.

Jiravanichpaisal, P., Miyazaki, T., and Limsuwan, C., 1994. Histopathology, Biochemistry, and Pathogenicity of *Vibrio harveyi* Infecting Black Tiger Prawn *Penaeus monodon*. *J Aquat Anim Health* 6:27-35.

Jiravanichpaisal, P., Puanglarp, N., Petkon, S., Donnuea, S., Söderhäll, I., and Söderhäll, K., 2007. Expression of immune-related genes in larval stages of the giant tiger shrimp, *Penaeus monodon*. *Fish & Shellfish Immunology* 23:815-824.

Kraxberger-Beatty, T., McGarey, D.J., Grier, H.J., and Lim, D.V., 1990. *Vibrio harveyi*, an opportunistic pathogen of common snook, *Centropomus undecimalis* (Bloch), held in captivity. *J Fish Dis* 13:557-560.

Landau, S. and Everitt, B., 2004. *A handbook of statistical analyses using SPSS*. Chapman and Hall/ CRC Press LLC, Boca Raton, Florida.

Lavilla-Pitogo, C.R., Leaño, E.M., and Paner, M.G., 1998. Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. *Aquaculture* 164:337-349.

Levy, S.M., Falleiros, A.M., Moscardi, F., and Gregorio, E.A., 2011. The role of peritrophic membrane in the resistance of *Anticarsia gemmatalis* larvae (Lepidoptera: Noctuidae) during the infection by its nucleopolyhedrovirus (*Ag*MNPV). *Arthropod Struct Dev* 40:429-434.

Ley, R.E., Peterson, D.A., and Gordon, J.I., 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837-848.

Li, M., Wang, B., Zhang, M., Rantalainen, M., Wang, S., Zhou, H., Zhang, Y., Shen, J., Pang, X., Zhang, M., Wei, H., Chen, Y., Lu, H., Zuo, J., Su, M., Qiu, Y., Jia, W., Xiao, C., Smith, L.M., Yang, S., Holmes, E., Tang, H., Zhao, G., Nicholson, J.K., Li, L., and Zhao, L., 2008. Symbiotic gut microbes modulate human metabolic phenotypes. *PNAS* 105:2117-2122.

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408.

Lorenzon, S., de Guarrini, S., Smith, V.J., and Ferrero, E.A., 1999. Effects of LPS injection on circulating haemocytes in crustaceans *in vivo*. *Fish Shellfish Immun* 9:31-50.

Lovett, D.L. and Felder, D.L., 1990. Ontogenetic Changes in Enzyme Distribution and Midgut Function in Developmental Stages of *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *Biol Bull* 178:160-174.

Luo, T., Yang, H., Li, F., Zhang, X., and Xu, X., 2006. Purification, characterization and cDNA cloning of a novel lipopolysaccharide-binding lectin from the shrimp *Penaeus monodon*. *Dev Comp Immunol* 30:607-617.

Martin, G.G., Simcox, R., Nguyen, A., and Chilingaryan, A., 2006. Peritrophic membrane of the penaeid shrimp *Sicyonia ingentis*: structure, formation, and permeability. *Biol Bull* 211:275-285.

Medzhitov, R. and Janeway, C.A., Jr., 2002. Decoding the patterns of self and nonself by the innate immune system. *Science* 296:298-300.

Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* 2:317-322.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695-700.

Nya, E.J. and Austin, B., 2010. Use of bacterial lipopolysaccharide (LPS) as an immunostimulant for the control of *Aeromonas hydrophila* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). *J Appl Microbiol* 108:686-694.

Okumura, T., 2007. Effects of lipopolysaccharide on gene expression of antimicrobial peptides (penaeidins and crustin), serine proteinase and prophenoloxidase in haemocytes of the Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immun* 22:68-76.

Oxley, A.P., Shipton, W., Owens, L., and McKay, D., 2002. Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. *J Appl Microbiol* 93:214-223.

Paulsen, S.M., Lunde, H., Engstad, R.E., and Robertsen, B., 2003. In vivo effects of B-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immun* 14:39-54.

Rawls, J.F., Samuel, B.S., and Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *PNAS* 101:4596-4601.

Rengpipat, S., Tunyanun, A., Fast, A.W., Piyatiratitivorakul, S., and Menasveta, P., 2003. Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. *Dis Aquat Organ* 55:169-173.

Ribeiro, F., 2010. *Development morphology and oncogeny of penaeid prawns postlarva*. Nottingham University Press, Nottingham, UK.

Rungrassamee, W., Klanchui, A., Chaiyapechara, S., Maibunkaew, S., Tangphatsornruang, S., Jiravanichpaisal, P., and Karoonuthaisiri, N., 2013. Bacterial Population in Intestines of the Black Tiger Shrimp (*Penaeus monodon*) under Different Growth Stages. *PLoS ONE* 8:e60802.

Shockey, J.E., O'Leary, N.A., de la Vega, E., Browdy, C.L., Baatz, J.E., and Gross, P.S., 2009. The role of crustins in *Litopenaeus vannamei* in response to infection with shrimp pathogens: An *in vivo* approach. *Dev Comp Immunol* 33:668-673.

Smith, V.J., Brown, J.H., and Hauton, C., 2003. Immunostimulation in crustaceans: does it really protect against infection? *Fish Shellfish Immun* 15:71-90.

Somboonwiwat, K., Marcos, M., Tassanakajon, A., Klinbunga, S., Aumelas, A., Romestand, B., Gueguen, Y., Boze, H., Moulin, G., and Bachere, E., 2005. Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp Penaeus monodon. *Dev Comp Immunol* 29:841-851.

Soonthornchai, W., Rungrassamee, W., Karoonuthaisiri, N., Jarayabhand, P., Klinbunga, S., Soderhall, K., and Jiravanichpaisal, P., 2010. Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Dev Comp Immunol* 34:19-28.

Soto-Rodriguez, S.A., Gomez-Gil, B., Lozano, R., del Rio-Rodriguez, R., Dieguez, A.L., and Romalde, J.L., 2012. Virulence of *Vibrio harveyi* responsible for the "Bright-red" Syndrome in the Pacific white shrimp *Litopenaeus vannamei*. *J Invertebr Pathol* 109:307-317.

Sritunyalucksana, K., Gangnonngiw, W., Archakunakorn, S., Fegan, D., and Flegel, T.W., 2005. Bacterial clearance rate and a new differential hemocyte staining method to assess immunostimulant activity in shrimp. *Dis Aquat Organ* 63:89-94.

Takahashi, Y., Kondo, M., Itami, T., Honda, T., Inagawa, H., Nishizawa, T., Soma, G.-I., and Yokomizo, Y., 2000. Enhancement of disease resistance against penaeid acute viraemia and induction of virus-inactivating activity in haemolymph of kuruma shrimp, *Penaeus japonicus*, by oral administration of *Pantoea agglomerans* lipopolysaccharide (LPS). *Fish Shellfish Immun* 10:555-558.

Tanticharoen, M., Flegel, T.W., Meerod, W., Grudloyma, U., and Pisamai, N., 2009. Aquacultural biotechnology in Thailand: the case of the shrimp industry. *Int J Biotechnology* 10:588-603.

Tendencia, E.A. and de la Pena, L.D., 2001. Antibiotic resistance of bacteria from shrimp ponds. *Aquaculture* 195:193-204.

Vandenberghe, J., Verdonck, L., Robles-Arozarena, R., Rivera, G., Bolland, A., Balladares, M., Gomez-Gil, B., Calderon, J., Sorgeloos, P., and Swings, J., 1999. Vibrios associated with *Litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. *Appl Environ Microbiol* 65:2592-2597.

Vargas-Albores, F., Yepiz-Plascencia, G., Jimenez-Vega, F., and Avila-Villa, A., 2004. Structural and functional differences of *Litopenaeus vannam*ei crustins. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 138:415-422.

Vaseeharan, B. and Ramasamy, P., 2003. Abundance of potentially pathogenic micro-organisms in *Penaeus monodon* larvae rearing systems in India. *Microbiol Res* 158:299-308.

Wang, L., Li, F., Wang, B., and Xiang, J., 2012. Structure and partial protein profiles of the peritrophic membrane (PM) from the gut of the shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol* 33:1285-1291.

Wang, X.-W. and Wang, J.-X., 2013. Diversity and multiple functions of lectins in shrimp immunity. *Developmental & Comparative Immunology* 39:27-38.

Wang, X.W., Zhang, X.W., Xu, W.T., Zhao, X.F., and Wang, J.X., 2009. A novel C-type lectin (FcLec4) facilitates the clearance of *Vibrio anguillarumin vivo* in Chinese white shrimp. *Dev Comp Immunol* 33:1039-1047.

Weis, W.I., Taylor, M.E., and Drickamer, K., 1998. The C-type lectin superfamily in the immune system. *Immunological Reviews* 163:19-34.

Wilkenfeld, J.S., Lawrence, A.L., and Kuban, F.D., 1984. Survival, metamorphosis and growth of Penaeid shrimp larvae reared on a variety of algal and animal foods. *J World Maricult Soc* 15:31-49.

Wyban, J., 2007. Thailand's white shrimp revolution, p. 56-58, Global Aqua Culture Advocate, USA.

Xu, J. and Gordon, J.I., 2003. Inaugural Article: Honor thy symbionts. *PNAS* 100:10452-10459.

Yogeeswaran, A., Velmurugan, S., Punitha, S.M.J., Babu, M.M., Selvaraj, T., Kumaran, T., and Citarasu, T., 2012. Protection of *Penaeus monodon* against white spot syndrome virus by inactivated vaccine with herbal immunostimulants. *Fish Shellfish Immun* 32:1058-1067.

Chapter 6

Project Output Summary

6.1. ผลงานที่ตีพิมพ์ในวารสารวิชาการนานาชาติ

2 publications in international peer-reviewed journals

Rungrassamee W, Klanchui A, Chaiyapechara S, Maibunkaew S, Tangphatsornruang S, Karoonuthaisiri N*. 2013. Bacterial population in intestines of the black tiger shrimp (*Penaeus monodon*) under different growth stages. PLoS ONE 8(4): e60802. (doi:10.1371/journal.pone.0060802) (Impact Factor = 4.1)

Rungrassamee W*, Maibunkaew S, Karoonuthaisiri N, Jiravanichpaisal P. 2013. Application of bacterial lipopolysaccharide (LPS) to improve black tiger shrimp survival under *Vibrio harveyi* exposure. Developmental and Comparative Immunology. (Impact factor =3.3) (*accepted)*

(*corresponding author)

1 manuscript in preparation

Rungrassamee W, Maibunkaew S, Karoonuthaisiri N. Comparison of bacteria population in intestines of black tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) under different growth stages. (*in preparation*)

6.2. ผลงานวิจัยอื่น ๆ

1 National Conference (poster)

Rungrassamee W*, Maibunkaew S, Karoonuthaisiri N. 2012.

Comparison of bacteria population in intestines of black tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) under different growth stages. The 38th Congress on Science and Technology ofThailand on “Science for the Future of Mankind” (STT2012), October 17-19, 2012 atEmpress Convention Centre, Chiangmai.