ผลของโพรไบโอติกต่อระบบภูมิคุ้มกันของกุ้งและการป้องกันการติดเชื้อ *Vibrio parahaemolyticus*

นางสาวสุดารัตน์ ชมวงค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2559

EFFECT OF PROBIOTIC ON SHRIMP IMMUNE SYSTEM AND PROTECTION AGAINST *Vibrio parahaemolyticus* INFECTION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2016

Thesis Title	EFFECT OF PROBIOTIC ON SHRIMP IMMUNE SYSTEM AND PROTECTION AGAINST *Vibrio parahaemolyticus* INFECTION
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สุดารัตน์ ชมวงค์ : ผลของโพรไบโอติกต่อระบบภูมิคุ้มกันของกุ้งและการป้องกันการติดเชื้อ *Vibrio parahaemolyticus* (EFFECT OF PROBIOTIC ON SHRIMP IMMUNE SYSTEM AND PROTECTION AGAINST *Vibrio parahaemolyticus* INFECTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. อัญชลี ทัศนาขจร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ปิติ อ่ำพายัพ , 77 หน้า.

แบคทีเรียกรดแลคติค เป็นเชื้อโพรไบโอติกที่มีศักยภาพต่อการเพาะเลี้ยงสัตว์น้ำ และสามารถพบได้ในลำไส้ของสัตว์ที่มีสุขภาพดี จากการแยกแบคทีเรียกรดแลคติคในลำไส้ของกุ้งขาว *Litopenaeus vannamei* บนอาหารเลี้ยงเชื้อ MRS ได้แบคทีเรียกรดแลคติคจำนวน 89 ไอโซเลต เมื่อนำมาวิเคราะห์ลำดับนิวคลีโอไทด์ 16S rDNA พบว่าเชื้อแบคทีเรียส่วนใหญ่ที่แยกได้จากลำไส้กุ้งเป็นสายพันธุ์ของแบคทีเรียกรดแลคติค ได้ทำการคัดเลือกโพรไบโอติกจำนวน 2 ชนิด ได้แก่ *Lactobacillus plantarum* และ *Lactococcus lactis* เพื่อนำมาทดสอบฤทธิ์การต้านเชื้อก่อโรคในหลอดทดลอง ผลการทดลองแสดงให้เห็นว่าน้ำเลี้ยงเชื้อของทั้งสองโพรไบโอติกมีฤทธิ์ต้านเชื้อ *Vibrio parahaemolyticus* ที่เป็นสาเหตุของโรคตายด่วน (VP_{AHPND}) ได้ จากการทดลองให้อาหารเสริมด้วยโพรไบโอติกทั้งสองชนิดกับของลูกกุ้ง พบว่าสามารถเพิ่มอัตราการรอดตายของลูกกุ้งเมื่อติดเชื้อ VP_{AHPND} นอกจากนี้ยังศึกษาผลของโพรไบโอติกทั้งสองสายพันธุ์ต่อระบบภูมิคุ้มกันของกุ้งโดยการวัดกิจกรรมของระบบภูมิคุ้มกัน และระดับการแสดงออกยีน ผลการทดลองแสดงให้เห็นว่าการให้ลูกกุ้งกินโพรไบโอติกส่งผลให้กิจกรรมเอนไซม์ ฟีนอลออกซิเดสและ SOD เพิ่มสูงขึ้น นอกจากนั้นพบว่าระดับการแสดงออกของยีน *Lv*proPO1 และ *Lv*proPO2 ยกเว้นยีน *Lv*Pacifastin เพิ่มขึ้นในลำไส้อย่างมีนัยสำคัญภายหลังจากให้กินโพรไบโอติก *L. lactis* และ *L. plantarum* จากการตรวจสอบการแสดงออกของยีนที่เกี่ยวข้องกับระบบโพรฟีนอลออกซิเดสในกุ้งต่อความเกี่ยวข้องการติดชื้อ VP_{AHPND} พบว่าระดับการแสดงออกของยีน *Lv*proPO1 ลดลงที่ 24 และ 48 ชั่วโมง และมีการแสดงออกกลับเข้าสู่สภาวะปกติที่ 72 ชั่วโมง ผลการศึกษานี้แสดงให้เห็นว่าโพรไบโอติกทั้งสองชนิดสามารถเพิ่มระบบภูมิคุ้มกันของกุ้ง และยังเพิ่มการต้านทานการติดเชื้อ VP_{AHPND} ที่เป็นสาเหตุของโรคตายด่วน

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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5672218323 : MAJOR BIOTECHNOLOGY

KEYWORDS: PROBIOTIC / LACTIC ACID BACTERIA / SHRIMP / VIBRIO PARAHAEMOLYTICUS / IMMUNITY

SUDARAT CHOMWONG: EFFECT OF PROBIOTIC ON SHRIMP IMMUNE SYSTEM AND PROTECTION AGAINST *Vibrio parahaemolyticus* INFECTION. ADVISOR: PROF. DR. ANCHALEE TASSANAKAJON, CO-ADVISOR: DR. PITI AMPARYUP, 77 pp.

Lactic acid bacteria (LAB) are proposed as potential probiotic candidates in aquaculture and also known to be present in the gut of healthy aquatic animals. To isolate LAB from the gut of the white shrimp *Litopenaeus vannamei*, intestines were collected and the isolation of 89 LAB isolates was carried out using the MRS selective agar plate. The 16S ribosomal DNA (16S rDNA) sequence analysis reveals that most of the bacterial isolates identified from shrimp guts are the strain of LAB. Two candidate probiotics, *Lactobacillus plantarum* and *Lactococcus lactis* were selected for *in vitro* antimicrobial activity assay. The results indicated that cell-free supernatant of two probiotics contained antimicrobial substances actively against *Vibrio parahaemolyticus* causing Early mortality syndrome (EMS)/Acute Hepatopancreatic Necrosis Disease (AHPND) (VP_{AHPND}). Administrations of two probiotic-supplemented feed enhanced the survival rate of shrimp upon infection with VP_{AHPND}. In order to investigate the effect of the two probiotic strains on the shrimp innate immune system, immune activity and mRNA levels were monitored. The results indicated that oral administration of probiotic increased the level of prophenoloxidase and SOD activities. Moreover, expression levels of *Lv*proPO1 and *Lv*proPO2, but not *Lv*Pacifastin-like in midgut are significantly up-regulated after probiotic *L. plantarum* and *L. lactis* feeding. To examine the involvement of proPO-related genes in VP_{AHPND} infection, temporal gene expressions were analyzed and showed that the *Lv*proPO1 transcript level was downregulated at 24-48h and then returned to non-stimulated levels at 72 h. These finding suggests that two candidate probiotic LAB could enhance the immune system of shrimp *L. vannamei* and increase the resistance to VP_{AHPND} infection.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor Professor Dr. Anchalee Tassanakajon, and my co-advisor Dr. Piti Amparyup for their guidance, supervision, encouragement and supports throughout my study.

My gratitude is also extended to Associate Professor Dr. Teerapong Buaboocha, Assistant Professor Cheewanun Dachoupakan Sirisomboon and Dr. Kallaya Sritunyalucksana-Dangtip for serving as thesis committees, for their valuable comments and also useful suggestions.

Many thanks to all members and friends of the Biochemistry Department especially in R728 for their helps in the laboratory and friendships that help me enjoy and happy throughout my study.

Finally, I would like to express my deepest gratitude to my parents and members of my family for their love, care, understanding and encouragement extended throughout my study.

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LIST OF ABBREVIATIONS

A	absorbance
bp	base pair
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
DNA	deoxyribonucleic acid
EF1α	elongation factor 1 alpha
mg	milligram
min	minute
ml	milliliter
mM	millimolar
OD	optical density
°C	degree Celsius
ORF	open reading frame
PCR	polymerase chain reaction
PO	phenoloxidase
RT	reverse transcription
SP	serine proteinase
SPH	serine proteinase homolog
WSSV	white spot syndrome virus
YHV	yellow head virus
µg	microgram
µl	microliter

SP	serine proteinase
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CHAPTER I
INTRODUCTION

1.1 General information

The cultivation of penaeid shrimp species is an important economic activity worldwide and a valuable source of protein for human consumption. A world shrimp survey by The Global Aquaculture Alliance reported the shrimp production declined to 3.4 million tonnes in 2012 and 3.0 million tonnes in 2013. In 2014, production of shrimp increased substantially to 3.7 million tonnes. The shrimp industry in Asia seems to be on the path of recovery following the substantial production declines in 2012 and 2013 caused by Early Mortality Syndrome. (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) (Anderson et al., 2016). Before EMS/AHPNS disease outbreak, Thailand was the world's largest exporter of farmed shrimp. With the losses, shrimp prices in places like the United States and Europe spiked, restaurants took shrimp specials off their menus, and countries that typically bought shrimp from Thailand looked elsewhere.

Shrimp farming has immense economic importance for Thailand. The disease outbreaks have caused by the major shrimp pathogens of virus including white spot syndrome virus (WSSV) (Fig 1.1), yellow head virus (YHV) and infactious myonecrosis virus (IMNV) and bacteria in the genus vibrio. Several bacteria disease outbreaks were also due to vibriosis including *Vibrio alginolyticus*, *V. damsela*, *Vibrio harveyi*, and EMS/AHPND-causing *V. parahaemolyticus* (Fig 1.2) (S et al., 2012); (Lee et al., 1996); (Liu et al., 1996); (Lightner, 2011)and (Flegel, 2012). Vibrio colonize the exoskeleton, gills and intestines of penaeids (Kannapiran et al., 2009), the hemolymph of crustaceans (Sizemore et al., 1975) and the hepatopancreas of shrimp (Gomez-Gil et al., 1998) Vibrio species are considered to be a secondary and opportunistic pathogen, and causes mortality of shrimp under poor environmental conditions (CHIU et al., 2007); (Liu et al., 2004b)

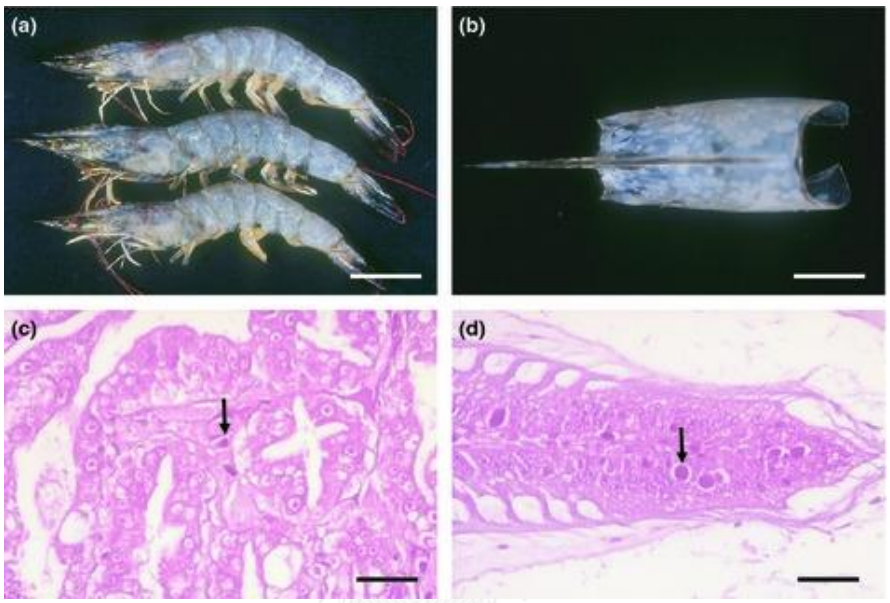


FIGURE 1.1 White spot disease in shrimp. (a) White spot syndrome virus (WSSV)-infected shrimp (*Litopenaeus vannamei*) with white spots on the cuticle. Scale bar = 2 cm. (b) moribund-shrimp cephalothorax with distinct white spots. Scale bar = 1.5 cm. (c) Hepatopancreas showing a hypertrophied nucleus and basophilic viral inclusion bodies (arrow). Haematoxylin and eosin (H&E) stain, scale bar = 50 μm. (d) Hypertrophied nucleus and basophilic viral inclusion bodies (arrow) in tail fan tissue. H&E stain, scale bar = 50 μm (L Cheng et al., 2013).

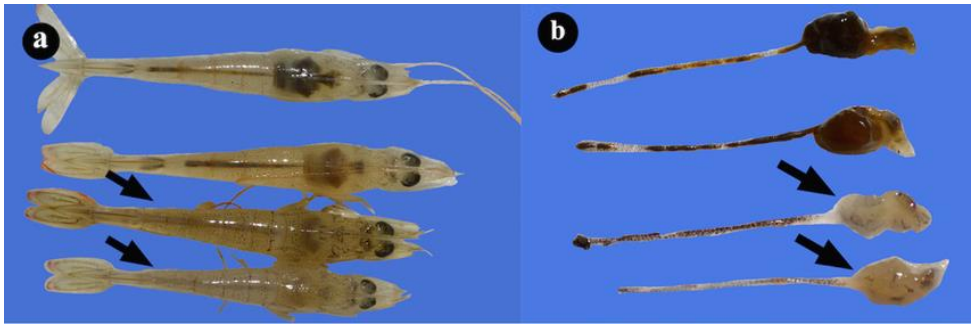


FIGURE 1.2 EMS/AHPNS disease in shrimp. (a) Photographs of hepatopancreases of healthy shrimp (*L. vannamei*) and shrimp naturally infected with EMS/AHPND (arrows). (b) The hepatopancreas without external membrane shows atrophy and white color (Sonia A. et al., 2015).

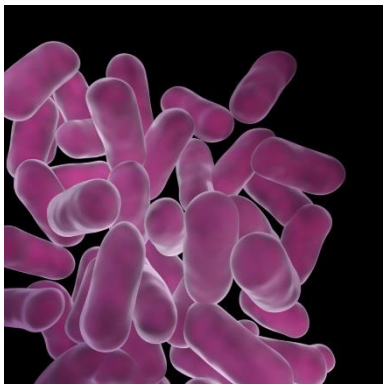
In shrimp aquaculture, antibiotics are applied to treat disease outbreaks and as a prophylactic (Austin, 1993) The use of antibiotics can affect microbial communities within receiving waters by eliminating sensitive species of microorganism (Samuelson et al., 1992). An important aspect of these results was the specificity of the host response, which depends on the bacterial species that colonize the digestive tract (Rawls et al., 2004). Possible modifications in gastrointestinal microbiota due to antibiotic treatment could alter this presumably beneficial host-microbiota relationship.

1.2 Probiotics

Probiotic technology is a top trend that is becoming a driving force in the natural products and is the alternative method for shrimp aquaculture that is having importance not only in the controlling of potential pathogens but also in other multi-dimensional functions such as stimulating of immune system, improving of the balance of the intestinal microbiota, survival, and digestive enzymatic activity and enhancement of the growth performance in shrimp (Kongnum and Hongpattarakere, 2012); (Liu et al., 2004a).

The term probiotic, meaning a live microbial adjunct that has a beneficial effect on the host by modifying the host associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response toward disease, or by improving the quality of ambient environment (Verschuere et al., 2000). The concept of probiotic activity has its origin in the knowledge that active modulation of the gastrointestinal tract could confer antagonism against pathogens, help development of the immune system, provide nutritional benefits and assist the intestine mucosal barrier (Vaughan et al., 2002).

Lactic acid bacteria (LAB) are among one of the most commonly proposed potential probiotic (e.g. *Lactobacillus planetarium* and *Lactococcus lactis*) (Fig 1.3) in aquaculture and also known to be present in gut of various animals (Sugimura et al., 2011; (Vijayabaskar et al., 2008). LAB can be seen as a live, dead or component of a microbial cell, which is administered via the feed or to the rearing water, benefiting the host by improving disease resistance, health status, growth performance, feed utilization, stress response or general vigour, which is achieved via improving the hosts microbial balance or the microbial balance of the ambient environment (Merrifield et al., 2010b). Moreover, LAB can perform well in various aquatic environments: freshwater (Rahiman and Pool, 2010), brackish water and sea water (Vijayan et al., 2006). Generally, LAB are live microbial feed supplements or water additives in the form of mono, multiple strains or in combination with prebiotics or other immunostimulants, which are administered to improve the rearing water quality, to enhance the physiological and immune responses of aquatic animals, and to reduce the use of chemicals and antibiotics in aquaculture.



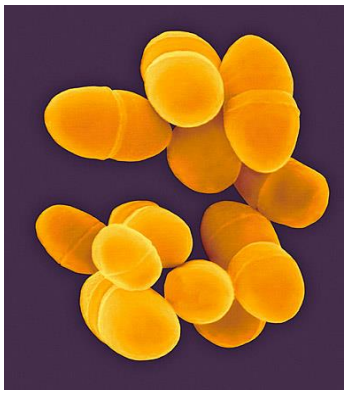


Figure 1.3 Micro-morphological image of *Lactobacillus plantarum* strain (Arasu et al., 2016) and *Lactococcus latis* (Gray et al., 2010)

1.3 Modes of action of probiotics

1.3.1 Colonization capacity

The effects of probiotics have proved to enhance disease resistance, growth performance and improve gut morphology and microbial balance. Factors known to influence the colonization of microorganisms can be grouped as follows: (i) host-related factors: body temperature, redox potential levels, enzymes, and genetic resistance. For example, bacteria may enter through the mouth, either with water or food particles, and pass down the alimentary tract, at which point some of them are retained as part of a resident microflora. Others are destroyed by the digestive process or pass through the gut, and are eliminated via the faeces. In addition, bacterial growth may be inhibited by any antimicrobial compound produced by the host. (ii) Microbe-related factors: effects of antagonistic microorganims, proteases, bacteriocins, lysozymes, hydrogen peroxide, formation of ammonia, diacetyl, and alteration of pH

values by the production of organic acids (Dopazo et al., 1988); (Gram et al., 1999); (Chythanya et al., 2002); (Sugita et al., 2002) ; (Gullian et al., 2004). For example, lactic acid bacteria are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms. In another experiment that was performed by (Rengpipat et al., 2003) the growth and resistance to *Vibrio* in black tiger shrimp (*Penaeus monodon*) fed with a *Bacillus* probiotic (BS11) were studied. It was found that the growth and survival rates of shrimps fed on the probiotic supplement were significantly greater than those of the controls.

1.3.2 Enhance immune responses

Probiotics help in achieving natural resistance and high survivability of larvae and post larvae of animal. Rengpipat et al., 2000 showed that *Bacillus* sp. (strain 11) can be provided for disease protection by activating the *Penaeus monodon* immune defenses. Simulation of immune response is increased by antibody activity and macrophage activity as reported by Marteua and Rambound (1993). (Liu Y. et al., 2010) reported that shrimp (*L.vannamei*) larval development, metamorphosis, immuno-stimulation and stress response was significantly accelerated after the addition of the probiotic (*B. subtilis* E20) to the larval rearing water at a level of 10^9cfu/L and increase of phenoloxidase (PO) activity and phagocytic activity of shrimp after being treated with *B. subtilis* E20. (CHIU et al., 2007) demonstrated that the probiotic bacteria *L. plantarum* can reduce the mortality of white shrimp, *L. vannamei*, challenged with a

virulent strain of *V. alginolyticus* and enhance the immune ability of shrimp, *L. vannamei* (CHIU et al., 2007).

1.3.3 Antimicrobial activity

Bacteriocins are proteins produced by certain types of bacteria that can antagonize other species which are related to the producer bacterium. Lactic acid bacteria and *Bacillus* are among the most common known to produce these compounds that may inhibit the growth of competing bacteria (Gildberg et al., 1997) and (Ali et al., 2000). Nisin is one of the famous bacteriocins, which is a ribosomally synthesized antimicrobial peptide produced by certain strains of *Lactococcus lactis* which has been proved to act against human *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and others (Balcazar et al., 2007).

1.3.4 Application of probiotics in shrimp aquaculture

Properties of probiotic are mediated by several effects that are dependent on the probiotic itself, the dosage employed, treatment duration and route, and frequency of delivery. Some probiotics exert their beneficial effects by elaborating antibacterial molecules such as bacteriocins that directly inhibit other bacteria or viruses, actively participating in the fight against infections, whereas others inhibit bacterial movement across the gut wall or translocation, enhance the mucosal barrier function by increasing the production of innate immune molecules, or modulate the inflammatory/immune

response (Hacini-Rachinel et al., 2009). Several studies have demonstrated that pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) signaling pathways, immune responses, and the secretion of antimicrobial peptides such as defensins and chemokines by the epithelium play important roles in probiotic mechanisms of action (Sherman PM et al., 2009; Mayra MA et al., 1993). In shrimp aquaculture, a probiotic *Bacillus* S11, isolated from the gastrointestinal tract (GIT) of shrimp *P. monodon* brood stock caught in the gulf of Thailand, demonstrated effective probiotic protection of shrimp from the pathogenic bacterium *Vibrio harveyi* infection (Ringo, 1998). Moreover, after probiotic feeding, shrimp exhibited significant increases in growth, survival, and external appearance above control group.

Recently, two strains including *Lactobacillus plantarum and Lactococcus lactis* of lactic acid bacteria (LAB), isolated from the guts of shrimp, have been reported to effectively act as potential probiotics playing important role in improving shrimp immune status, growth performance and disease resistance against shrimp pathogens, *Vibrio harveyi* and *V. penaeicida* (Kongnum and Hongpattarakere, 2012; Maeda et al., 2014). However, little is known about the inhibitory effect of LAB probiotics isolated from gut of healthy shrimp *Litopenaeus vannamei* against major shrimp pathogenic bacterium together with the efficacy as a probiotic supplement in stimulation of immune genes and resistance to the pathogenic Gram-negative marine bacterium *Vibrio parahaemolyticus* causing early mortality syndrome (EMS)/acute hepatopancreatic necrosis disease (AHPND) strain *in vivo*.

1.4 Immunity of shrimp

Shrimps possess an innate immune defense system also known as natural or non-specific defense system – includes both cellular and humoral immune responses, which work in jointly coordination and cross-talk for the detection, sensing and elimination of all foreign organisms potentially hazardous for the host (Jiravanichpaisal et al., 2006; Tassanakajon A et al., 2013) (Fig 1.4). Cellular defense components include all reactions performed directly by hemocytes (phagocytosis, encapsulation, nodule formation). On the other hand, humoral components include the activation and release of molecules stored within hemocyte cells, such as proteins in the prophenoloxidase (proPO) system (or melanization process), antimicrobial peptides or proteins, protease inhibitors, anticoagulant proteins, and agglutinins, etc. (Jiravanichpaisal et al., 2006); (Holmblad and Soderhall, 1999).

1.4.1 Cellular immune response

1.4.1.1 Phagocytosis, encapsulation and nodule formation

Phagocytosis is the most common reaction of defense cell mechanisms. By this process, hemocyte cells ingest and destroy invading pathogens or foreign particles (Secombes, 1996) Encapsulation and nodule formation are processes by which several hemocytes cooperate with each other aiming to stop the action of invading organisms, when the host is attacked by either extremely-large particles or numerous tiny particles, to be ingested then destroyed by individual cells (Söderhäll, 1992)

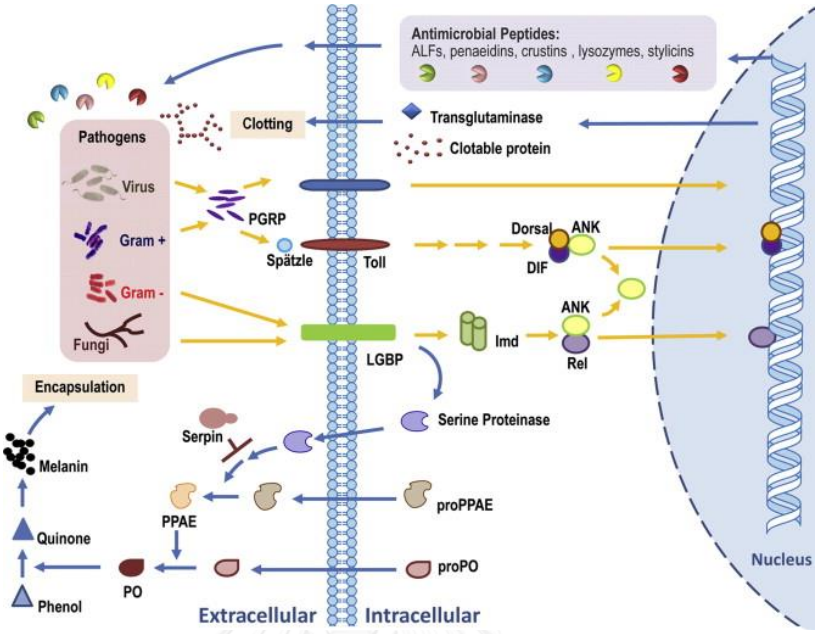


Figure 1.4 A schematic model of the shrimp immune system. For abbreviations and explanation see the text (Tassanakajon A et al., 2013).

1.4.2 Humeral immune response

1.4.2.1 Antimicrobial peptides (AMPs)

AMPs are effectors of the innate immune system and function as a first line of defense to fight against invading microorganisms (Hacini-Rachinel et al., 2009). Therefore, AMPs are critical for shrimp to fight against the pathogenic invasion. AMPs are typically small in size (approximately 150-200 amino acid residues), and have an amphipathic structure with cationic or anionic properties.

Several families of shrimp AMPs, such as penaeidins, crustins, anti-lipopolysaccharide factor (ALFs) and stylicins, have been identified and characterized

(Hacini-Rachinel et al., 2009); (Tassanakajon et al., 2010) They are produced and stored in the hemocyte, which is the key cell in the shrimp immune system (Somboonwiwat et al., 2005). Several recombinant proteins or synthetic peptides of shrimp AMP family members have been tested *in vitro* for their antimicrobial activities against various microorganisms (Table 1.1), where it is clear that different types and/or isoforms exhibit a different but diverse spectrum of activities and specificities.

Penaeidins, a unique family of AMPs specific to shrimps, have been isolated in several penaeid shrimp species, (Tassanakajon et al., 2010); (Woramongkolchai et al., 2011); (Sugita et al., 2002); (PenBase, 2006) The signatures of penaeidins are an unconstrained proline-rich domain (PRD) at the N-terminus and six cysteine residues at the C-terminus that form three disulfide linkages. Generally, penaeidins exhibit mainly antifungal and anti-Gram-positive bacterial properties. (Tassanakajon et al., 2010)

Crustin, a cationic peptide containing a cysteine-rich region and a whey acidic protein (WAP) domain, is another member of AMPs that is unique to the Crustacea, being found in crabs, shrimps and several other crustaceans (Tassanakajon et al., 2010). In shrimps, crustins have been isolated from several shrimp species including *L. vannamei* and *L. setiferus*, *M. japonicus*, *F. chinensis* and *P. monodon*. Most shrimp crustins are Type II crustins that display a strong antimicrobial activity against Gram-positive but not Gram-negative bacteria. However, an exception to this Crustin-like*Pm* (named Crustin*Pm*7), which has a strong antibacterial activity against both Gram-positive and Gram-negative bacteria, including *V. harveyi* (Amparyup et al., 2007);

(Tassanakajon et al., 2010) Other than antibacterial activity, crustin*Pm*1 and crustin*Pm*7 are able to agglutinate bacterial cells and thus might be important for their antibacterial action (Krusong K et al., 2012)

ALFs, firstly identified from horseshoe crab, are antimicrobial peptides that have been identified and characterized in many crustaceans, including shrimps, lobsters and crabs (Nagoshi et al., 2006); (de le Vega E et al., 2008); (Imjongjirak C et al., 2007 and (Imjongjirak et al., 2011). ALFs are amphipathic peptides that contain two-highly conserved-cysteine residues that form a stable disulfide loop harboring a highly conserved cluster of positively charged (Lys and Arg) residues. The antimicrobial activity of the shrimp ALFs (ALF*Pm*3, *Lv*ALF) has been reported to be a broad antimicrobial activity against bacteria, fungi and virus (Somboonwiwat et al., 2005) and (de la Vega E et al., 2008)

TABLE 1.1 The range of activity of shrimp AMPs.

Family	Isoform	Antimicrobial activity	Other activity	Reference
Crustins				
	CruFc	Gram-positive bacteria		Zhang et al., 2007
	Fc-crus 2	Gram-positive bacteria		Nagoshi et al., 2006
	Fc-crus	Gram-positive bacteria		Nagoshi et al., 2006
	crustin*Pm*1	Gram-positive bacteria	Agglutination	Krusong et al.,2012; Supungul et al., 2008
	crustin*Pm* 5	Gram-positive bacteria		Vatanavicharn et al.,2009
	crustin*Pm* 7	Gram-positive bacteria Gram-negative bacteria	Agglutination	Krusong et al.,2012 ; Amparyup et al., 2008
	SWD*Fc*	Gram-positive bacteria; Gram-negative bacteria; Fungi	Protease inhibitory activity against subtilicin A and protein K	Jia et al., 2008
	SWD*Pm*	Gram-positive bacteria	Protease inhibitory activity against subtilicin A	Amparyup et al., 2008

TABLE 1.1. The range of activity of shrimp AMPs.(Cont.)

Family	Isoform	Antimicrobial activity	Other activity	Reference
Penaeidin				
	Crusli*keFc*1	Gram-positive bacteria		Zhang et al., 2007
	*Litvan*Pen2	Gram-positive bacteria; Fungi		Destoumieux et al.,1999
	*Litvan*Pen3	Gram-positive bacteria; Fungi		Destoumieux et al., 2000
	*Litvan*Pen4	Gram-positive bacteria; Fungi		Cuthbertson et al., 2004
	*Fenchi*Pen5	Gram-negative bacteria; Gram-positive bacteria; Fungi		Kang et al., 2007
	*Penmon*Pen	Gram-positive bacteri		Ho et al., 2004
	*Penmon*Pen3	Gram-positive bacteria; Fungi	Cytokine	Li et al., 2010; Destoumieux et al., 1999
	*Penmon*Pen5	Gram-positive bacteria; Fungi; virus		Woramongkolchai et al., 2011; Hu et al., 2016

TABLE 1.1 The range of activity of shrimp AMPs. (Cont.)

Family	Isoform	Antimicrobial activity	Other activity	References
Lysozyme				
	P. vannamei	Gram-negative bacteria		Supungul et al., 2010
	M. japonicas	Gram-negative bacteria		Ponprateep et al., 2012
	F. chinensis	Gram-positive bacteria; Gram-negative bacteria		Mai et al., 2010
	P. monodon	Gram-negative bacteria		Sotelo-Mundo et al., 2003; Bu et al., 2008
	F. merguiensis	Gram-positive bacteria; Gram-negative bacteria		Ye et al., 2009
	L stylirostris	Gram-positive bacteria; Gram-negative bacteria		Mai et al., 2009; de Lorgeril et al., 2008

TABLE 1.1 The range of activity of shrimp AMPs. (Cont.)

Family	Isoform	Antimicrobial activity	Other activity	References
Antilipopolysaccharide factors				
	ALF*Pm2*	Gram-positive bacteria; Gram-negative bacteria		Tharntada et al, unpublished data
	ALF*Pm3*	Gram-positive bacteria; Gram-negative bacteria; Fungi; virus	LPS and LTA binding activity	Somboonwiwat et al., 2005; Somboonwiwat et al., 2008; Tharntada et al., 20
	*Ls*ALF1	Virus		de la Vega et al., 2008
	*Mj*ALF1		LPS neutralizing activity	Nagoshi et al., 2006

1.4.2.2 Prophenoloxidase (proPO) activating system

Prophenoloxidase (proPO) system or melanization is major innate defense system in invertebrates (Amparyup et al., 2013) This important process is controlled by the key enzyme phenoloxidase (PO) (Cerenius and Soderhall, 2004) The conversion of proPO to the active form of the enzyme (PO) can be brought about by minuscule amounts of foreign molecules such as lipopolysaccharide, peptidoglycan and beta-1,3-glucans from microorganisms (Söderhäll and Cerenius, 1998) Generally, proPO is synthesized in the hemocyte cells (Aspan et al., 1995) whereas in haemocyanin is known to be synthesized in the hepatopancreas. Recently, proPO has been shown to be transported and deposited in the cuticle of the silkworm, *B. mori* (Brey et al., 1995) where it may be involved in the sclerotization of the cuticle and in defence against invading parasites. However, localizations of phenoloxidase in the gut or intestine have been reported in invertebrates.

In insects (*Drosophila*, *Bombyx mori*, and *Helicoverpa armigera*), proPO in insect gut is the main factor involved in detoxification of plant phenolics (Jiang H, 1998) ; (Satoh D et al., 1999) and (Lee SY et al., 1998) Insect proPO is released into the foregut lumen, and is then activated by an unknown proteinase (Wu X. L. et al., 2015) Active PO then oxidizes plant phenolics into nontoxic intermediates. Moreover, silkworm *B. mori* proPO, which is produced by hindgut cells and secreted into the hindgut contents, is an important regulator of the bacterial flora in hindgut and feces (Shao Q et al., 2012).

In penaeid species, the melanization mediated by proPO system has been studied extensively in shrimp *P. monodon* (Amparyup et al., 2013) and (Tassanakajon et al., 2017). To date, several genes in the proPO system in *P. monodon* have been functionally characterized including two proPO genes (*Pm*proPO1 and *Pm*proPO2), two clip-domain serine proteinases (*Pm*ClipSP2 and *Pm*Snake), two proPO-activating enzymes (*Pm*PPAE1 and *Pm*PPAE2), two pattern recognition proteins (*Pm*LGBP and *Pm*MasSPH1) and four protein/proteinase inhibitors (*Pm*Pacifastin-like, *Pm*SERPIN3 *Pm*SERPIN8, and *Pm*MIP).

Moreover, a model of the proPO / melanization cascade has been proposed in *P. monodon* as shown schematically in Fig. 1.5. Initially, the *Pm*LGBP or *Pm*MasSPH1 act as pattern recognition proteins for detection of LPS and β-1,3-glucan or PGN, respectively, plays a role in the microbe recognition and the activation of the proPO cascade. This complex then sequentially activates the serine proteinase cascade (*Pm*ClipSP2 and *Pm*Snake) that can convert the two inactive PPAEs to active PPAEs (*Pm*PPAE1 and *Pm*PPAE2). Stimulation of active PPAEs leads to the activation of proPO to active PO (*Pm*proPO1 and *Pm*proPO2), resulting in the production of melanin and reactive oxygen compounds. However, to control the activation cascade of proPO system, the proteinase or protein inhibitors, including *Pm*Pacifastin-like, *Pm*SERPIN3, *Pm*SERPIN8, and *Pm*MIP are presumed to contribute as the negative regulators of this cascade. Additionally, gene knockdown of the proPO genes suggests the immune-crosstalk between the proPO activating system and the antimicrobial peptide synthesis pathways which enables the innate immune system to enhance the magnitude of the shrimp immune response (Amparyup et al., 2013) and (Tassanakajon et al., 2017)

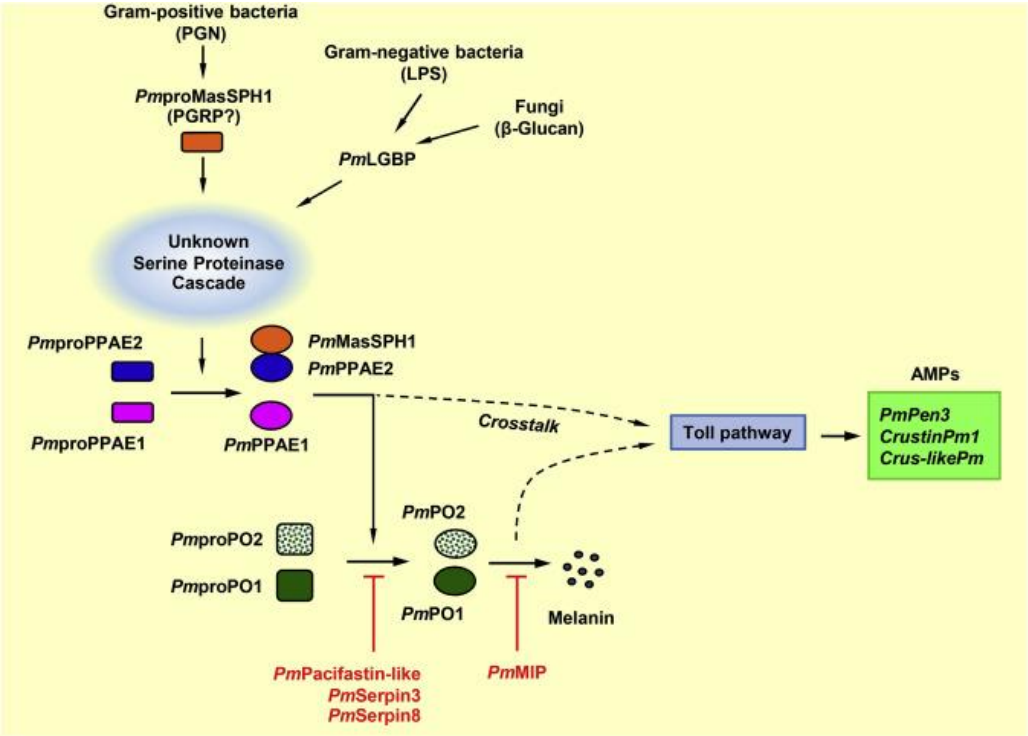


Figure 1.5 A schematic model of the melanization mediated by proPO system in shrimp. For abbreviations and explanation see the text (Tassanakajon et al., 2013).

Objectives

The objective of this thesis is to isolate the LAB from intestines of healthy shrimp *Litopenaeus vannamei* and to characterize the probiotic properties and host's immune system against shrimp pathogen. The LAB strains were isolated from intestines of healthy shrimp *Litopenaeus vannamei*. The inhibitory effect against microorganisms together with the efficiency as a probiotic supplement in induction of shrimp immune-related genes and resistance to the pathogenic Gram-negative marine bacterium *Vibrio parahaemolyticus* causing early mortality syndrome (EMS)/acute hepatopancreatic necrosis disease (AHPND) strain *in vivo* were also evaluated.

CHAPTER II
MATERIALS AND METHODS

2.1 Screening of lactic acid bacteria (LAB) from guts of shrimp

The healthy cultured white shrimp (*Litopeneaus vannamei*) (weight of 10 ± 2 g) were collected from shrimp farms located in Samut Prakan province in Thailand. To isolate probiotic LAB from guts of shrimp, white shrimp *L. vannamei* were aseptically washed with 70% ethanol before opening the ventral surface with sterile scissors.

Subsequently, the whole digestive tracts were homogenized and serially diluted with the sterile saline solution (0.85% (w/v) NaCl) before being spread on the de Man, Rogosa and Sharpe (MRS) agar plates. The MRS plates were incubated at 30 ℃ for 48h to 72 h. Single bacterial colony were then picked and re-streaked again on the new MRS agar plates and then were incubated at 30 ℃ for 48h to 72 h.

2.1.1 Analysis of 16S rDNA sequences for identification of selected lactic acid bacteria (LAB)

The LAB isolates were grown in the MRS broth at 30 ℃ for 24h to 48h. The cultured bacteria cells were transferred to 1.5 mL microcentrifuge tubes and then centrifuged at 8,000 rpm for 5 minutes. The bacterial cell pellets were resuspended in lysozyme solution (10 mM Tris-HCl, pH 8.0 containing 20μg/ml lysozyme,Sigma Aldrich) and future incubated for 15 minutes at room temperature. The resulting mixture of bacterial cell were boiling at 100℃ for 30 min and supernatants were directly used for further experiments.

To amplify the 16s ribosomal DNA (16s rDNA) gene from bacterial cells, Universal Bacterial Primers for 16S rDNA-specific primer pairs, UFUL (5' GCCTAACACATGCAAGTCGA 3') and URUL (5' CGTATTACCGCGGCTGCTGG 3') were used. The PCR cycle parameters were predenaturing at 94 ℃ for 3 minutes, followed by 35 cycle of 94 ℃ for 1 minute, 55℃ for 1 minute and 72℃ for 1 minute. The final extension was carried out at 72℃ for 5 minutes. The PCR products were electrophoretically analyzed through a 1.5% agarose gel and visualized ethidium bromide staining. To minimize the number of

clones, the approximately 500-bp PCR products were digested with *Sau3AI* and *RsaI* restriction enzyme (NEB) at 37°C for overnight. The digestion patterns of 16s rDNA PCR products were analyzed by 1.5% agarose gel electrophoresis. Then, the selected PCR amplicons of different clones were purified by NucleoSpin® Gel and Clean-up (MN) and directly commercially sequenced (Macrogen Inc., Korea). The obtained 16S rDNA nucleotide sequences were analyzed using the BLAST programs in the GenBank database.

2.1.2 16S rRNA phylogenetic analysis of the bacteria in guts of shrimp

The 16s rDNA sequences of obtained LABs were used for further comparative sequence analysis. The sequence data was proofread and edited and then compared to the public Genbank sequence databases (National Center for Biotechnology Information NCBI) using the BLASTN program. The 16s rDNA sequences with a ≥ 98% match to a database sequence were considered to be of the same species. Multiple sequence alignments were performed using the CLUSTAL W.

2.1.3 *In-vitro* screening of the antimicrobial activity of shrimp gut lactic acid bacteria (LAB) against microorganisms

The antimicrobial activity of the selected probiotic LAB was tested against four Gram-positive bacteria (*Staphylococcus aureus*, *Aerococcus viridans*, *Bacillus megaterium* and *B. subtilis*) and three Gram-negative bacteria (*Escherichia coli*, shrimp pathogenic bacterial *EMS/AHPEN-causing strain of V. parahaemolyticus* and shrimp pathogenic bacterial *V. harveyi*). Cell-free culture supernatants (CFCS) of the selected LAB were prepared by growing the cells in MRS broth at 30 °C for 24 h and then harvesting the cells by centrifugation at 11,000 × g for 10 minutes at 4°C. The CFCS was filtered through 0.45 µm filters.

For antimicrobial activity experiment, twenty microliters of each two-fold serially diluted CFCS was added to each well of a 96-well microtiter plate. Wells were then added with 80 µl of a suspension of mid-log phase bacteria (10^5 CFU/ml) in Poor Broth (1% (w/v) tryptone, 0.5% (w/v) NaCl, pH 7.5). The negative control was added with 20 µl of MRS broth, then incubated with 80 µl of Poor Broth. Cultures were grown at 30

°C for 24 h. The bacterial growth was evaluated by measuring the absorbance at 600 nm. The antimicrobial activity was determined as the minimal inhibitory concentration (MIC), the lowest concentration that caused 100% inhibition of bacterial growth

2.2 Effect of probiotics on the immune defense against pathogenic EMS/AHPEN-causing strain of *V. parahaemolyticus* infection

2.2.1 Shrimp feed preparation

Two probiotic LAB strains (*Lactobacillus plantarum* and *Lactococcus lactis*) isolated from shrimp guts were used in this study. Probiotic bacteria were cultured in sterilized 1000 ml flask containing 500 ml of the sterile MRS broth for 24h at 30°C. The bacterial cultures at 600 nanometre (OD_{600}) = 1.6 were collected by centrifugation at 6000 rpm for 5 min at 4°C, then washed twice in 50 ml of the sterile saline solution. The probiotic cells were adjusted to the final concentration of approximately 2-4 $x10^8$ colony forming units (CFU/ml). Shrimp feed were then mixed with prepared LAB cells, yield a final concentration of 2-4 × 10^8 CFU/g feed, and further coated with 1% fish oil. For the control feed, shrimp feed were mixed with sterile saline solution and coated with 1% fish oil. Supplemented and control feed were stored at 4°C and used upto 3 days.

2.2.2 Shrimp feeding experiments

The juvenile shrimp, *L. vannamei* (wet weight approximately 2-4 grams) were obtained from a hatchery in Chachoengsao Province, Thailand. They were transported to the Center of excellence for Molecular Biology and Genomics of Shrimp (CEMS LAB), Chulalongkorn university and maintained in tanks with running aerated water at 25±2°C, salinity at 20 parts per thousand (ppt). For the experiments, ten white shrimp (wet weight approximately 3±2 grams) were placed in 12-L plastic boxes containing 6 L of the saline sea water. Three replicates were carried out. All shrimps were fed daily at 09:00 h, 12:00 h and 18:00 h. For gene expression analysis, three shrimp of each experiments were randomly collected at 0h, 1d and 16d after feeding. The digestive tracts (foregut, midgut, and hindgut) of shrimp were collected and stored at -80°C until further analysis.

2.2.3 Determination of probiotics in shrimp gut by colonies plate count

Shrimps were dissected using sterilized surgical scissors to remove foregut, midgut and hindgut. To avoid possible external contamination while removing organs, the surface of each shrimp was previously cleaned using 70% ethanol. The intestine of 3 shrimps were pooled, placed in a sterile tube containing 1.5 ml of 0.85% NaCl and homogenization. Bacteriological determination was made using serial dilution in sterile saline solution followed by plating triplicates on Rogosa and Sharpe agar (MRS). After incubation (24 h and 48 h at 30 °C for MRS plates) colonies were counted and recorded. All the results are presented as colony-forming units per gram of fresh digestive tract (CFU/g).

2.2.4 Protective effect of probiotic feed supplement on the defense against EMS/AHPEN-causing strain of *V. parahaemolyticus* infection

EMS/AHPND-causing *V. parahaemolyticus* strains was re-streaked on the Tryptic soy agar (TSA) plate containing 1.5% (w/v) NaCl and incubated 30 °C overnight. A single bacterial colony was then picked and further cultured in Tryptic soy broth (TSB) containing 1.5% (w/v) NaCl at 30 °C with vigorous shaking until OD_{600} reached 0.6–0.8. Healthy shrimp (2–5 g wet body weight)(Ten shrimp per a group) were fed with LAB-supplemented or control feed as above in session 2.2.1. At 16 days post feeding, shrimp were immersed with 10^4 CFU/ml of EMS/AHPEN-causing strain of *V. parahaemolyticus*. The numbers of dead shrimp from each group were recorded daily until 10 days post the immersion. The experiment was carried out in triplicate. Statistical analysis of the mortality test was performed using a one-way ANOVA test.

2.3 Effect of probiotic on immune-related expression genes in guts of shrimp

2.3.1 Effect of probiotic on immune parameters of shrimp

The phenolooxidase activity (PO) assay

The PO activity was determined as described by Amparyup et al. (2009). The hemolymphs of the experimental group and the control group of white shrimp were withdrawn using a 1 ml sterile syringe containing 300 microliter of 10 mM Tris-HCL, pH8.0 without the anticoagulant. The total protein concentration of the individual

shrimp hemolymph was quantified using the Bradford protein assay kit (Bio-Rad) according to the manufacturer's instructions. Subsequently, 0.25 mg of total protein was added into 10mM Tris-HCl, pH 8.0 to final volume 200ul on the microplate and then mixed with 13 µl of 3 mg/ml dopamine (DOPA) as the PO substrate. PO activity was defined as ΔA_{470} per mg total protein/min. The results were analyzed from independent three experiments using a one-way analysis of variance (ANOVA).

The superoxide dismutase activity (SOD) assay

The SOD activity of hemolymph was quantified using the reduction of nitro blue tetrazolium (NBT) to formazan as a measure of superoxide anion (O^-_2) formation following a method from Song and Hisieh (1994). Protein concentration of the shrimp hemolymph of the experimental group and the control group was quantified as described above.

In brief, 0.25 mg of total protein was analyzed for SOD activity using 100ul of NEB (0.3% NBT) as the substrate. After incubation for 30 min at room temperature, the NBT solution was removed by centrifuged at 12,000 rpm for 10 minutes at 4°C. Then, 100ul of the absolute methanol was added to stop the reaction. After the mixture was discarded, the microplates were washed twice with 100ul of 70% methanol and was centrifuged at 12,000rpm for 10 minutes at 4°C. The microplates were air dried at room temperature for 30 minutes at room temperature. The intracellur formazan was solubilized by adding 120 ul of 2M potassium hydroxide (KOH) to solubilize the cell membrane and then by adding 140 ul of dimethyl sulfoxide (DMSO) to dissolve the cytoplasmic formazan. The absorbance (OD_{630}), recorded as an index of the specific activity (units per mg of protein), was calculated. The results were expressed as relative enzyme activity.

2.3.2 Shrimp tissue preparation, RNA extraction and cDNA synthesis

The five tissues including hemocyte, hapatopancrease, foregut, midgut and hindgut of three shrimp were collected and stored at -80°C until further analysis.

Total RNA of each shrimp tissues were extracted using Tri Reagent® (Molecular Research Center) according to the manufacturer's instructions. For DNase treatment,

total RNA was treated with the RNase-free DNase as described in manufacturer's protocol (Promega). After re-extraction with Tri Reagent®, total RNA was dissolved in DEPC-treated water and kept at -80°C.

For the first strand cDNA synthesis, one microgram of the DNase I-treated total RNA, which extracted from the shrimp tissues were reverse-transcribed to the first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol.

2.3.3 Tissue distribution analysis using reverse transcriptase PCR analysis

Tissue distribution from white shrimp (hemocyte, hepatopancrease, forgut, midgut and hindgut) analysis of the selected immune-related transcripts including prophenoloxidases (*Lv*proPO1 and *Lv*proPO2, *Lv*Pacifastin-like) and antimicrobial peptides (*Lv*ALF and *Lv*crustin-like) were examined using the RT-PCR analysis. The RT-PCR was initially performed by pre-denaturation at 94°C for 1 minute followed by 25 or 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C (or 60°C) for 30 seconds and extension at 72°C for 30 seconds. The final extension was carried out at 72°C for 5 minutes. Eight microliters of the amplification products were electrophoretically analyzed through 1.5 to 1.8% agarose gels. The electrophoresed band was visualized under a UV transilluminator after ethidium bromide staining.

2.3.4 Cloning of the full-open reading frame (ORF) of the pacifastin proteinase inhibitor from the white shrimp *L. vannamei*

It is likely that the pacifastin proteinase inhibitor plays an important role in the negative regulation of the proPO-activation system. Then, the full-ORF cDNA of this genes was cloned by RT-PCR. To obtain the full-ORF cDNA of a pacifastin proteinase inhibitor in the hemocyte and midgut from the white shrimp *L. vannamei* (*Lv*Pacifastin-like), a pair of primers Pacifastin-like-ORF-F and Pacifastin-like-ORF-R were designed based on the full-length cDNA of the Pacifastin-like of shrimp *Penaeus monodon* (Table 1) and used for the PCR amplification.

The cDNAs of white shrimp hemocyte and midgut were used as template in a 50 µl of PCR reaction volume using the Q5® High-Fidelity DNA polymerase (NEB) as described in manufacturer's protocol. The PCR cycles consisted of pre-denaturation at 98 °C for 30 seconds, followed by 30 cycles of 98 °C for 10 seconds, 55 °C for 30 seconds, and 72 °C for 3 minutes. The expected PCR products (approximately 2214 bp) were cloned into pGEM®-T easy vector (Promega) and commercially sequence (Macrogen Inc., Korea) on both strands to obtain the complete *Lv*Pacifastin-like genes from hemocyte and gut of white shrimp.

2.3.5 Expression analysis by semi-quantitative PCR

Expression analysis of innate immune-related genes using semi-quantitative RT-PCR. The cDNA from treatments were amplified with gene specific primer including proPO group (*Lv*proPO1, *Lv*proPO2 and *Lv*Pacifastin) and anitimicrobial peptide (*Lv*ALF and *Lv*crustin-like).The EF-1α gene was used as an internal control. All PCR reactions were performed according to the following protocol: 1 µl cDNA was mixed with 2.5 µl buffer, 2.5 µl dNTPs (1µM dNTP), 0.5 µl Taq polymerase (5 IU/µl), 2.5 µl each of gene-specific primer (2µM), and 13.5 µl distilled water. PCR product were visualized by agarose gel electrophoresis and ethidium bromide staining. Band intensity in every treatments was normalized with EF-1α before calculated for the relative genes expression.

2.3.6 Expression analysis by quantitative real-time PCR

Expression levels of the immune-related genes that expressed in shrimp guts including as *Lv*proPO1, *Lv*proPO2 and *Lv*Pacifastin-like were examined using quantitative real-time PCR analysis. The elongation factor 1 alpha (EF-1α), which is a housekeeping gene, was used as internal control.

The PCR amplification was performed in a reaction volume of 10 µl containing 5 µl of 2X SYBR Green Master Mix (Roch). The specific primer pairs were used at a final concentration of 0.2 µM. The thermal profile for quantitative real-time PCR was 95°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 20 seconds. The real-time RT-PCR assay was carried out in 96 well plate and each sample was run in triplicate using a

LightCycler®480 Instrument II system (Roche). The relative expression level between the experimental group and the control group were statistically tested using one way analysis of variance (ANOVA) followed by a Duncan's new multiple range test. Significant comparisons were considered when the *P* value was < 0.05.

2.4 Effect of EMS/AHPEN-causing strain of *V. parahaemolyticus* infection on the expression of immune-related genes in shrimp gut

2.4.1 Pathogenic bacterial challenge experiment

Juvenile shrimp, *L. vannamei* (5 g body weight) were purchased from local hatcheries and maintained in aerated 12-L plastic box containing 6 liters artificial seawater (Marinium) at 20 ppt salinity. The pathogenic bacteria (EMS/AHPND-causing strain of *V. parahaemolyticus*) was prepared as mentioned in session 2.2.4. Briefly, 50 ml of fresh pathogenic bacteria culture containing approximately 108 cfu/ml, used for immersion of 25 individual shrimp (2-3 g body weight) was added into 6 liters of 20 ppt artificial seawater to obtain a final bacterial density of approximately 2 x102 cfu/ml with proper aeration. For the control group, shrimp were immersed in 6 liters of 20 ppt artificial seawater contained 50ml of sterile TSB supplemented with 1.5% NaCl. Three replicates of each experiment were performed at the same time. Three shrimp were collected form each replicate at 0,24, 48 and 72 hours after pathogenic bacterial immersion for tissue collection and further analysis of gene expression. Shrimp which were not given any infection (time 0) served as the control.

2.4.2 RNA extraction and cDNA synthesis

Midgut of shrimp were separately collected. Total RNA was extracted and synthesized the first stand cDNA as above in session 2.3.2.

2.4.3 Expression analysis by quantitative real-time PCR

Gene expression analysis of prophenoloxidase *Lv*proPO1, *Lv*proPO2 and *Lv*Pacifastin-like transcripts in the pathogenic bacterial infected shrimp and the control shrimp was performed using quantitative real-time PCR as mentioned in session 2.3.6.

Table 2.1 Primer sequences and the expected size of the PCR product of gene in *L. vannamei*

Gene/Primer	Sequence	Size (bp)
Prophenoloxidase system (proPO)		
*1. Lv*proPO1	F: 5'-CATTCCGTCCGTCTGCCGA-3'	221
	R: 5'- CAGGCGGGTAGATCAGGTGC-3'	
2. *Lv*proPO2	F: 5'-TCTCAGCGTGAACTCGCCTTAC-3'	221
	R: 5'-GATCCTGCTCGGTGTACGGTCT-3'	
3. *Lv*Pacifastin-like	F: 5'-TGTAGCTGCATGAACGTACAGGA -3'	237
	R: 5'-ACTTCCACTGTTGGCTGAGAATGC -3'	
Antimicrobial peptide (AMP)		
3. Anti-lipopolysaccharide (*Lv*ALF)	F: 5'-CCGCTTCACCGTCAAACCTTAC-3'	196
	R: 5'-GCCACCGCTTAGCATCTTGTT -3'	
4. *Lv*Cruslike-like	F: 5'-CCGCTTCACCGTCAAACCTTAC-3'	237
	R: 5'-GCCACCGCTTAGCATCTTGTT -3'	
House-keeping gene		
5. *Elongation factor-1α* (*EF- α*)	F: 5'-GGTGCTGGACAAGCTGAAGGC-3'	150
	R: 5'-CGTTCCGGTGATCATGTTCTTGATG-3'	

CHAPTER III
RESULTS

3.1. Screening and isolation of lactic acid bacteria (LAB) in guts of shrimp

To identify the LAB from the digestive tract of white shrimp *L. vannamei* using the MRS agar plate as the selective media, randomly 89 bacterial colonies of LAB were amplified by PCR using universal primers for the bacterial 16S rRNA gene. After analysis of PCR products on 1.5% agarose gel, the results indicated that the major PCR products of the expected size of approximately 500 bp in size were obtained (Fig. 3.1).

Then, these PCR products were screened by restriction enzyme analysis with *Sau3AI* and *RsaI* to reduce the numbers of clones subjected to DNA sequencing analysis. Figure 3.2 showed that the four different patterns of DNA fragments were obtained implying that the different strains of LAB also present in shrimp guts. The different patterns of PCR products were selected for further DNA sequencing analysis.

To identify the nucleotide sequence of the 16S rDNA sequence, the obtained DNA sequences were analyzed with NCBI database using the BLASTN program. The BLAST results showed that the majority isolates of bacteria, which grown on MRS agar from shrimp gut were *Lactobacillus*, *Lactococcus*, *Vagococcus*, and *Staphylococcus*. Multiple sequence analysis of the 16S rDNA sequence revealed that GutLAB01, GutLAB02, GutLAB03 and GutLAB04 were closest to the 16S rDNA sequence of *Lactobacillus plantarum* (100% identity), *Lactococcus lactis subsp. cremoris* (99% identity), *Vagococcus carniphilus* (100% identity) and *Staphylococcus aureus* (100% identity), respectively (Appendix 1).

Based on the identity of the GutLAB01 and GutLAB02 to two shrimp probiotic strains (*Lactobacillus plantarum* (KU892396) and *Lactococcus lactis* (LC129537) that have been previously reported in shrimp gut, in this thesis, two LAB candidates (GutLAB01 and GutLAB02) identified as *Lactobacillus plantarum* and *Lactococcus lactis* were subsequently selected and further tested for both antimicrobial properties and efficacy to function as an effective probiotics in shrimp.

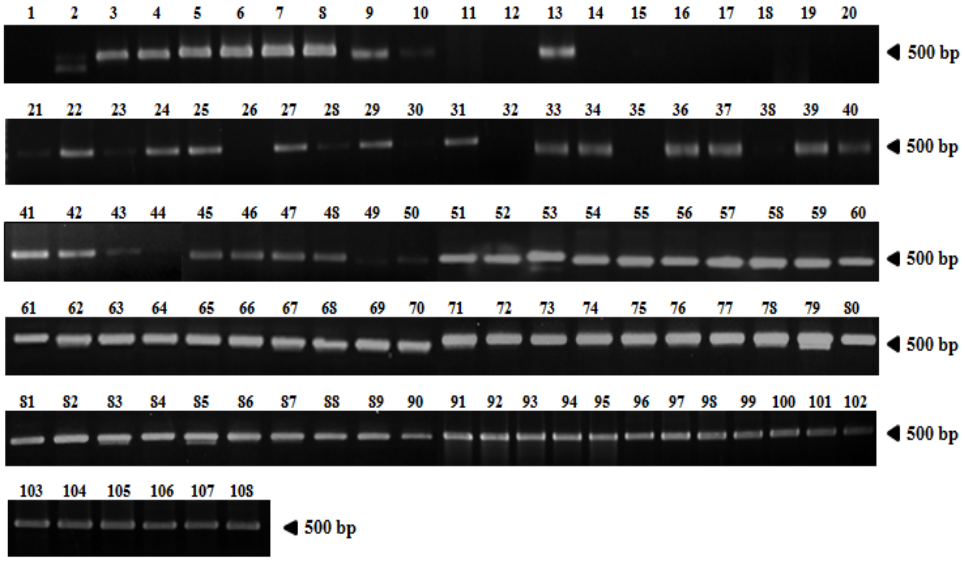


Figure 3.1 PCR amplification of 16S rRNA gene from lactic acid bacteria (LAB) strains isolated from the digestive tract of shrimp *L. vannamei*. The bacterial 16S rRNA gene was amplified from the obtained 108 strains of the LAB isolated from the MRS agar plate. The PCR amplicon of the expected molecular size of approximately 500 bp are indicated. Numbers illustrate individual bacterial isolates.

(A)

(B)

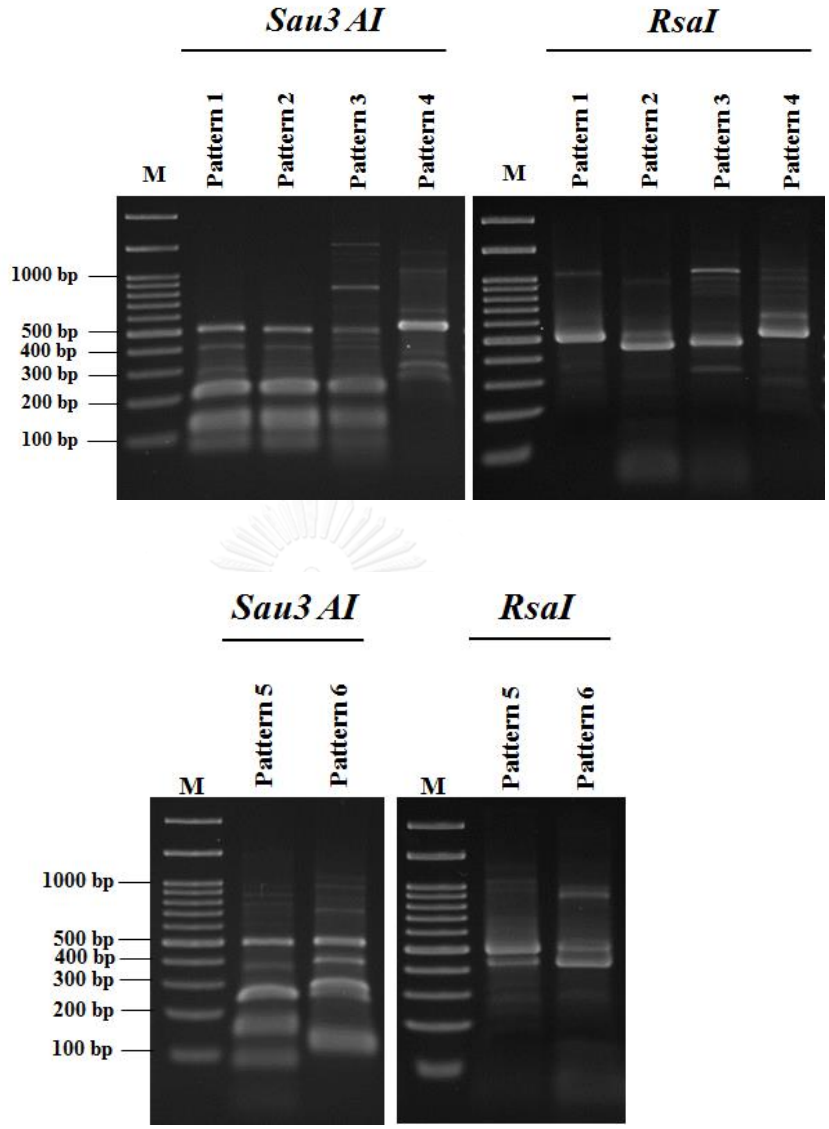


Figure 3.2 Restriction enzyme digestion analysis for the DNA fragment patterns of the 16S rRNA amplicons. The 16S rRNA amplicons (obtained as indicated in Figure 1) were separately digested with two restriction enzymes, *Sau*3AI and *Rsa*I. Each digested products was separated on a 1.5% agarose gel. Lane M indicates DNA marker.

3.1.1 Determination of the antibacterial activity of two LAB isolated from shrimp gut against microorganisms

The *in vitro* antibacterial activity of cell-free culture supernatants of two probiotic candidates, GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*), were tested against seven selected microorganisms composed of four Gram-positive bacteria (*Staphylococcus aureus*, *Aerococcus viridans*, *Bacillus megaterium* and *B. subtilis*) and three Gram-negative bacteria (*Escherichia coli*, shrimp pathogenic bacterial *Vibrio parahaemolyticus* EMS/AHPND and shrimp pathogenic bacterial *V. harveyi*).

As shown in Table 3.1, a strong antimicrobial activity of GutLAB01 cell-free culture supernatant was detected against *A. viridans* (MIC value of 3.125-6.25 µg/ml), followed by a pathogenic bacterium *V. harveyi* (MIC value of 6.25-12.5 µg/ml), and a lower activity was detected against *S. aureus*, *B. megaterium*, *B. subtilis*, *E. coli* and *V. parahaemolyticus* EMS/AHPND (MIC value of 12.5-25 µg/ml) (Table 3.1).

Moreover, GutLAB02 cell-free culture supernatant displayed a strong antimicrobial activity against the Gram-positive bacterium *A. viridans* and Gram-negative pathogenic bacterium *V. parahaemolyticus* EMS/AHPND (MIC value of 12.5–25 µg/ml), while a lower activity was detected against *S. aureus*, *B. megaterium*, *B. subtilis*, *V. harveyi* and *E. coli* (MIC value of 25-50 µg/ml).

For the control probiotics, two bacterial species (*B. megaterium* and *B. subtilis*) isolated from a probiotic product of Department of Fisheries (DOF1) after incubation on LB agar for 24 h at 30 °C and subsequent identification by 16s rRNA analysis. The antimicrobial activities of *B. megaterium* and *B. subtilis* prepared from cell-free culture supernatants were determined. Generally, no activities were detected against *S. aureus*, *B. megaterium*, *B. subtilis*, *E. coli* and pathogenic bacteria *V. harveyi* and *V. parahaemolyticus* EMS/AHPND (MIC value of >50 µg/ml) (Table 3.1). However, the antimicrobial activity of *B. megaterium* cell-free culture supernatant was detected against *A. viridans* (MIC value of 12.5-25 µg/ml).

Table 3.1 Antimicrobial activity of the LAB candidates against microorganisms.

Microorganism	Minimal inhibitory concentration (MIC in µg/ml)			
	Control Probiotics		Candidate Probiotics from shrimp gut	
	B. megaterium	*B. subtilis*	*L. lactis*	*L. plantarum*
Gram-positive bacteria				
Staphylococcus aureus	>50	>50	25-50	12.5-25
Aerococcus viridans	12.5-25	>50	6.25-12.5	3.125-6.25
Bacillus megaterium	–	>50	25-50	12.5-25
Bacillus subtilis	>50	–	25-50	12.5-25
Gram-negative bacteria				
Vibrio parahaemolyticus EMS/AHPND	>50	>50	12.5-25	12.5-25
Vibrio harveyi	>50	>50	12.5-50	6.25-12.5
Escherichia coli	>50	>50	25-50	6.25-25

MIC values are expressed as the interval of concentration [a]–[b], where [a] is the highest concentration tested at which microbial growth can be observed and [b] is the lowest concentration that causes 100% growth inhibition. Experiments were performed in triplicate.

3.2 Effect of probiotics on the immune defense against pathogenic EMS/AHPEN-causing strain of *V. parahaemolyticus* infection

3.2.1 Determination of probiotics in shrimp gut after probiotic administration

In order to test whether orally administered probiotics persist in the shrimp gut, three tissues of guts including foregut, midgut and hindgut at day 5 after administration of probiotic GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*) at the concentration of 2.4×10^8 CFU/g feed for 16 days were aseptically homogenized in PBS buffer. Then the tissue suspensions from three parts of shrimp guts were separately subjected to culture in the MRS medium for bacterial viability assay (Fig. 3.3).

The results (Fig. 3.4) indicated that the count of intestinal LAB in midgut (2.2575×10^4 cfu/ml) of shrimp was significantly ($p<0.05$) higher than that in the foregut (1.142×10^4 cfu/ml) and hindgut (0.2475×10^4 cfu/ml), respectively. In control group, viable bacteria from shrimp gut lysates including foregut, midgut and hindgut were not detected on the MRS agar plates.

To confirm the nucleotide sequences of probiotic strains in shrimp guts that feeding with probiotic GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*), three bacterial isolates of each specimen of shrimp guts were randomly selected and subjected to the PCR analysis using universal primers for the bacterial 16S rRNA gene. Then, the PCR products were subject to DNA sequencing analysis. After Blast N analysis, the 16S rRNA gene sequencing analysis demonstrated that bacterial isolates from guts were members of the LAB and exhibited maximum similarity to 16S rRNA sequence of *Lactobacillus plantarum* and *Lactococcus lactis*. For sequence alignment analysis of 16S rRNA genes isolated from gut of probiotic feeding shrimp, the results show 100% identity to GutLAB01 (*Lactobacillus plantarum*) or GutLAB02 (*Lactococcus lactis*) (Appendix 2). These results suggesting that two probiotics were successfully localized in shrimp guts.

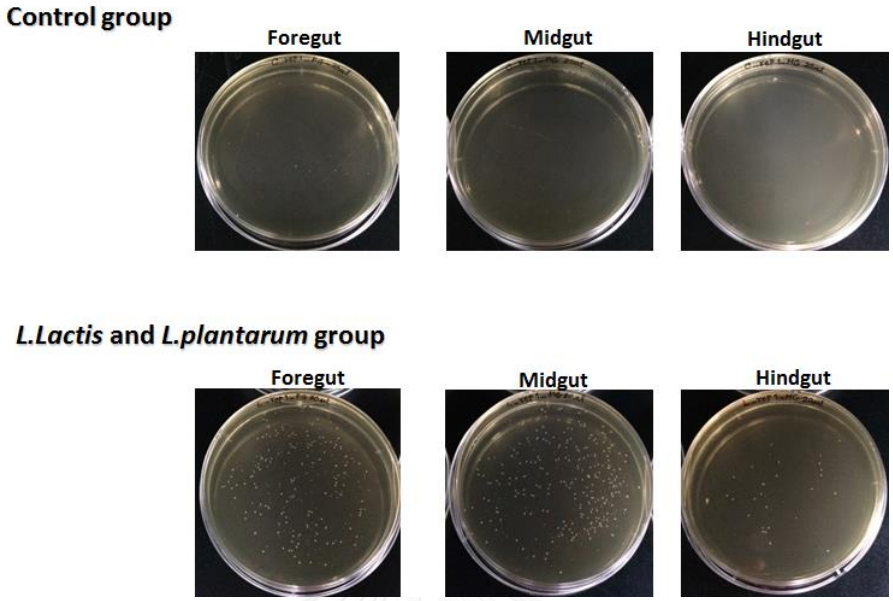


Figure 3.3 Bacterial viability assay of *L. vannamei* gut at day 5 post probiotic administrations. Shrimp were fed with two probiotics GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*) for 16 days. Controls were tissue lysates from control shrimp fed with normal feed without any probiotics. Tissue lysates were prepared from foregut, midgut and hindgut of the probiotic-treated and control shrimp at day 5 post probiotic administrations before being spreaded on the MRS agar plate. The viable probiotic colonies were photographed. Images shown are representative of three independent samples.

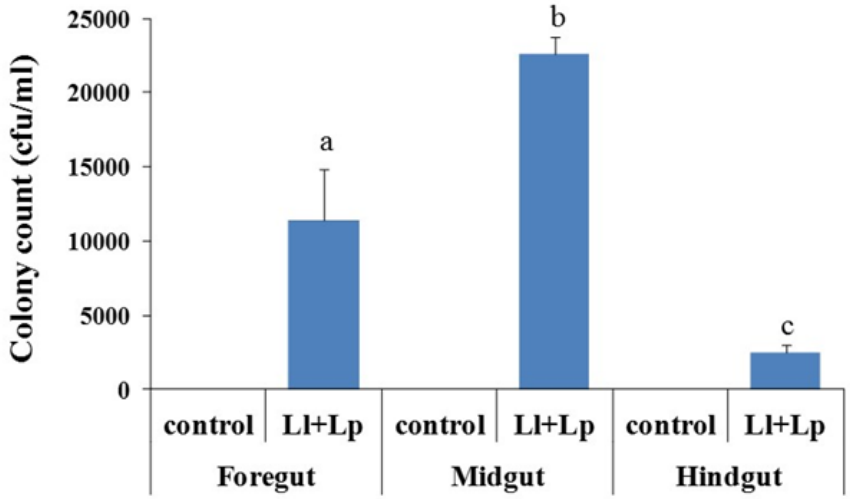


Figure 3.4 Viable colony count from *L. vannamei* gut administered with the lactic acid bacterial probiotics. Shrimp were fed with two probiotics, GutLAB01 and GutLAB02 (Ll + Lp), for 16 days. Controls were bacterial counts from the gut lysates of control shrimp previously fed with normal feed. Tissues of foregut, midgut and hindgut were dissected from the probiotic-treated and control shrimp at day 5 post administration before being spreaded on the MRS agar plate. Viable probiotic colonies detected on the MRS agar plates were then counted. The data are shown as the means ± S.D. (error bars), derived from triplicate experiments. Different letters (above each bar) indicate a significant difference between mean of the samples ($p < 0.05$).

3.3 Effects of probiotics supplemented dietary on the mortality rate of shrimp against pathogenic EMS/AHPEN-causing strain of *V. parahaemolyticus* infection

In order to evaluate the hypothesis that potential probiotic LAB have immunomodulatory effects for prevention of the pathogenic bacterium *V. parahaemolyticus* EMS/AHPND infection, white shrimp *L. vannamei* (1 to 2g) were fed with probiotics-supplemented diet for 16 days before being challenged with 10^4 CFU/ml of *V. parahaemolyticus* EMS/AHPND.

As shown in Figure 3.5, juvenile shrimp fed separately with GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*) at the concentration of 2.4×10^8 CFU/g feed in *each* probiotic displayed a cumulative mortality of 40% and 50% on day 10 post infection, respectively, whereas shrimp fed with combination of GutLAB01 and GutLAB02 at the concentration of 2.4×10^8 CFU/g feed in *each* probiotic showed the lowest cumulative mortality of 36.7% on day 10 post infection.

For control shrimp fed with control probiotics *B. megaterium* and *B. subtilis*, a cumulative mortality of 50% on day 10 post infection was observed whereas the control group fed with normal feed showed the high level of infection of 90% cumulative mortality on day 10 post challenge (Fig. 3.5).

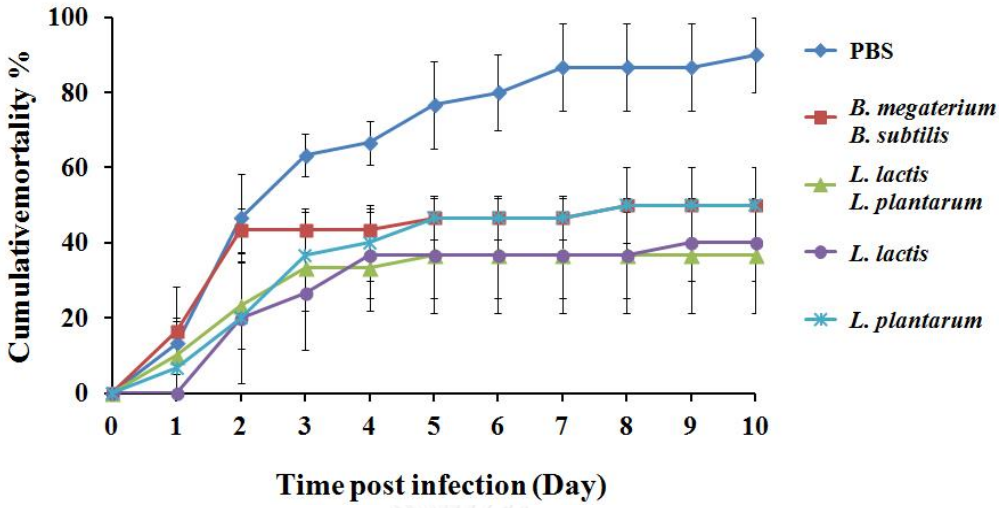


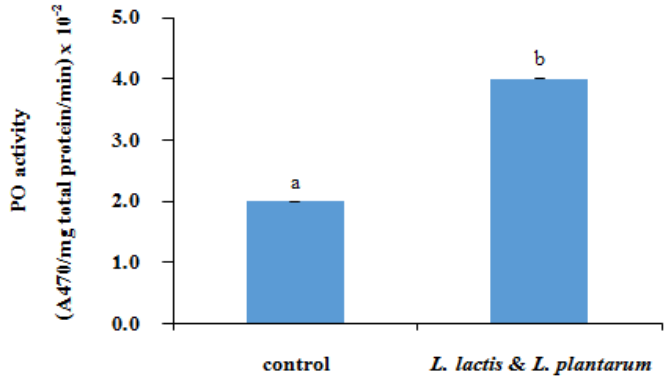
Figure 3.5 Effects of probiotics supplemented diet on the cumulative mortality of shrimp *L. vannamei* challenged with *V. parahaemolyticus* EMS/AHPND. Juvenile shrimp were fed separately with GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*) or fed with the combination of GutLAB01 and GutLAB02 for 20 days prior to challenge with *V. parahaemolyticus* EMS/AHPND. The numbers of dead shrimp from each group were recorded daily until 10 days post infection. Control were shrimp fed with combination of control probiotics (*Bacillus megaterium* + *B. subtilis*) or normal feed for 20 days prior to challenge with *V. parahaemolyticus* EMS/AHPND. The data are shown as the means ± S.D. (error bars). Cumulative mortality rate are derived from three independent experiments.

3.4 Examination of the immune parameter (phenoloxidase (PO) and superoxide dismutase (SOD) *activities* in shrimp fed probiotic-supplemented diet

In order to reveal the shrimp immune enhancement of shrimp received the diet containing probiotic GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*), two important immune parameters, the PO activity and SOD activity, in the hemolymph of the experimental groups of shrimp that fed with GutLAB01 and GutLAB02, on day 16 after feeding were analyzed and compared with the control group of shrimp that received the diet without any probiotic.

The enzymatic activities of the PO and SOD in the probiotic-feeding are shown in Figure 3.6A and 3.6B, respectively. The significant differences in PO and SOD activities were observed in shrimp that received the probiotics GutLAB01 and GutLAB02, compared with the control shrimp. PO and SOD activities of shrimp feeding the probiotics increased about two fold compared with the activities of control shrimp.

(A)



(B)

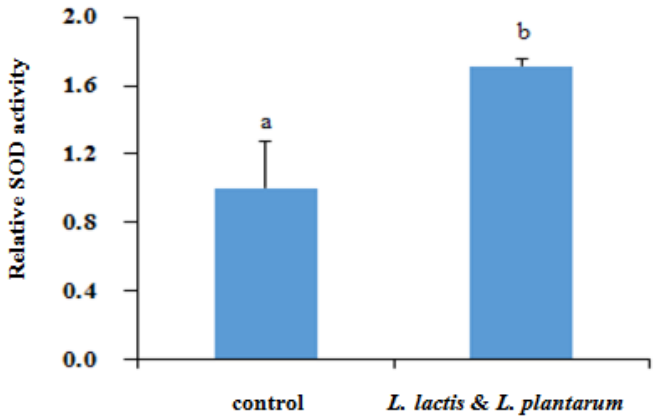


Figure 3.6 Evaluation of the effect of probiotic-supplement on the change of immune parameters in hemolymph of shrimp *L. vannamei*. (A) Phenoloxidase (PO) and (B) superoxide dismutase (SOD) activities in hemolymph of shrimp fed with the diet containing probiotic GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*) for 16 days. Control group were shrimp fed with the diet without any probiotic. Hemolymph was collected from the experimental and control shrimp at day 16 post probiotic administrations. PO and SOD activities were then assayed using L-DOPA and NBT, as substrates, respectively. The data from three independently replicated experiments are shown as the means ± S.D. (error bars). Different letters (above each bar) indicate a significant difference between mean of the samples ($p < 0.05$).

3.5 Effects of probiotc supplemented dietary on the immune-related gene expression in shrimp

3.5.1 Selection of immune-related genes in shrimp

Based on the major immune system of shrimp, two major groups of immune genes including the proPO system (*Lv*proPO1, *Lv*proPO2 and *Lv*Pacifastin-like) and antimicrobial peptides (*Lv*ALF and *Lv*crustin-like), were selected for evaluation of the gene expression in shrimp *L. vannamei*.

Previously, several proPO system-related genes and antimicrobial peptides from shrimp *L. vannamei* have been identified and functionally characterized (Amparyup et al., 2013). Two key enzymes, *Lv*proPO1 (GenBank accession number: EU284136) and *Lv*proPO2 (EU373096) in shrimp proPO system and two major genes, *Lv*ALF (DQ208702) and *Lvcrustin* (FE049920) of shrimp AMPs were found to be important for the immune defense in shrimp. However, to date, no information of a *key* negative regulator, pacifastin of the shrimp proPO has been provided. In crustaceans, Pacifastin have been reported from the crayfish *Pacifastacus leniusculus* (Liang et al., 1997) and the crabs *Eriocheir sinensis* (Gai et al., 2008) and *Portunus trituberculatus* (Liu et al., 2015c ; Wang et al., 2012). Pacifastin is an important proteinase inhibitor in the proPO system that composed of two different protein subunits (pacifastin-inhibitor light chain domain and the transferrin-like heavy chain domain (Liang et al., 1997).

3.5.2 Cloning of Pacifastin-like gene of shrimp *L. vannamei*

In this thesis, to obtain the full-length cDNA of the Pacifastin-like gene of shrimp *L. vannamei*, gene specific primers (*Pm*Pacifastin-like-ORF-F and *Pm*Pacifastin-like-ORF-R) was used for PCR amplification using cDNA from hemocytes and midgut of *L. vannamei* as templates. The PCR products (approximately 2200 bp) (Fig. 3.7) were cloned and sequenced on both strands to obtain the complete *Lv*Pacifastin-like sequence.

After BLAST analysis, the results found that *Lv*Pacifastin-like mRNAs were successfully cloned and showed high homology to the pacifastin light chain of shrimp *P. monodon* (89% similarity) and crayfish *Pacifastacus leniusculus* (48% similarity). The full-length cDNA of Pacifastin-like in shrimp hemocyte (*Lv*Pacifastin-likeHC) and midgut (*Lv*Pacifastin-likeMG) was composed of an open reading frame (ORF) of 2214 bp that encoded 737 deduced amino acid residues. The mature protein (721 amino acids) without the signal peptide (1-19 amino acids) had an estimated molecular weight of approximately 78.3 kDa and an isoelectric point of 5.5. SMART analysis of the *LV*Pacifastin-like protein revealed thirteen pacifastin light chain domains (PLDs) *located at N-terminus* and two kunitz domains *located at C-terminus* of protein (Fig 3.8).

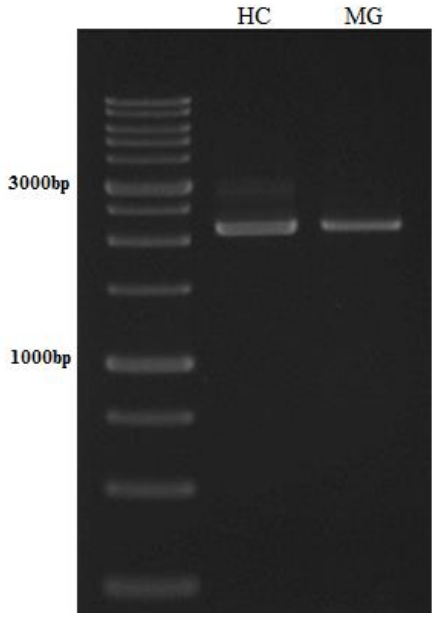


Figure 3.7 PCR amplification of the full-length cDNA of the Pacifastin-like gene from shrimp *L. vannamei*. *Lv*Pacifastin-like gene was amplified by PCR using cDNA from hemocytes (HC) and midgut (MG) of *L. vannamei* as template. The PCR amplicon of approximately 2200 bp of the *Lv*Pacifastin-like are indicated. Lane M indicates DNA marker.

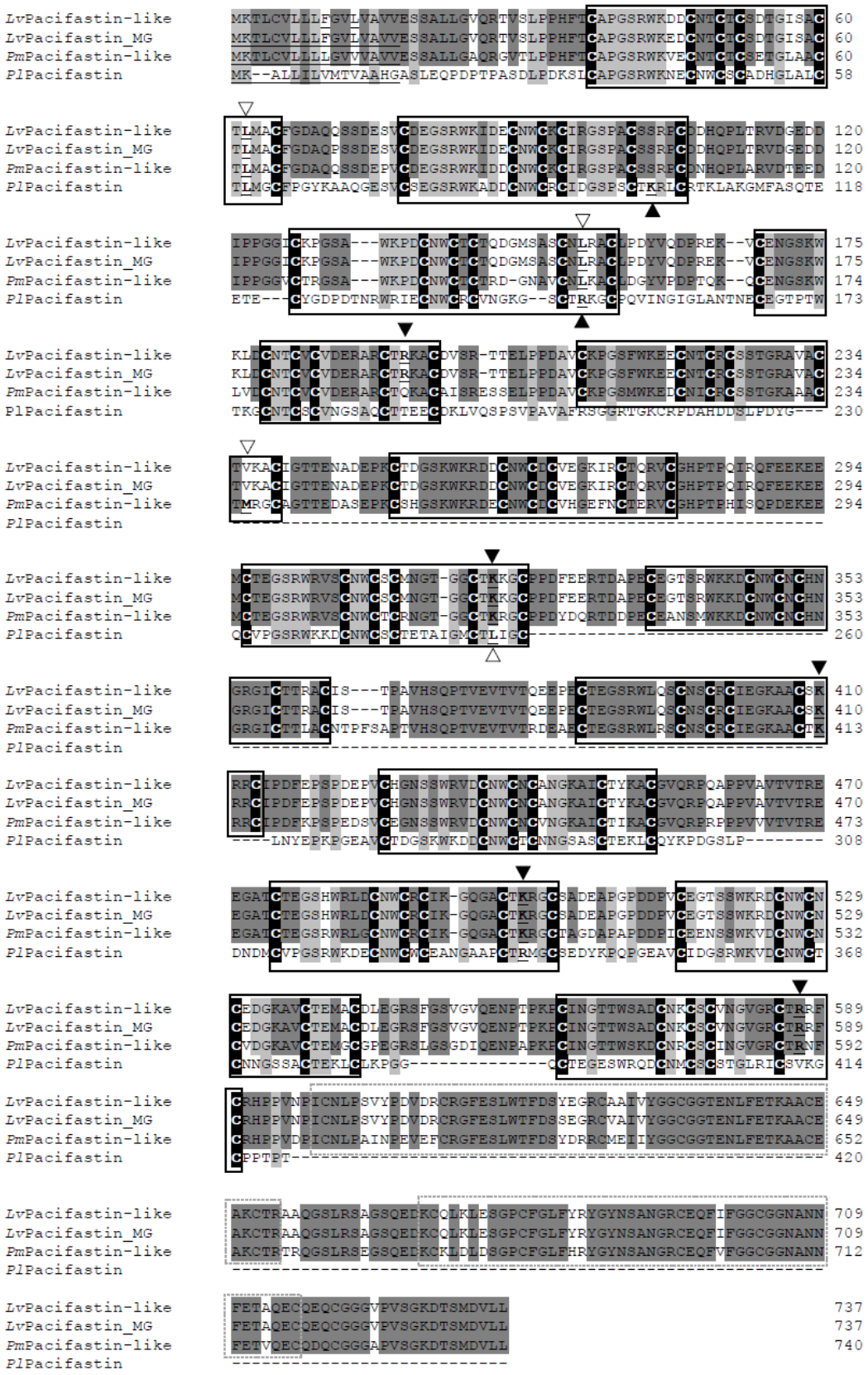

LvPacifastin-like
LvPacifastin_MG
PmPacifastin-like
PlPacifastin

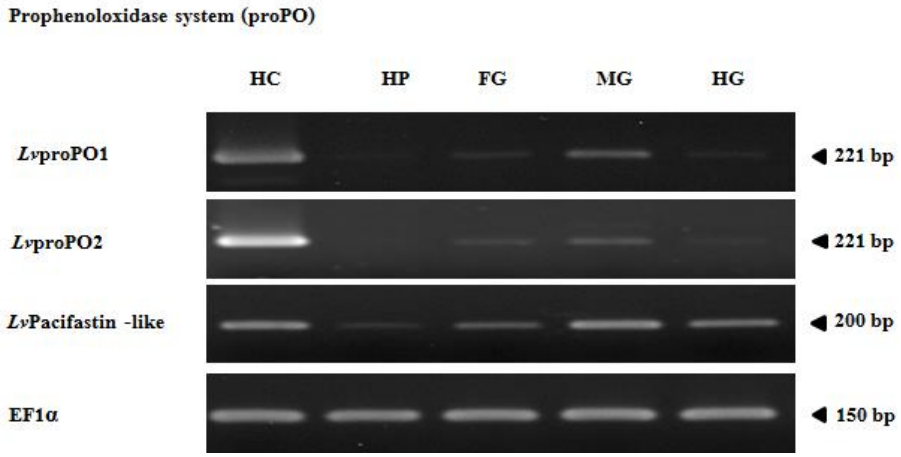
Figure 3.8 Multiple amino acid sequence alignment of Pacifastin-like from hemocyte (*Lv*Pacifastin-like) and midgut (*Lv*Pacifastin_MG) of *L. vannamei* with *P. monodon* *Pm*Pacifastin-like and *Pacifastacus leniusculus Pl*Pacifastin. Numbers of the deduced amino acids are indicated on the right margin. The thirteen conserved pacifastin light chain domains (PLDs) are boxed, whereas the conserved cysteine are marked by black highlight. The P1 residues in PLDs are in bold and underlined. The enzyme specificity regarding to the P1 position for trypsin and chymotrypsin are indicated by the black and white arrowheads, respectively. Two kunitz domains at C-terminus are indicated by dashed boxes. The putative signal peptides are underlined. Conserved amino acids between *L. vannamei* and *P. monodon* are shaded by dark grey color. Identical amino acid residues among three species are shaded by grey color.

3.5.3 Tissue distribution analysis of the shrimp immune-related genes

To assess the gene expression of immune-related gens in shrimp tissues, five tissue types including hemocyte, hepatopancrease, foregut, midgut and hindgut were collected from white shrimp. After RNA extraction and cDNA synthesis, cDNA of three shrimp were pooled for each tissue types.

In this study, three proPO-related genes (*Lv*proPO1, *LvproPO2* and *LvPacifastin*-like) and two antimicrobial peptides (*Lv*ALF and *Lvcrustin*-like), were selected for gene expression analysis. The results of tissue distribution indicated that the expression levels of genes in the shrimp proPO system were the highest in the hemocyte, followed by a moderate level in the midgut, and a relatively low level in the foregut, hindgut and hepatopancreas, respectively (Fig. 3.9A). For the group of genes in shrimp antimicrobial peptides, the results indicated that the transcripts were expressed in a wide range of shrimp tissues, with the highest expression levels in the shrimp hemocyte (Fig.3.9 B).

(A)



(B)

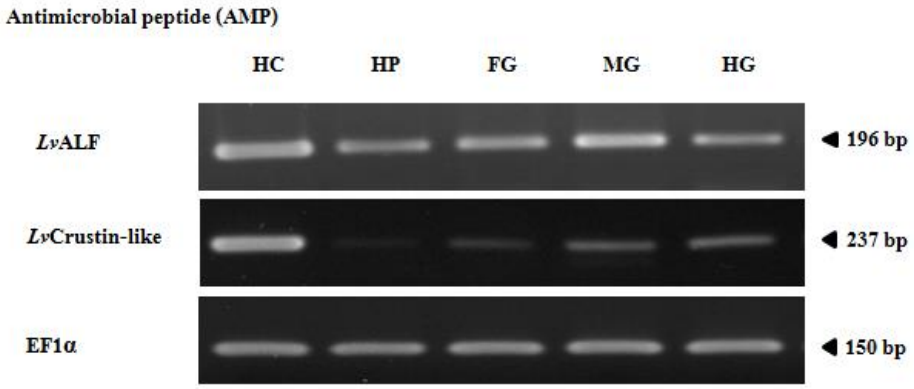


Figure 3.9 Tissue distribution analysis of genes associated with the (A) proPO system (*Lv*proPO1, *Lv*proPO2 and *Lv*Pacifastin-like) and antimicrobial peptides (*Lv*ALF and *Lv*crustin-like) by semi-quantitative RT-PCR. Total RNA was extracted from the indicated five tissues of *L. vannamei* including hemocytes (HC), hepatopancreas (HP), foregut (FG), midgut (MG) and hindgut (HG) and RT-PCR amplification was then performed. EF1-α was amplified and used as an internal control. The expected molecular sizes of each PCR amplicon are indicated. The experiment was performed three times and results shown are representative of three independent experiments.

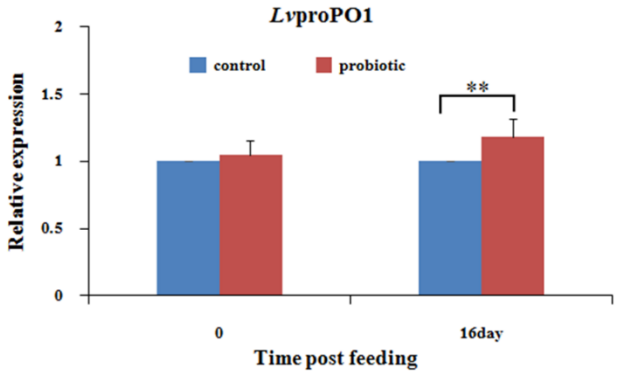
3.5.4 Expression profiles of the shrimp immune-related genes in shrimp guts using the semi-quantitative RT-PCR

Based on tissue distribution analysis of immune-related genes in gut tissues of shrimp, midgut tissue was selected for the expression profiling experiments. Shrimp were divided into two groups, each *group* has *three* replicates with ten shrimp in each *replicate.* Each group of shrimp were fed with GutLAB01 and GutLAB02 for 16 days.

Preliminary expression profile of immune-related genes in midgut tissues were determined by the semi-quantitative RT-PCR. The results indicated that the expression levels of *Lv*proPO1 and *Lv*proPO2 transcripts in the shrimp midgut were significantly up-regulated ($P < 0.05$) in shrimp fed on probiotic-supplemented diets for 16 days compared to the control group (Figure 3.10A and 3.10B).

However, there was no significant difference of the gene expression of *Lv*Pacifastin-like and antimicrobial peptides (*Lv*ALF and *Lv*crustin-like) in shrimp midgut compared to the control treatment (Figure 3.10C, 3.11A and 3.11B, respectively). Results suggested that probiotic GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*) strains may modulate the proPO-related genes in the shrimp midgut. Thus, the genes in shrimp proPO system were selected for the further study to determine the effects of *Lactobacillus* probiotics on gene expression in midgut of shrimp using the real-time RT-PCR analysis.

(A)



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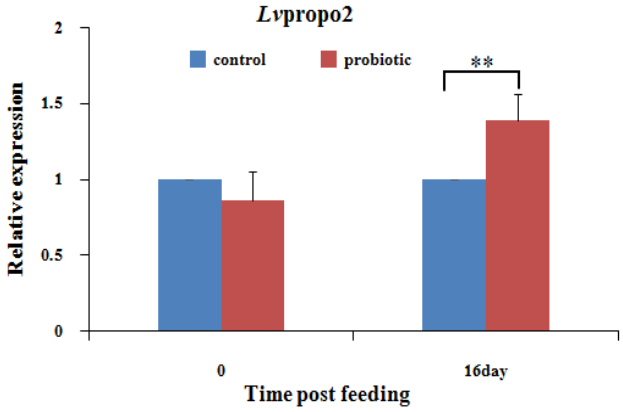


(C)

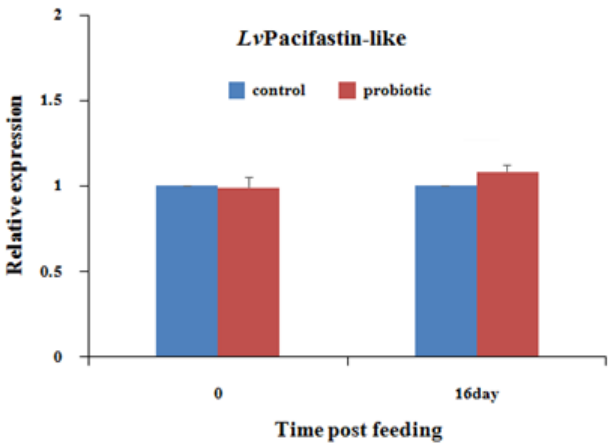
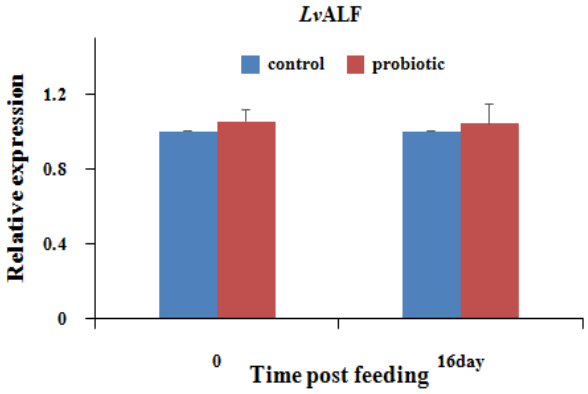


Figure 3.10 Effect of probiotic feeding on the expression of three proPO system-related genes including (A) *Lv*proPO1, (B) *Lv*proPO2, and (C) *Lv*Pacifastin-like, in midgut of shrimp *L. vannamei*. The relative mRNA expressions of three proPO system-related genes in gut of shrimp was determined at day 0 and 16 post probiotic feeding by semi-quantitative RT-PCR using EF1-α as an internal control. Control group were shrimp fed with the normal diet *without* any *probiotic supplement.* The data are from three independently replicated experiments and shown as the means ± S.D. (error bars). Asterisk indicates a statistical significant difference between means (**p < 0.05).

(A)



(B)

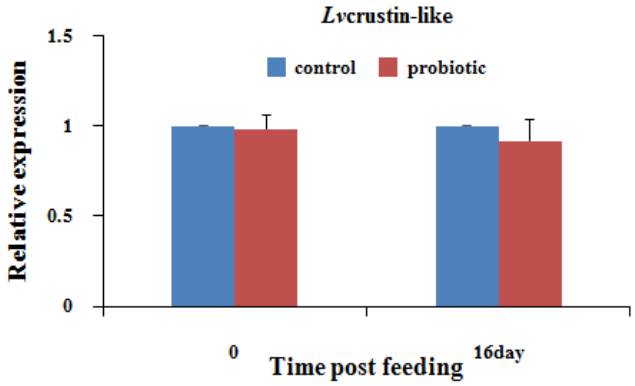


Figure 3.11 Effect of probiotic feeding on the expression of two antimicrobial peptides genes including (A) *Lv*ALF, and (B) *Lv*crustin-like, in midgut of shrimp *L. vannamei*. The relative mRNA expressions of two antimicrobial peptides genes in gut of shrimp was determined at day 0 and 16 post probiotic feeding by semi-quantitative RT-PCR using EF1-α as an internal control. Control group were shrimp fed with the normal diet without any probiotic supplement. The data are from three independently replicated experiments and shown as the means ± S.D. (error bars).

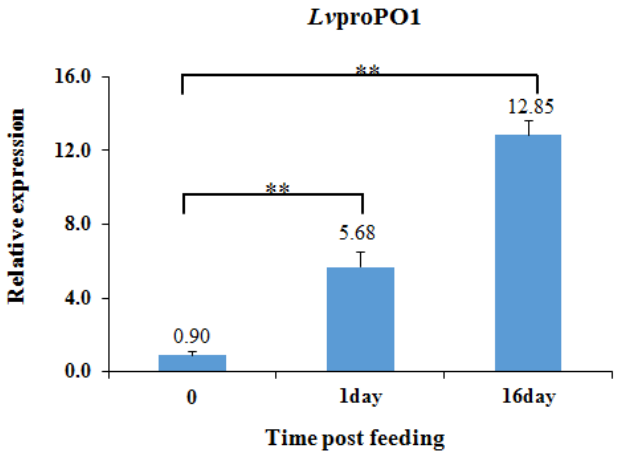
3.5.5 Expression profiles of the shrimp proPO-related genes in shrimp guts using the real-time PCR analysis

After probiotic feeding, the total RNA of the mid gut of shrimp was isolated from probiotic-treated and control shrimp at day 0, day 1, and day 16. Then, the pooled cDNA of three individual shrimp at each time point were prepared.

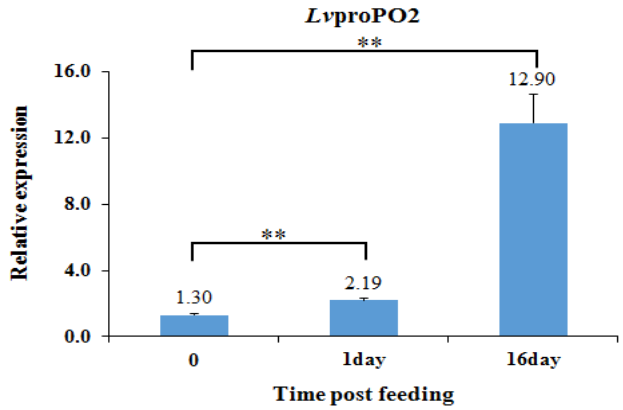
As shown in Figure 3.12, the mRNA expression level of *Lv*proPO1 and *Lv*proPO2 of shrimp fed with probiotic containing diets, GutLAB01 (*Lactobacillus plantarum*) and GutLAB02 (*Lactococcus lactis*), were found to be increased significantly. However, the gene expression of *Lv*Pacifastin-like in midgut was slightly increased after probiotic *L. lactis* and *L. plantarum* feeding (Figure 3.12C).

For the *Lv*proPO1, the mRNA expression in midgut dramatically increased by 6.3-fold at day 1 and 14.3-fold at day 16, as compared with day 0 post feeding (Figure 3.12A). Likewise, the mRNA expression of *Lv*proPO2 in midgut also shown to be dramatically increased by 9.9-fold at day 16, as compared with day 0 post feeding (Figure 3.12B). Thus, *Lv*proPO1 and *Lv*proPO2 expression levels in midgut are transiently significantly up-regulated after probiotic *L. lactis* and *L. plantarum* feeding.

(A)



(B)



(C)

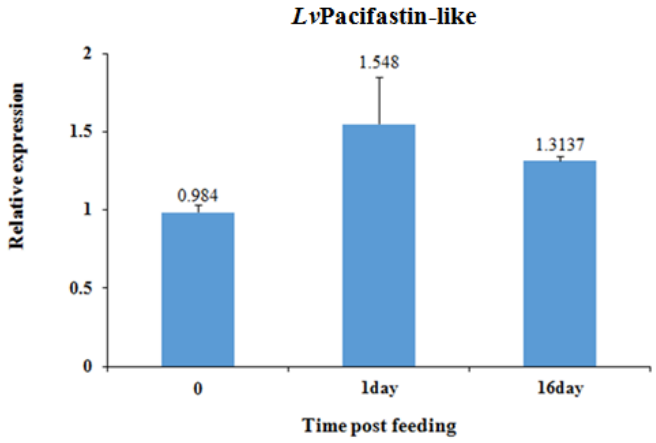


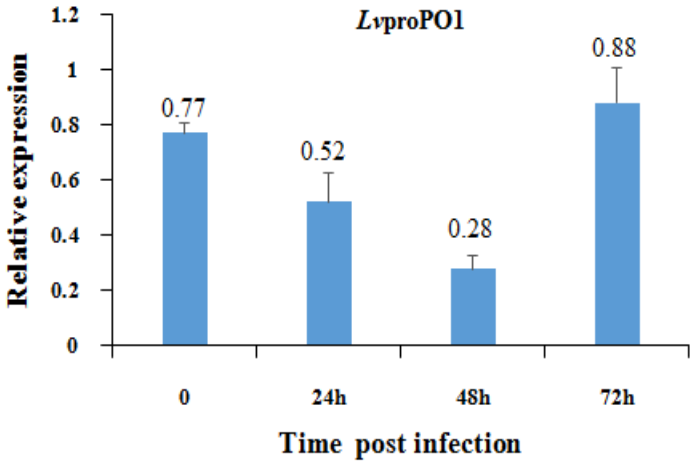
Figure 3.12 Time-course analysis of the effect of probiotic feeding on three proPO system-related genes including (A) *Lv*proPO1, (B) *Lv*proPO2, and (C) *Lv*Pacifastin-like, in midgut or shrimp *L. vannamei*. Total RNA was isolated from the midgut of the probiotic-treated and control shrimp at day 0, 1, and 16 post feeding. Control group were shrimp fed with the normal diet without any probiotic supplement. The relative expressions of *Lv*proPO1, *Lv*proPO2, and *Lv*Pacifastin-like transcripts were evaluated by SYBR Green real-time PCR. Relative expression levels of mRNA were calculated according to Pfaffl (2001) using EF1α as the internal reference gene. The average relative expressions are representative of three independent repeats±S.D. (error bars).

3.6 Effects of EMS/AHPEN-causing strain of *V. parahaemolyticus* infection on immune-related gene expression in shrimp gut

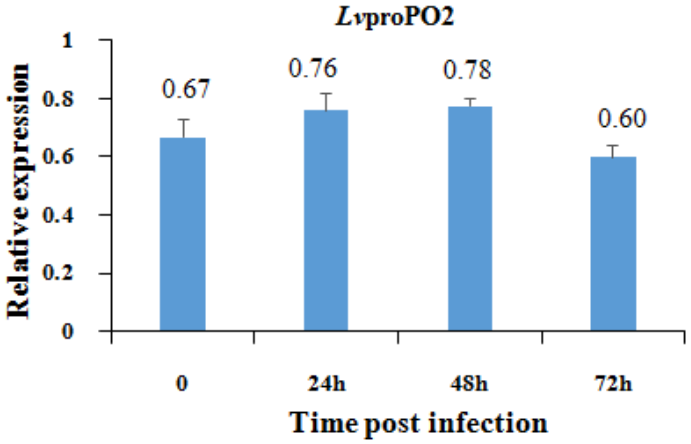
To examine whether *Lv*proPO1, LvproPO2 and *Lv*Pacifastin-like genes are involved in *Vibrio parahaemolyticus EMS/AHPND* infection, cDNAs of midgut of shrimp collected 0 h, 24 h, 48h and 72h after infection were subjected to real-time PCR.

The results showed that *Lv*proPO1 transcripts in midgut significantly decreased by 0.7-fold at 24h, 0.4-fold at 48h post-bacterial challenge, and then returned to non-stimulated levels at 72 h (Fig. 3.13A). However, no significant change in the *Lv*proPO2 transcription levels in midgut was evident in the bacterial injected shrimp, compared with the control group (Fig. 3.13B). For *Lv*Pacifastin-like gene expression in midgut, by 24 to 72 h post-challenge, *Lv*Pacifastin-like mRNA were dramatically decreased by 0.2 to 0.6 fold to below control levels (Fig. 3.13C).

(A)


LvproPO1
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
0.77
0.52
0.28
0.88
0
24h
48h
72h
Time post infection

(B)


LvproPO2
Relative expression
1
0.8
0.6
0.4
0.2
0
0.67
0.76
0.78
0.60
0
24h
48h
72h
Time post infection

(C)

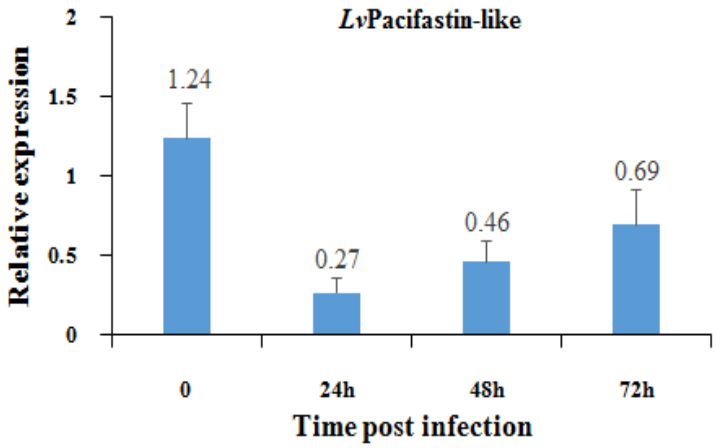


Figure 3.13 Time-course analysis of the effect of *Vibrio parahaemolyticus* EMS/AHPND infection on three proPO system-related genes, (A) *Lv*proPO1, (B) *Lv*proPO2, and (C) *Lv*Pacifastin-like, in midgut or shrimp *L. vannamei*. Total RNA was isolated from the midgut of the *V. parahaemolyticus* EMS/AHPND infected- and control- shrimp at 0, 24, 48 and 72h post infection. The relative expressions of *Lv*proPO1, *Lv*proPO2, and *Lv*Pacifastin-like transcripts were evaluated by SYBR Green real-time PCR. Relative expression levels of mRNA were calculated according to Pfaffl (2001) using EF1α as the internal reference gene. The average relative expressions are representative of three independent repeats $\pm$ S.D. (error bars)

CHAPTER IV
DISCUSSION

Probiotics that isolated and applied within the same organisms is extremely crucial because they have already adhered to the gut wall and, thus, are well-adapted to grow and also to compete with pathogens (Ghosh et al., 2007).

To date, Lactic acid bacteria (LAB) are traditionally used for probiotics in humans and animals, that beneficial for human and animal health by activating innate immunity (Ichikawa et al., 2012; Kawashima et al., 2013, Ouwehand et al., 2002; Irianto and Austin ., 2002). LAB are Gram-positive, catalase-negative, form no spores, and are immotile. However, research on the LAB probiotic effects in shrimp infection models are limited.

Isolation of LAB from shrimp gut with high antimicrobial activity

The purpose of this study is to find the novel probiotic to promote the shrimp health and to prevent EMS disease by screening LAB from shrimp gut. In this work, 100 clones of isolated LAB from digestive tract of shrimp *L. vannamei* were screened by PCR using universal primers for bacteria 16S rDNA. The protective effect of probiotic against pathogen and innate-immunity stimulating activity of LAB in shrimp were evaluated. The results indicated that most isolates in shrimp gut obtained were *Lactococcus*, *Lactobacillus*, *Vagococcus* and the non-LAB *Staphylococcus*. This result is in accordance with the previous studies, which indicated that the very common and prevalent LAB found in the digestive tract of aquatic animals is coccoid LAB (Ringø and Gatesoupe, 1998; Ringø, 2004).

Previously, (Kongnum and Hongpattarakere, 2012) reported the isolation of *Lactobacillus plantarum* from the digestive tract of wild shrimp *Litopenaeus vannamei* and also showed that *L. plantarum* administration of shrimp through feeding significantly increased survival rate against *V. harveyi* infection (Kongnum and Hongpattarakere, 2012). Moreover, Maeda et al. (2014) reported that *Lactococcus lactis* -containing diet-fed shrimps, which isolated from kuruma shrimp (*Marsupenaeus*

japonicus) intestine, displayed significantly increased survival rate post *V. penaeicida* infection and also significant up-regulation of lysozyme transcript in the intestine and hepatopancreas of kuruma shrimp (Maeda et al., 2014). However, mode of action of probiotics is still limited and should be further studied. Similarly, in this study, two LAB (*Lactobacillus plantarum* and *Lactococcus lactis*) were found in white shrimp intestine, suggesting these LAB are candidate probiotics that co-locatized in shrimp gut. Therefore, these probiotics were selected for further analysis

Lactobacillus and *Lactococcus* have been reported to produce bacteriocins, which are bacterial antimicrobial peptides and served as antibacterial agents to eliminate growth of competing microbes or pathogens (Klaenhammer., 1988). In the present study, the antimicrobial activity of two LAB isolates (*L. plantarum* and *L. lactis*), were examined *in vitro*. Two candidate probiotic bacteria, *L. plantarum* and *L. lactis* displayed a strong antimicrobial activity against Gram-positive bacterium *A. viridians* and Gram-negative bacterium EMS/AHPEN-causing strain of *V. parahaemolyticus*. This data is in agreement with the previously reported which shown that *L. plantarum* completely inhibited Gram-negative bacterium *V. harveyi* (Kongnum and Hongpattarakere, 2012), while *L. lactis* exhibited bactericidal activity against the tested gram-negative bacterium *V. penaeicida* (Maeda et al., 2014). Although two probiotics exhibit antimicrobial activity, bacteriocins of these probiotics are still not known. Further study on the antimicrobial activity of these bacteriocins from *L. plantarum* and *L. lactis* shoud be understanding the mechanisms of action from the bacteriocins.

Shrimp acquired tolerance to bacterial infection by ingesting *L. plantarum* and *L. lactis*

For probiotics application in aquaculture, feed-incorporated probiotic can improve resistant to pathogens (Verschuere et al., 2000). Here, the potential role of probiotics, *L. plantarum* and *L. lactis*, in shrimp defense response were also tested against pathogenic bacterium *V. parahaemolyticus* EMS/AHPND, a specific virulence strain of *V. parahaemolyticus* that is recently identified as a causative agent of the early mortality syndrome (EMS), which later known as an acute hepatopancreatic necrosis disease (AHPND), that causes severe losses of shrimp production and usually

occurs within approximately 35 days after stocking shrimp post-larvae in shrimp ponds (Joshi et al., 2014; Tran et al., 2013).

Prior to the study of the effects of two LAB on the shrimp defense responses, the toxicity of these probiotic strains were evaluated. It was observed that *L. plantarum* and *L. lactis* were non-pathogenic to shrimp since no shrimp died after the feeding of bacterial inoculum at concentration of 10^7cells/shrimp.

For feeding experiments, the result indicated that the shrimp fed with two LAB (GutLAB01 *Lactobacillus plantarum* and GutLAB02 *Lactococcus lactis*) exhibited tolerance to the lethality of *V. parahaemolyticus* EMS/AHPND infections as compared with the control diet-fed group. In Kuruma shrimp *M. japonicus*, a significantly increased resistance to the *V. penaeicida* infection was observed in the shrimp fed with the probiotic-supplemented diet when compared with the control group (Maeda et al., 2014). The obtained result thus suggests that activation of the shrimp innate immune response by ingesting two LAB induces tolerance against microbial infection.

Innate-immunity activation by LAB in shrimp

Previously, LAB were reported to have high immunity stimulating activity in human and animal (Ichikawa et al., 2012; Kawashima et al., 2013). Moreover, multiple studies demonstrate the ability of probiotics on innate immune activation in several species of crustaceans (Panigrahi et al., 2004; Gullian et al., 2004). However, the activity of LAB in stimulating innate immunity in shrimp gut is still limited. In the current study, the results observed significant improvement of PO and SOD activity when shrimp fed with dietary supplemental two LAB, indicating increase in shrimp immunity. This finding is similar to some previous reports which observed significant improvement of PO and SOD when animals are fed with dietary supplemental probiotics (Chiu et al., 2007).

The prophenoloxidase (proPO) activation system plays an important role in the invertebrate immune system in allowing a rapid response to pathogen infection, in which PO is a key enzyme (Amparyup et al., 2013). Superoxide dismutases (SOD) that is responsible for scavenging superoxide radicals and is involved in protective

mechanisms in tissue injury following oxidative process and phagocytosis, is the major antioxidant defense system in shrimp.

To examine the gene expression in shrimp gut using semi-quantitative RT-PCR, the higher expression of *Lv*proPO1 and *Lv*proPO2 genes were observed in the probiotic diet group while the relative expression of antimicrobial peptide (*Lv*ALF and *Lv*Crustin-like) gene of shrimp between control and probiotic-treated groups were not statistically different. These results suggested that dietary probiotic did not show positive influence on expression of antimicrobial peptide genes, which was contrary to some previous studies which observed significant increase of gene expression.

To confirm the gene expression level of proPO genes, the effect of probiotic supplemented on the expression of two proPO genes and a pacifastin-like in shrimp were evaluated using real-time PCR analysis. *L. plantarum -L. lactis* containing diet-fed displayed a significant up-regulation of *Lv*proPO gene expression in midgut after feeding for 16 days. This data is corresponded with the expression of prophenoloxidase gene in Kuruma shrimp which was shown to increase significantly in the intestine after probiotic feeding (Maeda et al., 2014).

This study indicates that the two LAB activates the shrimp immune system, which may allow shrimp to combat a lethal infection by *V. parahaemolyticus* EMS/AHPND. This effect is mediated by an increase of the PO and SOD activities and the transcriptional level of *Lv*proPO1 and *Lv*proPO2. This study also demonstrate that two LAB are suitable probiotic for shrimp health improvement.

Previously, several reports showed the effectiveness of probiotics in preventing the disease and in activation of host immune responses and is dependent on several factors such as strain-specific differences of probiotics and the dosage of probiotics. (Kandasamy et al., 2017). Thus, comparative analysis of the host-probiotic interaction of different probiotics is essential for future probiotic application in shrimp disease control.

Generally, the expression levels of the proPO genes were differentially expressed when shrimp was challenged with pathogenic microbes, suggested that the

proPO system plays an important role in immunity (Suwansonthichai et al., 2003). In this study, similar and strong down-regulation of *Lv*proPO1 mRNA level was observed in the midgut of shrimp infected with *V. parahaemolyticus* EMS/AHPND infections compared to the control group. This finding suggested that *L. plantarum* and *L. lactis* probiotic supplements could enhance the shrimp immune response for protection of pathogenic *V. parahaemolyticus* bacterial infections through stimulation of the proPO system.

Results of this research showed that shrimp fed with LAB probiotic exhibit the increasing of PO activity and also proPO mRNA expression. The enhancement mechanisms of gut immune functions by probiotic in fish are well documented (Balcazar et al., 2007), but immune mechanism of host-probiotic interaction has been less studied in crustacean including shrimp. In teleost fishes, probiotics can positively activate several immune parameters (monocytes, macrophages and neutrophils) and stimulate the elevation of immunoglobulin level (Panigrahi et al., 2004). Although the host-probiotic mechanism to stimulate the immune system of shrimp is not well clear, it has been reported that shrimp fed with the probiotic exhibited significant increase in PO activity (Chiu et al., 2007;Yeh et al., 2014; Tseng et al. 2009). One possible mechanism in activation of shrimp proPO system in the present study is that peptidoglycans of probiotic LAB stimulate the shrimp immune response including proPO system.

Although proPO system/melanization plays a major role in the immune defense of hemocyte in invertebrates, several studies have reported positive role of melanization in regulation of bacterial pathogens and detoxification in gut system (Shao et al., 2012; Wu et al., 2015). Additionally, to preliminary check whether there is phenoloxidase activity in shrimp guts of shrimp, guts were stained using the dopamine as a substrate with or without addition of phenylthiourea (PTU), a strong PO activity inhibitor. The results indicated that the shrimp gut was stained black using dopamine substrate. When PTU was added, melanization of the shrimp gut was clearly inhibited (Appendix 3). Taken together, this observation demonstrates that gut content melanization is a result of PO activity, and accordingly that PO might be present in the

shrimp gut. However, the mechanism and interaction of melanization and probiotic in shrimp gut needs to be further elucidated.

In summary, lactic acid bacteria (LAB), *Lactobacillus plantarum* and *Lactococcus lactis* were successfully isolated from gut of shrimp *L. vannamei*. Moreover, two candidate probiotic bacteria show strong antimicrobial properties against Gram-positive bacteria and Gram-negative bacteria, including pathogenic bacterium *V. parahaemolyticus* EMS/AHPND. In addition, supplementation of two probiotics into diet improved the resistance of *L. vannamei* against the *V. parahaemolyticus* EMS/AHPND infection by induction of shrimp innate immunity as observed by increasing of as well as higher immune parameters (PO and SOD activities) and also transcript levels of proPO mRNA compared to the control group. These findings suggest that *Lactobacillus plantarum* and *Lactococcus lactis* could serve as a potential probiotic for shrimp aquaculture.

CHAPTER V
CONCLUSION

1. Two LAB (*Lactobacillus plantarum* and *Lactococcus lactis*) were successfully isolated and identified from digestive tract of the white shrimp *L. vannamei*.

2. *In vitro* and *in vivo* antimicrobial analyses revealed that *L. lactis* and *L. plantarum* are active against the microbial pathogens especially the Gram-negative marine bacterium *V. parahaemolyticus* that caused EMS/AHPND in shrimp.

3. Administer of feed-supplemented with *L. lactis* and *L. plantarum* could enhance shrimp immune response as indicated by the enzymatic activities of PO and SOD and also the relative mRNA expression level of shrimp proPO genes.

4. The full length cDNAs of *Lv*Pacifastin-like from shrimp hemocyte and midgut were successfully identified and contains an open reading frame (ORF) of 2,214 bp encoding a predicted protein of 737 amino acids including thirteen pacifastin light chain domains (PLDs) located at N-terminus and two kunitz domains located at C-terminus of protein.

5. Gene expression analysis of the proPO-related genes in shrimp midgut after *V. parahaemolyticus* that caused EMS/AHPND infection indicated that *Lv*proPO1 transcripts, but not *Lv*proPO2 significantly decreased at 24 to 48h, and then returned to non-stimulated levels at 72 h. However, gene expression of *Lv*Pacifastin-like mRNA was dramatically decreased after infection.

6. Our data demonstrates that *L. lactis* and *L. plantarum* could be the effective probiotics with a great potential to control and prevent shrimp from microbial pathogens.

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APPENDIX

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GutLAB02                        GGAAACGAATGCTAATACCGCATAATAACTTTAAACATAAGTTTTAAGTTTGAA--AGAT
LactococcusLactisLC129537       GGAAACGAATGCTAATACCGCATAATAACTTTAAACATAAGTTTTAAGTTTGAA--AGAT
GutLAB01                        GGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGG
LactobacillusPlantarumKU892396  GGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGG
GutLAB04                        GGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATGGTTCAAAAGTGAAA-GACG
StaphylococcusAureusAP017377    GGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATGGTTCAAAAGTGAAA-GACG
GutLAB03                        GGAAACAGGTGCTAATACCGCATAATTTGTTTTTCCGCATGGAAGAATAATAAA--AGAC
VagococcusCarniphilusKT728717   GGAAACAGGTGCTAATACCGCATAATTTGTTTTTCCGCATGGAAGAATAATAAA--AGAC
                                ******    ********** ****    **    *  * *     *   * **

GutLAB02                        GCAATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCT
LactococcusLactisLC129537       GCAATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCT
GutLAB01                        CTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGAGGTAACGGCT
LactobacillusPlantarumKU892396  CTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGAGGTAACGGCT
GutLAB04                        GTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCT
StaphylococcusAureusAP017377    GTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCT
GutLAB03                        GCTTCGGTGTCACTGTTGGATGGGCCCGCGCTGCATTAGTTAGTTGGTGGGGTAACGGCC
VagococcusCarniphilusKT728717   GCTTCGGTGTCACTGTTGGATGGGCCCGCGCTGCATTAGTTAGTTGGTGGGGTAACGGCC
                                      *****    ****   *****  * ***** *** ****  ***** ***

GutLAB02                        CACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
LactococcusLactisLC129537       CACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
GutLAB01                        CACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGAC
LactobacillusPlantarumKU892396  CACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGAC
GutLAB04                        TACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGAC
StaphylococcusAureusAP017377    TACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGAC
GutLAB03                        TACCAAGACCATGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
VagococcusCarniphilusKT728717   TACCAAGACCATGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
                                 **** * * * *** * ****************** ********** *** ********

GutLAB02                        ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTG
LactococcusLactisLC129537       ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTG
GutLAB01                        ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTG
LactobacillusPlantarumKU892396  ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTG
GutLAB04                        ACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTG
StaphylococcusAureusAP017377    ACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTG
GutLAB03                        ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTG
VagococcusCarniphilusKT728717   ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTG
                                **** *** ********************************  ****** ****** ***

GutLAB02                        ACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGA
LactococcusLactisLC129537       ACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGA
GutLAB01                        ATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGA
LactobacillusPlantarumKU892396  ATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGA
GutLAB04                        ACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGA
StaphylococcusAureusAP017377    ACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGA
GutLAB03                        ACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGA
VagococcusCarniphilusKT728717   ACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGA
                                *  ******************** *****  ***** ***************  **  **

GutLAB02                        AGAACGATGATGAGAGTGGAAAGCTCCTCATGTGACGGTATCTACCCAGAAAGGGACGGC
LactococcusLactisLC129537       AGAACGTTGGTGAGAGTGGAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGC
GutLAB01                        AGAACATATCTGAGAGTAACT-GTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGC
LactobacillusPlantarumKU892396  AGAACATATCTGAGAGTAACT-GTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGC
GutLAB04                        AGAACATATGTGTAAGTAACT-GTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGC
StaphylococcusAureusAP017377    AGAACATATGTGTAAGTAACT-GTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGC
GutLAB03                        AGAACAAGTGAGAGAGTTACT-GTTCTCACCTTGACGGTATCTAACCAGAAAGCCACGGC
VagococcusCarniphilusKT728717   AGAACAAGTGAGAGAGTTACT-GTTCTCACCTTGACGGTATCTAACCAGAAAGCCACGGC
                                *****      *  ***     *  *      ********  **  *******  *****

GutLAB02                        TAACTACGTGCCAGCAGCCGCGGTAATACG
LactococcusLactisLC129537       TAACTACGTGCCAGCAGCCGCGGTAATACG
GutLAB01                        TAACTACGTGCCAGCAGCCGCGGTAATACG
LactobacillusPlantarumKU892396  TAACTACGTGCCAGCAGCCGCGGTAATACG
GutLAB04                        TAACTACGTGCCAGCAGCCGCGGTAATACG
StaphylococcusAureusAP017377    TAACTACGTGCCAGCAGCCGCGGTAATACG
GutLAB03                        TAACTACGTGCCAGCAGCCGCGGTAATACG
VagococcusCarniphilusKT728717   TAACTACGTGCCAGCAGCCGCGGTAATACG
                                ******************************
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Appendix 1 Multiple sequence alignment of the 16S rDNA sequence from four lactic acid bacteria (LAB) strains (GutLAB01, *Lactobacillus plantarum*; GutLAB02, *Lactococcus lactis subsp. Cremoris*; GutLAB03, *Vagococcus carniphilus*; GutLAB04, *Staphylococcus aureus*) isolated from shrimp gut with that of other 16s rDNA sequence of *Lactobacillus plantarum* (KU892396), *Lactococcus lactis* (LC129537), *Vagococcus carniphilus* (KT728717) and *Staphylococcus aureus* (AP017377). Asterisk (*) indicate positions with fully conserved residue.

(A)

```
GutLAB01      GGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGG
2SLAB12       GGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGG
              ************************************************************

GutLAB01      CTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGAGGTAACGGCT
2SLAB12       CTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGAGGTAACGGCT
              ************************************************************

GutLAB01      CACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGAC
2SLAB12       CACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGAC
              ************************************************************

GutLAB01      ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTG
2SLAB12       ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTG
              ************************************************************

GutLAB01      ATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGA
2SLAB12       ATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGA
              ************************************************************

GutLAB01      AGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCT
2SLAB12       AGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCT
              ************************************************************

GutLAB01      AACTACGTGCCAGCAGCCGCGGTAATACG
2SLAB12       AACTACGTGCCAGCAGCCG----------
              ****************************
```

(B)

```
GutLAB02        GGAAACGAATGCTAATACCGCATAATAACTTTAAACATAAGTTTTAAGTTTGAAAGATGC
LAB12UFUL       ------------------------------------AAGTTTTAAGTTTGAAAGATGC
                                                    **********************

GutLAB02        AATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCA
LAB12UFUL       AATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAGGCTCA
                ************************************************************

GutLAB02        CCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACAC
LAB12UFUL       CCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACAC
                ************************************************************

GutLAB02        GGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGAC
LAB12UFUL       GGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGAC
                ************************************************************

GutLAB02        CGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAG
LAB12UFUL       CGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAG
                ************************************************************

GutLAB02        AACGATGATGAGAGTGGAAAGCTCCTCATGTGACGGTATCTACCCAGAAAGGGACGGCTA
LAB12UFUL       AACG--------------------------------------------------------
                ****

GutLAB02        ACTACGTGCCAGCAGCCGCGGTAATACG
LAB12UFUL       ----------------------------
```

Appendix 2 Nucleotide sequence alignment for comparison of the 16S rDNA sequence of the lactic acid bacteria (2SLAB12 and LAB12UFUL) isolated from gut of *L. vannamei* feeding with probiotic GutLAB01 *(Lactobacillus plantarum*) or GutLAB02 *(Lactococcus lactis*) with that of the sequence of the probiotics (A) GutLAB01 and (B) GutLAB02. Asterisk (*) indicate the positions with fully conserved residue.

Appendix 3 Detection of the PO enzyme activity in shrimp guts. Gut was stained black by addition of dopamine without PTU (a) or with PTU (b), indicating that PO is presented in gut tissue.

VITA

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