Department of Environmental and Aquatic Sciences

Use of customised probiotics for western king prawn (*Penaeus latisulcatus* Kishinouye, 1896) culture

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DECLARATION

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PREAMBLE

The purpose of this research is to establish a protocol to select, isolate, purify and apply the probiotics, *Pseudomonas synxantha* and *P. aeruginosa* which can be beneficial to culture the juveniles of the western king prawns (*Penaeus latisulcatus*). The usefulness of these customised probiotics was accomplished by evaluating the physiological and immune responses of *P. latisulcatus* when they were exposed to the probiotics.

This thesis consists of nine chapters. Chapter 1 is in the form of an introduction which briefly highlights the current published knowledge and status of probiotics in aquaculture. This chapter also justifies and underlines the need to undertake the current research.

Chapter 2 in the form of 'literature review' briefly reviews the challenges and opportunities of the prawn aquaculture worldwide. This chapter introduces the contribution of the western king prawn species in the aquaculture sector. The chapter also introduces the status, distribution and prospects of the role of probiotics in aquaculture. The various modes of action and application methods of probiotics in aquaculture are reviewed in this chapter. Though the main focus in this chapter is on the penaeid prawns, yet the relevant information from other species is also included in order to have a comparative perspective. Some of the relevant materials and methods *viz.* prebiotics, ozone application, tryptone soya broth, *Artemia* and *Vibrio harveyi* used in this research are also discussed in this chapter.

Chapters 3, 4, 5, 6, 7 and 8 detail the main research of this thesis and attempt to evaluate the selection, isolation, effectiveness and application protocol of the customised probiotics in the cultivation of the juveniles of *P. latisulcatus* by conducting series of laboratory based experiments. All these chapters form an essential component of this research and can be viewed as independent experiments bound by a common theme. These chapters (except chapter 6 and 7, which are under review and in press in *Appendix 7* and 8, respectively) are published in separate peer-reviewed journals (*Appendices 3, 4, 5* and *6*). As these chapters are published in

separate journals, the readers may find some minor repetition in ‘Introduction’ and ‘Materials and Methods’ sections in them.

Chapter 3 details a procedure to customise probiotics from the commercial probiotic products using five inhibition test methods. A protocol for selecting appropriate probiotics using inhibition test methods was established in this chapter. Two probiotic strains, which are consequently identified at species level, are chosen for further studies.

Chapter 4 includes an experiment that compares the two application methods for the application of the customised probiotics. The first method is the direct application of the probiotics onto the rearing medium and the second is supplementing them with the formulated feed. This experiment also aimed to test the effectiveness of the use of customised probiotics for the cultivation of *P. latisulcatus* by evaluating the physiological and immune responses of the prawns when exposed to these probiotics.

Chapter 5 presents an experiment to compare the effectiveness of the customised probiotics with two other commercially available prebiotics ß-1,3-D-glucan and Bio-Mos®, in which tools based on physiological and immune responses, intestinal morphology are used to evaluated the probiotics. The appropriate application methods for these immunostimulants (prebiotics and probiotics) on prawns are also discussed in this chapter.

Chapter 6 incorporates four trials to investigate the disease-resistant capacity of probiotic-fed prawns as an alternative to antibiotics or chemical uses in aquaculture. The prawns were fed with or without customised probiotics before challenging them with the harmful pathogenic bacterium, *V. harveyi*.

In chapter 7, the application of the customised probiotics is further tested by conducting an experiment in which *Artemia* were encapsulated with the customised probiotics. Seven trials are used to determine an appropriate method for the introduction of the customised probiotics into the prawn aquaculture system. The encapsulation capacity of *Artemia* nauplii with probiotics is discussed in this chapter.

Chapter 8 summarises the research on prebiotics with a focus on the current research. The chapter also overviews the current research on prebiotics and prebiotics. It also

discusses the justification in the use of the customised prebiotics. The data collected from this research are also compared to present a more complete picture in the use of customised prebiotics. An assessment of the role of the customised-probiotics in the prawn culture is also discussed. This research demonstrates that the customisation of the probiotics for specific species is a long cumbersome process, which needs to be transferred and tested in commercial prawn operations. In chapter 9, the main conclusions are highlighted which are then followed by the recommendations for future research.

ABSTRACT

In recent decades, a rapid increase in fish production from the aquaculture sector has led to degradation of the environment due to indiscriminate use of chemical additives and veterinary medicines. Consequently, antimicrobial resistance among pathogenic bacteria has increased and environmental problems associated with these chemicals and antibiotics have become a burden for sustainable aquaculture development. Therefore, there is a need to alter the indiscriminate use of chemicals and antibiotics for the use in aquaculture by replacing these with prebiotics or other harmless substitutes. Different probiotics can confer huge functions and benefits to various hosts through the improvement in survival rates and enhancement of health. However, there is a need for special species-specific prebiotics in a particular culture environment.

Customising the species-specific probiotics for prawn culture was performed via several experiments. After the specific prebiotics, *Pseudomonas synxantha* and *P. aeruginosa* were isolated and tested from a commercial product, the emphasis was on determining the effectiveness of them on the cultivation of the juveniles' western king prawns, *Penaeus latisulcatus*. The customisation process began with trialling five inhibition test methods to determine the most effective detection method for the potential probiotic bacteria. *P. synxantha* and *P. aeruginosa* showed the highest inhibition against *Vibrio* spp. isolated from *P. latisulcatus* and pathogenic *Vibrio* isolated from other aquatic animals.

A series of experiments were conducted under laboratory conditions to investigate the physiological and immune responses of the juvenile *P. latisulcatus* exposed to the customised probiotics. The research results proved the suitability of these probiotics for the cultivation of *P. latisulcatus* as they conclusively met all the essential requirements for the appropriate probiotics. The application of these customised probiotics at 10^5 CFU/mL as "water additives" or "feed supplements" improved the specific growth rate, survival and the health of juvenile *P. latisulcatus*. These customised probiotics showed similar beneficial effects as observed with other commercial prebiotics, Bio-Mos® and ß-1,3-D-glucan. The supplementation of these probiotics with the formulated feed was more efficacious and more practical than

direct application into the rearing media. The prawns exposed to the combined probiotics were healthier than those exposed to the individual probiotics. In addition, *P. aeruginosa* was more effective than *P. synxantha* for improving prawn health.

P. latisulcatus were not adversely affected by the customised probiotics, as *P. latisulcatus* grew well in the presence of a high probiotic density of 10^7 CFU/mL. The application of the probiotics however, reduced the negative effects of the pathogen *V. harveyi* as the probiotic-fed prawns survived 100% when they were exposed to *V. harveyi* at 10^5, 10^7 and 10^3 CFU/mL for 24, 24 and 36 h, respectively. Hence customised probiotics are suggested as an alternative to antibiotic for disease control in prawn aquaculture**.** The prawn survival was also influenced by the concentrations of the pathogen and duration of the challenge. At a challenge-concentration of 10^3 CFU/mL of *V. harveyi*, the 100% survival-hours were shorter (12 h) in the control group (prawns not fed with probiotics) than in the probiotic-fed prawns (36 h). Further, prawns not fed with probiotics died at a faster rate (96 h) than the probiotic-fed prawns (156 h). The prawns died when the exposure to *V. harveyi* (even at 10^3 CFU/mL) was longer than 36 h. The probiotic-fed prawns could not completely resist the pathogenic effects of the *V. harveyi* despite the detection of the probiotics in the intestine of the prawns earlier than detection of *V. harveyi*. It is recommended that these customised probiotics can be used as a substitute to antibiotics in the cultivation of western king prawns.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

LIST OF ABBREVIATIONS

AHL	Animal Health Laboratory
ANOVA	Analysis of Variance
AusAID	Australian Agency for International Development
BA	Blood agar
BLIS	Bacteriocin-like inhibitory substance
CARL	Curtin Aquatic Research Laboratory
CFU/mL	Colony forming unit per millilitre
DHA	Docosahexaenoid acid
DHC	Differential haemocyte count
FCR	Feed conversion ratio
g	Gram
GC	Granulocyte(s), granular cell(s)
h	Hour(s)
HC	Hyalinocyte(s), hyaline cell(s)
HUFA	Highly unsaturated fatty acids
L	Litre
LSD	Least Significant Difference
MCA	MacConkey Agar
mg/L	Milligram per litre
mL	Millilitre
MSA	Marine Salt Agar
MSSA	Marine Sea Salt Agar
NS	Normal saline
OD	Optical density
ORP	Oxidation-Reduction potential
PC	Protein concentration
PCR	Polymerase chain reaction
PL	Postlarvae
PO	Phenoloxidase
ppt	Parts per thousand, ‰
ProPO	Prophenoloxidase
Qld	Queensland
ROC	Residual oxidant concentration
SA	South Australia
SE	Standard error
SGC	Semi-granulocyte(s), semi-granular cell(s)
SGR	Specific growth rate
SPSS	Statistical Package for the Social Science
TBC	Total bacterial count
TCBS	Thiosulfate-Citrate-Bile Salt Sucrose Agar
THC	Total haemocyte count
TSB	Tryptone soya broth
TSV	Taura syndrome virus
WA	Western Australia
WSSV	White spot syndrome virus
WKP	Western king prawns
YHV	Yellow head virus

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

1. Articles

1. Hai, NV, Fotedar, R & Buller, N 2007, 'Selection of probiotics by various inhibition test methods for use in the culture of western king prawns, *Penaeus latisulcatus* (Kishinouye)', *Aquaculture*, vol. 272, pp. 231-239.
2. Hai, NV, Buller, N & Fotedar, R, 2009, 'Effects of probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the growth, survival and immune parameters of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896)', *Aquaculture Research*, vol. 40, pp. 590-602.
3. Hai, NV & Fotedar, R 2009, 'Comparison of the effects of the immunostimulants Bio-Mos®, ß-1,3-D-glucan and selected probiotics on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896)', *Aquaculture*, vol. 289, pp. 310-316.
4. Hai, NV, Buller, N & Fotedar, R 2009, 'The use of customised probiotics in the cultivation of western king prawns (*Penaeus latisulcatus* Kishinouye, 1896)', *Fish & Shellfish Immunology* 27, pp. 100-104.
5. Hai, NV, Buller, N & Fotedar, R in press, 'Encapsulation capacity of *Artemia* nauplii with customised probiotics for use in the cultivation of western king prawns (*Penaeus latisulcatus* Kishinouye, 1896)', *Aquaculture Research*.
6. Hai, NV 2009, 'Prebiotics Bio-Mos® and ß-glucan in aquaculture', *The Alltech Young Scientist Competition 2009*, Lexington, Kentucky, USA.
7. Hai, NV, Buller, N & Fotedar, R Unpublished, 'Effects of customised probiotics on the physiological and immunological responses of juvenile western king prawns (*Penaeus latisulcatus*) challenged with *Vibrio harveyi*'.

2. Abstracts

1. Hai, NV & Fotedar, R 2009, 'Effects of Bio-Mos®, a mannan oligosaccharide, on the cultivation of western king prawns, *Penaeus latisulcatus* Kishinouye 1896, under laboratory conditions. Alltech's 25th Annual Symposium, May 17-20, 2009, Lexington, Kentucky, USA.

2. Hai, NV & Fotedar, R 2009, 'Are *Pseudomons synxantha* and *P. aeruginosa* the appropriate probiotics for the cultivation of western king prawns (*Penaeus latisulcatus* Kishinouye 1896)?' *World Aquaculture Society Conference*, September 25-29, 2009, Veracruz, Mexico.
3. Fotedar, R, Hai, NV, Sang, HM & Tuan, NV 2009, 'Physiological responses of the selected immunostimulants on aquatic species', *World Aquaculture Society Conference*, September 25-29, 2009, Veracruz, Mexico.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND INFORMATION

Although prawn prices have shown a steady decline in recent years, prawn farming has continued to be attractive to local farmers, investors and local governments because of its high profitability and the market's demand for its products (Cao 2007). Aquaculture has acted as an engine for economic growth in many rural areas (Ahmed *et al.* 2007).

Among 110 species of 12 genera belong to family penaeidae (Flegel 2007), *Penaeus*, *Litopenaeus*, *Marsupenaeus*, *Metapenaeus* and *Fenneropenaeus* are commonly cultured worldwide. Black tiger prawns, *Penaeus monodon*, remain the most widely farmed species (Ahmed *et al.* 2007) making a major contribution to global prawn production (Deachamag *et al.* 2006) and being the species of choice because of its rapid growth, low cost food requirements and ability to reach a marketable size compared to the other commonly farmed species (Kenway *et al.* 2006). Howver, the global production of *P. monodon* has declined due to outbreaks of bacterial and viral diseases (Scholz *et al.* 1999). Therefore, attempts are being focused on the other prawn species such as western king prawns (*Penaeus latisulcatus*).

The western king prawn is widely distributed throughout the Indo-West Pacific region and are a popular species in Australia. They have been cultured not only in China (Wang *et al.* 2004), Japan (Shokita 1970, 1984), Thailand (Ling 1973) and India (Kathirvel and Selvaraj 1987; Kathirvel *et al.* 1986), but also in Australia with many recent attempts to culture this species in Western Australia (Hai *et al.* 2009a; Hai *et al.* 2009b; Hai *et al.* In press; Hai and Fotedar 2009; Hai *et al.* 2007; Prangnell 2007; Prangnell and Fotedar 2005; 2006a; 2006b; 2004a; Sang and Fotedar 2004b). This specie has become an important cultured species due to its established markets in Asia and Australia (Andrews and Bowen 1992), and its tolerance to a wide range of salinity and temperature (Penn 1980; Ramasamy and Pandian 1984), including

tropical, subtropical and temperate areas. Hence, the species is becoming the major candidate for farming in Australia as it suits Australia's wide range of climates.

The production of the penaeid prawn has increased rapidly in recent decades due to the intensification of technology, but prawn farmers have experienced production decline infectious diseases (Liu *et al.* 2004; Yu and Song 2000). As a result, prawn production has been constrained by environmental degradation, stress associated with intensification of prawn farming, and disease outbreaks (Bachère 2000; Lavilla-Pitogo *et al.* 1998; Pritchard *et al.* 2000). In the prawn aquaculture industry, it was falsely assumed that practical problems would be overcome by focusing on simpler approaches to husbandry and nutrition (Benzie 1998). In fact, there is an interaction between physiological, biological and environmental aspect in management and control of diseases for a successful production.

Disease problems have emerged as major constraints in aquaculture production. Outbreaks of infectious diseases are causing significant economic losses in prawn farming industry (Chotigeat *et al.* 2007; Sarathi *et al.* 2007). The major losses in the aquaculture industry are due to general problems associated with the poor water quality management and the uncontrolled spread of opportunistic bacteria (Decamp *et al.* 2008). However, the prawn mortalities cannot be attributed to specific obligate pathogenic bacteria, but rather to a proliferation of opportunistic pathogenic bacteria (Munro *et al.* 1994). Opportunistic bacteria are present in the culture facilities, in prawns, and in live feed (Decamp *et al.* 2008). However, the bacterial diseases are mainly due to *Vibrio* spp. (Lavilla-Pitogo *et al.* 1998). Luminescent vibriosis caused by *Vibrio harveyi* has been a disease of great concern, causing massive mortalities in the prawn aquaculture industry (Lavilla-Pitogo *et al.* 1990). Thus, it is imperative to control these microbial diseases in order to improve prawn welfare and economical returns (Skjermo *et al.* 2006).

Conventional prophylactic treatments to deal with disease infections involve the application of chemicals and antibiotics. First of all, the prophylactic application of antibiotics is expensive and detrimental to the environment (Decamp *et al.* 2008). Second, the potential negative consequences of indiscriminate use with chemicals and antibiotics can develop into drug resistant bacteria (Skjermo and Vadstein 1999)

and reduce efficacy of antibiotic treatments for human and animal diseases (Moriarty 1997). Concern about antibiotic resistant microorganisms (Amabile *et al.* 1995) has led to suggestions for alternative disease prevention means, including the use of non-pathogenic bacteria called probiotic biocontrol agents (Austin *et al.* 1995; Moriarty 1997; Skjermo and Vadstein 1999). However, there is some doubt about the effective and safe use of probiotics due to ineffective bacterial strains/species, inadequate species/strains numbers (Balcázar *et al.* 2006a; Moriarty *et al.* 2005), unrealistic claims and lack of scientific evidence as well as inappropriate delivery methods (Temmerman *et al.* 2003). Therefore, there is a need to develop suitable probiotics for specific species. Complicated task and empirical and fundamental research requirements in full-scale trials as well as appropriate monitoring tools and controlled production are needed to produce probiotics for effective use in aquaculture (Decamp *et al.* 2008). Hence, the selection of probiotics for specific marine aquatic species has to be carefully considered in order to make them species specific.

The modern concept of probiotics as micro-organisms and substances contributing to intestinal microbial balance was proposed by Parker (1974). Probiotics as live microbial feed supplements are known to improve human and livestock health. They can be defined as microbial cells that are administered through the gastrointestinal tract and kept alive, with the aim of improving the health of the hosts (Gatesoupe 1999). Many beneficial effects may be expected from probiotics such as competition with pathogens for nutrition and adhesion sites, and the stimulation of the immune system (Gatesoupe 1999). Probiotics provide several modes of action including competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement of water quality; enhancement of immune response of host species, and the enhancement of nutrition of the host species through the production of supplemental digestive enzymes (Thompson *et al.* 1999; Verschuere *et al.* 2000). Probiotics are commonly used in aquaculture in the promotion of disease resistance and as an alternative to chemicals, antibiotics and biocides (Moriarty 1999). In aquaculture, probiotics are normally isolated and selected from strains in the species habitat environment. These microbes consist of vibrionaceae, pseudomonads, lactic acid bacteria, *Bacillus* spp. and yeasts (Gatesoupe 1999).

Non-pathogenic strains of *Vibrio*, *Aeromonas*, *Pseudomonas* and *Alteromonas* that have known antagonistic properties to pathogenic strains, are used as probiotic strains (Irianto and Austin 2002a; Moriarty 2003). In contrast, *Vibrio*, *Flavobacterium Pseudomonas*, *Alteromonas* and *Aeromonas* are pathogenic disease texa and cause severe prawn mortalities (Moriarty 2003; Moriarty *et al.* 2005). In Asia, some probiotic products are advertised to consist of *Clostridium* sp., *Pseudomonas putida* and *P. aeruginosa*, however, they are less effective against pathogens in aquatic species (Moriarty 2003). Moreover, *Pseudomonas* species present in some probiotic solutions are promoted as potential probiotics for marine prawns due to their growth inhibition to a number of pathogens such as *Salmonella* sp., *Staphylococcus aureus* and *Vibrio parahaemolyticus*, *V. harveyi*, *V. fluvialis*, *V. vulnificus, Photobacterium damselae* and *Aeromonas* spp. (Chythanya *et al.* 2002; Oblinger and Kreft 1990; Vijayan *et al.* 2006). Despite the increase in research on probiotics for aquatic animals as a result of the demand for environmentally friendly aquaculture (Gatesoupe 1999), no research has been conducted to produce specific probiotics for western king prawn culture. The main aim of this research project is to produce and evaluate customised species-specific probiotics for the laboratory cultivation of *P. latisulcatus*.

1.2 AIM

To customise probiotics for improving the survival, growth performance and enhancing immune responses of juvenile western king prawn (*Penaeus latisulcatus* Kishinouye, 1896).

1.3 OBJECTIVES

The aim of the research will be achieved by meeting the following specific objectives:

1. To select and isolate specific probiotics from commercially available probiotic product(s) to be used for the benefit of *P. latisulcatus* culture under laboratory conditions.

2. To establish a protocol from various inhibition test methods for selecting effective probiotics for use in the cultivation of wesern king prawn.
3. To compare the application methods of the customised probiotics for their effect on the physiological and immune responses of juvenile *P. latisulcatus*.
4. To investigate the effectiveness of the customised probiotics for juvenile *P. latisulcatus*.
5. To understand the modes of action of the customised probiotics during the cultivation of juvenile *P. latisulcatus*.
6. To compare the efficacy of the customised probiotics and prebiotics (ß-1,3-glucan and Bio-Mos®) on the physiological and immune responses of juvenile *P. latisulcatus*.
7. To evaluate the fundamental role of the customised probiotics on disease resistance against the pathogen *V. harveyi* in the cultivation of juvenile *P. latisulcatus*.
8. To determine the efficacy of enrichment of *Artemia* with the customised probiotics for the benefit of the juvenile *P. latisulcatus*.
9. To provide the selected research recommendations based on the outcomes of the current research that is aimed to understand the role of the customised probiotics in prawn aquaculture.

1.4 SIGNIFICANCE

The research outcomes aim to make an effective contribution in improving the prawn aquaculture industry worldwide by contributing to the understanding of the effective use of appropriate probiotics for western king prawn aquaculture. The protocol developed can also be used as a guideline for producing/customising appropriate probiotics in aquaculture in general. The specific significances of the current research are outlined below:

- The research will demonstrate the different mechanisms by which probiotics can play various roles in the prawn aquaculture industry.
- The research will compare and contrast the different application methods of the probiotics to the prawn culture.
- The research will reenforce the suggestion that an alternative to chemicals and antibiotics in the prawn aquaculture industry is warranted.

- The research will assist in creating a better understanding of the use of probiotics through their administration in live feeds used by the prawn larviculture industry.
- The research will illustrate how custom made probiotics can be developed for the prawn industry.
- To research will contribute to the basic knowledge of prawn immunity and physiology under the influence of probiotics.
- The research will assist in the development of a new environmentally friendly aquaculture industry with the judicious use of beneficial bacterial probiotics.
- The research will contribute to an increase in the production of *P. latisulcatus* via an improvement in the survival and growth without the use of chemicals and antibiotics.

CHAPTER 2

LITERATURE REVIEW

2.1 BRIEFT OVERVIEW OF PRAWN AQUACULTURE

2.1.1 Current status

The potential challenges to penaeid aquaculture must be recognised and identified as the constraints need to be addressed and overcome during the developmental process of prawn farming worldwide. The first challenge is to increase production to meet the increasing global demand for seafood products (Zhang 2007), which can no longer be supplied by captured fisheries due to dwindling aquatic stocks, pollution and overfishing (Ahmed *et al.* 2007). Besides the clear definitions of the developmental strategies and policies for specific areas or regions, sustainable development for penaeid prawns will depend upon feasible solutions for environmental problems and proper developmental planning (Cao 2007). Diversification of culture environments and farmed species, development of hatchery technologies for high commercial value species and/or high demand by consumers, development of domestic feed industry supplemented with appropriate additives, and provision of insurance and other related services are still unexplored economically in penaeid aquaculture (Hishamunda 2007).

The practical problems of growth and survival rate, disease prevention, maturation and larval quality or even chemicals/antibiotic residues in aquaculture products under conventional culture means have appeared more frequently. Water pollution and environmental deterioration that have been reaching warning alert levels in some countries affect aquaculture production directly or indirectly at various levels (Skjermo and Vadstein 1999). Intensification of aquaculture activities has increased the occurrence of apparent diseases (Shariff *et al.* 2001). Massive loss caused by diseases has closed some commercial farming and even large culture sites. Defective rearing conditions viz., high stocking density, excess or poor food quality are associated with mortalities, caused by either heterotrophic or pathogenic bacterial strains such as *Pseudomonas, Aeromonas* and *Vibrio* (Moriarty 2003; Moriarty *et al.*

2005). Disease outbreaks in all stages of development of prawns can be caused by *Vibrio* species such as *V. damsela* (Song *et al.* 1993), *V. alginolyticus*, *V. parahaemolyticus* (Lightner 1992), *V. penaeicida* (Costa *et al.* 1998) or *V. harveyi* (Karunasagar *et al.* 1994; Robertson *et al.* 1998). Viral diseases have emerged as the most serious enzootic pathogens. Moreover, difficulties in controlling diseases come from the various susceptibilities of aquatic animals to the diversity of pathogens (Bachère 2003).

As the regular or discriminative use of antibiotics and chemicals as preventative and curative means for diseases leads to drug-resistant bacteria and harmful effects on the environment (Bachère 2000, 2003). There is an increase in the need to control, prevent or minimise the devastating effects of disease in prawn culture without use of toxic chemicals or antibiotics (Smith *et al.* 2003). The use of immunostimulants such as prebiotics and probiotics has been considered as alternatives to antibiotics and chemicals used (Bachère 2003; Li *et al.* 2006; Meunpol *et al.* 2003; Rengpipat *et al.* 1998a; Vaseeharan and Ramasamy 2003).

Although immunostimulants have been recommended for disease control, outcomes are unpredictable and mixed. These results are attributed to a lack of an understanding of the close relationship between the hosts, pathogens and environmental stressors (Sánchez-Martínez *et al.* 2007). Doubts on the efficacy and safety of probiotics on the market comes from the use of ineffective bacterial species/strains, unrealistic claims, lack of scientific evidence, poor quality control during processing of products, and inappropriate delivery methods leading to contamination or reduced performance (Temmerman *et al.* 2003). On the other hand, vibriosis can be controlled by the use of antibiotics, vaccine and immunostimulants such as probiotics and prebiotics, but viral infection in prawns has no effective therapeutic or prophylactic counter measures (Itami *et al.* 1998). The question raised here is the function of biochemical compounds produced from immunostimulants for cost-effective control of disease infections in aquaculture, especially for long lasting protection in juvenile and adult prawns (Smith *et al.* 2003).

As the health and biotechnical performances can be improved by the prophylactic use of probiotics in cultured penaeid prawn species, these uses should be considered

to be a kind of risk insurance in providing notable benefits, when the culture is performing under optimal conditions and in the absence of opportunistic pathogens, and that it is also helpful if infectious diseases break out (Verschuere *et al.* 2000). Once it has been decided to apply probiotics on a large scale, production of large amounts of bacterial biomass/probiotics requires appropriate quality control to avoid contamination by other bacteria (Verschuere *et al.* 2000). Therefore, the development of appropriate probiotics/prebiotics for species-specific situations is not a simple task and requires empirical and fundamental research, full-scale trials, appropriate product monitoring and control means (Decamp *et al.* 2008). A question emerges as to how many opportunities are left or pathogens to grow and become a threat, when appropriate probiotics dominate other microbial communities, and one useful approach is to carefully monitor shifts in the overall microbial community (Verschuere *et al.* 2000).

Although some proposed methods for the control of disease in aquaculture such as the use of probiotics, immunostimulants and zootechniques have made some progress (Bachère 2003; Robert and Gérard 1999), antibiotics and chemicals have been used intensively as preventive and curative means (Bachère 2003; Barg and Lavilla-Pitogo 1996). The question is raised on the consequences of probiotics in nutrition and the well-being of the consumers (Hammes and Hertel 2002). Besides an appearance of antibiotic-resistant bacteria caused by regular use or misuse of antibiotics (Bachère 2003; Chelossi *et al.* 2003; Karunasagar *et al.* 1994; Molina-Aja *et al.* 2002; Sahul Hameed *et al.* 2003), residue from the antibiotics and chemicals has degraded the environment (Bachère 2003; Kautsky *et al.* 2000) and could pose a risk to humans (Kesarcodi-Watson *et al.* 2008; Schwarz *et al.* 2001).

Probiotics may contribute to solve at least a part or all of the problems mentioned above. For instance, probiotics as feed additives can improve the water quality, promote the survival and growth rates, and enhance the health and maturation of prawns. Moreover, probiotics can be used as alternatives to chemicals and antibiotics as prophylactic means for control and management of infectious diseases. Consequently, all achievements from the applications of probiotics can also serve for domesticated breeding programs for specific prawn species.

2.1.2 Future

Nutrient requirements for prawns such as proteins, lipids, carbohydrates, vitamins, cholesterol, essential fatty acids and minerals, are better known, hence feed can be more accurately formulated for specific species (Cuzon *et al.* 2004). Replacement of expensive proteins with readily available and cheap protein sources is possible in terms of economics and environmentally-friendly considerations. Although gut physiologies, digestive enzymes and habitats between various decapods can be similar, while their feeding habits may be different (Cuzon *et al.* 2004).

Environmental, ecological, biotechnical and nutritional aspects are considered into account for operating prawn farming as basic physiology and genetics of farmed species need to be understood. Fundamental work on physiology, genetics and immunology at a molecular level has been progressing to create new tools to assist producers in establishing adequate health management and prevention of diseases (Bachère 2003). Important progress has been made in the prevention and control of diseases, with reliable diagnostic methods based on molecular probe-detection techniques (Walker and Subasinghe 2000). Studies on the properties and function of penaeidins led to the understanding of physiology and their capacity to respond to pathological injuries (Bachère 2003). Molecular and biotechnological tools are available for investigating details of the genome and biology of penaeid prawns (Benzie 1998). Therefore, an integrated approach to control of diseases must be improved through the combination of ecological, environmental and biotechnical aspects of production (Bachère 2000). Molecular techniques will clearly improve the role of probiotics in this integrated management approach.

Although unplanned expansion of the penaeid prawn aquaculture in coastal areas in recent decades has seen an increase in production, this activity has been owed to its production ecosystems. These lessons need to be taken into account to avoid the mistakes experienced elsewhere (Hishamunda 2007). The development of appropriate management practices, the control of environmental variables, genetic improvement, understanding of virus physiology, modulation of the prawn immune system, all distribute to the control of white spot syndrome virus (WSSV) effectively in the prawn aquaculture so far. Therefore, successful farms are the ones using

specific-pathogen-free prawns, correctly monitoring samples and undertaking evaluation using polymerase chain reaction (PCR), histopathology, filing and using serological methods on newly introduced broostock, eggs, postlarvae and juvenile prawns (Sánchez-Martínez *et al.* 2007), in which the use of probiotics has taken a part in producing specific pathogen free prawns.

Generally, progresses in immunology and physiology of the cultured species provide potential approaches for disease control at various ontogentic stages of production of penaeids (Bachère 2003). There is little corresponding increase in scientific knowledge of prawn physiology, while prawn immunology is a key element in establishing strategies for the control of diseases in prawn aquaculture (Bachère 2000). Alternative prophylactic methods including the use of probiotics and prebiotics are proposed for the control of diseases in aquaculture as they enhance resistance against pathogens of prawns by improving prawns' immune systems and are considered one of the effective strategies in use for the control of prawn diseases (Huang *et al.* 2006). These methods help protect aquatic animals against infections (Bachère 2003; Verschuere *et al.* 2000; Vici *et al.* 2000) as probiotics are an effective addition to disease control strategies in aquaculture (Balcázar *et al.* 2006b; Irianto and Austin 2002a). It is possible to vaccinate *P. japonicus* with recombinant proteins against WSSV (Namikoshi *et al.* 2004), and together with probiotics, provide an effective alternative to the use of chemicals and antibiotics.

Interestingly, probiotics as an adjuvant for detoxification protocols (Brudnak 2002) may emerge as an idea for aquaculture. Although doubts on the use of probiotics such as limitations and inside effects of pharmaceutical agents, and consumer demand for natural products, the establishment of a consensus on probiotic product regulations is required to assist in the enforcement of guidelines and standards, and appropriate clinical studies on mechanisms of actions of strains (Reid 2006). Knowledge on the dynamics of bacterial activity, differences in efficacies of vaccines, different responses of various aspects of the immune systems, and limited duration of antibacterial activity increases manipulative advantages in prawn culture systems (Alabi *et al.* 2000).

Development of domesticated programs to relieve pressure on wild stocks, and to improve industry production through selective breeding programs will require the inclusion of appropriate genetic variation in the broodstock population (Taniguchi *et al.* 1994). Rearing large numbers of families from defined matings have been successful in *P. vannamei* (Wyban *et al.* 1993), *P. monodon* (Benzie 1997) and *P. stylirostris* (Bedier *et al.* 1996). Sperm quality of pond-reared *P. monodon* males was not different from wild males (Pratoomchat *et al.* 1993) indicating that husbandry methods can provide adequate conditions to achieve normal male fertility levels. The domesticated programs in biosecure tank systems leads to an alleviation of high pressure on wild-caught broodstock, enables control over pathogens and generates farmed stocks. These programs with improvements in broodstock diets and husbandry will meet the reproductive output needs from tank-reared prawns (Coman *et al.* 2006). Breeding programs using massive selection and inbreeding effects, produced a *L. vannamei* strain ofanimals free of the three most dangerous diseases of taura syndrome virus (TSV), WSSV and yellow head virus (YHV), resulting in a great resource for the prawn culture industry (De Donato *et al.* 2005). Browdy (1998) believed that if broodstock diet, health and other conditions in a maturation system are well maintained, the quality of spawns of captive matured females can be as good as that of wild females. In fact, under controlled/indoor conditions, probiotics will be served by favourable conditions so that they can manipulate toward proliferation of their populations to dominate other bacterial populations. Therefore, probiotics will be of great benefit for maturation of prawn broodstock under captive conditions.

Relevant and concrete knowledge, which will be continuously updated with the demand on quality food for human consumption, on aspects of biology and physiology to husbandry practices has been creating huge prospects for penaeid prawn aquaculture. Strategic advances for penaeid prawn aquaculture have anticipated the combination of endocrine control of reproduction, its effects on egg quality, molecular markers, maturation diets and practical husbandry methods (Benzie 1997). Finally, macro-government monitoring should be established to prevent disease spread and to increase production for sustainable development of the penaeid prawn aquaculture. It appears essential to open the network to complementary research on prawn pathology, physiology, genetics and environment (Bachère 2000), in which the roles of probiotics cannot be absented in the prawn

aquaculture industry. Western king prawn is one of the common cultured species in Australia.

2.2 WESTERN KING PRAWNS

2.2.1 Taxonomy, biology and environmental requirements

The nomenclature of *Penaeus (Melicertus) latisulcatus* (Kishinouye 1896) is as follows (*Taxonomy and nomenclature* Integrated Taxonomic Information System 2005):

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Subclass: Eumalacostraca
Superorder: Eucarida
Order: Decapoda
Suborder: Dendrobranchiata
Superfamily: Penaeoidae
Family: Penaedae
Genus: *Penaeus* (*Melicertus*)
Species: *Penaeus* (*Melicertus*) *latisulcatus*
Authority: Kishinouye 1896

Plate 2.1 Western king prawns

Western king prawn, called bamboo shrimp, is widely distributed into the Indo-West Pacific region (Dore and Frimodt 1987; Kangass 1999; Rothlisberg and Jackson 1987; Tseng 1987). The natural distribution depends on sediment particle size and organic carbon content (Branford 1981). This species prefers to live on sand, sandy mud, gravel substrates or in shallow marine water down to a 90 metre depth (Andrews and Bowen 1992; Dore and Frimodt 1987). They are nocturnal and bury in the sediment during the daytime (Kangass 1999; Rasheed and Bull 1992; Wassenberg and Hill 1994) as they are very sensitive to light (Wassenberg and Hill 1994). This species behaviour and physiology is similar to kuruma prawn (*Penaeus japonicus*) and witch prawns (*Penaeus canaliculatus*) (Dore and Frimodt 1987).

P. latisulcatus can survive at temperatures between 10 and 32 ^{o}C (Tseng 1987). Larvae can grow at a temperature range of 21-30 ^{o}C (Rothlisberg and Jackson 1987). The optimum temperature for culture of this species is 23-26 ^{o}C and 22-26 ^{o}C for optimum spawning (Tseng 1987). The optimum temperature range for maximum growth of the early stages of this species is 25-28 ^{o}C (Prangnell 2007). This species can spawn at a temperature as low as 17 ^{o}C (Penn 1980).

Salinity is an important influence on the *P. latisulcatus* distribution within temperate estuaries with larger prawns more susceptible to declining salinity (Potter *et al.* 1991). *P. latisulcatus* can tolerate a wide range of salinities of 20-25 ppt (Ramasamy and Pandian 1984). The maximum survival of this species is achieved at salinities between 25-45 ppt (Ramasamy and Pandian 1984), and 22-34 ppt (Sang and Fotedar 2004b). In addition, the isosmotic point of *P. latisulcatus* shows a positive relationship with size as the isosmotic point at a weight of 2.95 to 5.79 g ranges from 855 to 937 mOsm/kg (Sang and Fotedar 2004b). In fact, *P. latisulcatus* probably osmoregulate in a similar fashion as to the rest of penaeid prawns (Dall *et al.* 1990). *P. latisulcatus* are hyperosmoregulators when the medium salinity is below and hypoosmoregulators when the medium salinity is above the isomotic point, which ranges from 29 to 32 ppt (Sang and Fotedar 2004b). Attempts were made recently to culture this species in inland saline water in WA. For example, although *P. latisulcatus* PL and juveniles reared in potassium-deficient inland saline water have 100% mortality within 24 h and 11-13 days, respectively, *P. latisulcatus*

juveniles can tolerate sudden salinity decrease from 32 to 25 ppt and 27 to 20 ppt, and even tolerate sudden increases in medium potassium concentration from 78 to 284 mg/L and 78 to 365 mg/L in inland saline water (Prangnell 2007).

P. latisulcatus mature at the age of 1 to 2 years (Abdel Razek *et al.* 1996; Penn 1980). At the first year of life, females can reach maturity when they are as small as 10.8 cm in length (Abdel Razek *et al.* 1996). The mature prawns reach a carapace length of 23 mm for males and 25 mm for females (Penn 1980). Their spawning times depend on the location. For example, during summer in temperate water of Cockburn South, WA, and year round in warm water of Shark Bay and Exmouth Gulf, WA but only at an oceanic salinity of 35 ppt (Penn 1980). Their fecundity of 241,300 to 656,180 is correlated with the total length of 14.8 to 17.0 cm, respectively (Abdel Razek *et al.* 1996). Spawning takes place offshore and planktonic stages migrate to inshore towards the end of larval development, at which PL prefers seawater or higher salinity (Kangass 1999). They remain in estuaries for a year before moving offshore (Kangass 1999; Penn 1979; Potter *et al.* 1991). *P. latisulcatus* like all penaeid prawns, prefers benthic fauna and detritus (Dall *et al.* 1990; Rasheed and Bull 1992; Wassenberg and Hill 1987).

2.2.2 Outlook for the cultivation of western king prawns

P. latisulcatus have been considered as a prospective aquaculture species since 1987. The first culture of this species was in South Australia, Australia (Pownall 1974). Studies on this species have been made in China (Wang *et al.* 2004), Japan (Shokita 1970, 1984), Thailand (Ling 1973), India (Kathirvel and Selvaraj 1987; Kathirvel *et al.* 1986), and also in Australia (Hai *et al.* 2009a; Hai *et al.* 2009b; Hai and Fotedar 2009; Hai *et al.* 2007; Prangnell 2007; Prangnell and Fotedar 2005; 2006a; 2006b; 2004a; Sang and Fotedar 2004b). This species has become important cultured prawn species due to their established markets in Asia and Australia (Andrews and Bowen 1992), and their tolerance to a wide range of environmental conditions of salinity and temperature (Penn 1980; Ramasamy and Pandian 1984), which consists of various tropical, subtropical and temperate areas suitable for this species. Moreover, this species is becoming the major penaeid farmed prawn in Australia because of the wide geographical coverage of these environmentally suitable areas.

2.3 IMMUNE SYSTEMS

2.3.1 Non-specific immune systems

Research on innate immune systems reveals new insights into management and control of infectious diseases in aquaculture (Bachère 2003). Understanding the immune criteria as enhancement of non-specific defence responses against bacterial and viral injections is the most effective way for sustainable aquaculture production (Bachère 2003; Chang *et al.* 2003; Chang *et al.* 1999). According to Raa (1996), invertebrates are apparently dependent on non-specific immune mechanisms to cope with infections as they lack the specific immunological memory that is found in fish and warm-blooded animals. The immune system of crustacean is non-specific and relies on phagocytosis, encapsulation and agglutination (Smith and Soderhall 1983), comprises cellular and humoral factors, and cellular factors implicate functions such as phagocytosis, nodule formation and encapsulation (Rowley and Powell 2007). The immune parameters include prophenoloxidase (ProPO), total and differential haemocyte count (THC and DHC), protein concentration (PC), agglutinating and phenoloxidase (PO) activities in the serum, levels of glucose and lactate in the plasma, clotting time and bacteraemia (Maggioni *et al.* 2004). In this thesis, only THC, DHC, clotting time and bacteraemia are focused.

2.3.2 Immune parameters

The haemocytes are mainly involved with cellular immune reactions such as phagocytosis, melanisation and encapsulation of foreign intruders and in their subsequent destruction by the production of cytotoxic and microbicidal molecules (Campa-Cordova *et al.* 2002; Maggioni *et al.* 2004), in which phagocytosis is the most common defence mechanism in invertebrates (Dyrynda *et al.* 1995; Kondo *et al.* 1998; Söderhäll and Cerenius 1992). The THC and DHC can be used to assess the stress or health status of prawns, despite the large individual variability in the cell numbers (Jussila *et al.* 1997; Maggioni *et al.* 2004). Changes in THC and DHC may cause by increases in haematopoiesis or a proliferation in circulating haemocytes, which can increase sixfold under the stimulation of bacteria in prawns (Sequeira *et*

al. 1996). The changes in THC has been described in crustaceans in relation to several stressors such as hypoxia (Le Moullac *et al.* 1998b), acclimation in capacity (Sánchez *et al.* 2001), temperature variation (Cheng and Chen 2001; Le Moullac and Haffner 2000; Smith *et al.* 1995; Truscott and White 1990), type of diet or starvation (Le Moullac and Haffner 2000; Stewart *et al.* 1967), moult cycle (Evans *et al.* 1992; Le Moullac *et al.* 1997; Tsing *et al.* 1989), presence of xenobiotics (Smith and Johnston 1992; Smith *et al.* 1995) and injections (Cheng and Chen 2001; Rengpipat *et al.* 2000; Söderhäll *et al.* 1998).

In crustacean species, three morphologically different haemocyte types are identified such as hyalinocytes, semi-granulocytes and granulocytes (Bauchau 1981; Hose *et al.* 1990; Le Moullac *et al.* 1997; Sequeira *et al.* 1995; Söderhäll and Smith 1983). Granular cells are believed to be the main phagocytes in the haemolymph of crustaceans (Hose *et al.* 1990; Martin *et al.* 1996). Granulocytes are the only type observed when blood withdrawals do not damage these extremely labile cells (Durliat and Vranckx 1983). Hyaline cells, the most numerous in the haematopoietic organs, would be in a differentiating state from granulocytes in the blood stream, and would be the end product of the granule bursting process and may represent ageing or lysing granular haemocytes (Durliat and Vranckx 1983).

The responses of DHC to various stressors have been little studied (Jussila *et al.* 1997). Under various environmental stressors, these haemocyte types may have different responses such viz. prophenoloxidase activation, phagocytosis, coagulation, release of agglutinins and synthesis of melanin (Cerenius and Söderhäll 1995, 2004; Lanz *et al.* 1993). The DHC changes during the moult cycle (Bauchau 1981) or associates with sex in *Panulirus japonicus* (Sequeira *et al.* 1995). Haemocyte number or proportion of DHC affect by various factors such as sex, growth, moult, starvation, and parasite (Evans *et al.* 1992; Sequeira *et al.* 1995).

Clotting time is a primary mechanism of host defence against foreign organisms, involves cellular and plasma interaction (Fotedar *et al.* 2001). In crustaceans, plasma clotting results from the conversion of a plasma coagulogen into a gel which is crosslinked covalently by the action of a cellular transglutaminase (Fuller and Doolittle 1971). Foreign articles invading the haemolymph impair the clotting ability

(Durliat and Vranckx 1983). Elevated clotting times are associated with decline in THC or increase in prevalence of bacteria caused a higher number of damaged haemocytes (Durliat and Vranckx 1983). High clotting times indicate a decrease ability to confine pathogens and pointing to lower immune status (Fotedar *et al.* 2001). High clotting times of rock lobsters (*Panalirus cygnus*) exposed to air during transportation implicated low immunity (Fotedar *et al.* 2001). Clotting times increased significantly over the duration of holding (Fotedar *et al.* 2006). Short-term handling and exercise showed lower clotting times (Jussila *et al.* 2001), while prolonged clotting times resulted in decline in THC (Durliat and Vranckx 1983).

Aquatic animals contact a large number of bacteria in the water, sediment and food (Fotedar *et al.* 2001). Uninjured healthy blue crabs (*Callinectes sapidus*) do not have sterile haemolymph (Welsh and Sizemore 1985). Pathogenic bacteria colonise gills and guts of prawns and may cause opportunistic infectious diseases when self defence mechanisms are suppressed under stressors. The bacterial load in the haemolymph is called bacteraemia. High levels of bacteraemia suggest a decline in immune capacity and probably increase susceptibility to infection (Fotedar *et al.* 2001). Bacteraemia increased when rock lobsters were exposed to air (Fotedar *et al.* 2001), and when blue crab were subjected to the stresses of commercial capture, handling and transport (Welsh and Sizemore 1985).

It is believed that understanding the immune responses helps to prevent disease injections aquaculture (Bachère 2003; Chang *et al.* 2003; Chang *et al.* 1999), but the benefit of probiotics in enhancement of immune responses is still doubtful in invertebrates (Marques *et al.* 2006a). Probiotic-fed animals may not be able to resist all diseases (Sakai 1999). However, Bricknell and Dalmo (2005) claimed that a great deal can be done to improve larval survival against bacterial and viral pathogens by the judicious use of probiotics.

2.4 PROBIOTICS

2.4.1 Introduction

As antibiotics and diverse types of feed additives are known to be used for the control of bacteriosis, reduction of stress and improvement in the health of cultured prawns, their regular or indiscriminate use has led to problems of drug resistance (Balcázar *et al.* 2006a; Esiobu *et al.* 2002; Karunasagar *et al.* 1994; Weston 1996). The use of preventive yet environment-friendly approaches viz antibacterial peptides, probiotics and prebiotics are becoming increasingly important in aquaculture (Bachère 2003; Sakai 1999; Soltanian *et al.* 2007a; Vine *et al.* 2006).

The development of immunostimulant molecules helps prawns overcome stress conditions, including handling, grading, vaccination, net changing, sea water transfer, anti-parasite bath treatments (Bricknell and Dalmo 2005; Burrells *et al.* 2001), and unilateral eyestalk ablation (Maggioni *et al.* 2004). Probiotics stimulate various components of the cellular and humoral immune systems, and act as adjuvants to increase vaccine effectiveness (Sakai 1999). For example, probiotic *Bacillus* S11 provided disease protection by activating cellular and humoral immune defences (Rengpipat *et al.* 2000). Specific immune responses and protection can be induced in *P. monodon* by oral vaccination (Witteveldt *et al.* 2004a). Probiotics have an influence on the non-specific immune elements such as phagocytic cell activity, natural killer cell activity, lysozyme levels, complement levels and total immunoglobulin levels, in which probiotics mainly facilitate the function of phagocytic cells and increase their bactericidal activities (Sakai 1999). Therefore, the use of probiotics is becoming increasingly important in aquaculture (Marques *et al.* 2006b).

The use of probiotics is a new preventative approach or strategy for microbial management to achieve less mortalities caused by infectious diseases (Bricknell and Dalmo 2005; Skjermo and Bergh 2004), and to maintain the good health of cultured organisms (Marques *et al.* 2006a). Probiotics become important for heightening the activity of non-specific defence mechanisms and conferring protection against diseases (Jeney and Anderson 1993). The concept of probiotics as organisms and

substances contributing to intestinal microbial balance was provided by Parker (1974). Probiotics is the use of microbial supplements to benefit their host (Fuller 1989). A probiotic is a mono or mixed culture of live microorganisms that beneficially affects the host by improving the properties to the indigenous microflora (Havenaar *et al.* 1992). Moriarty (1998) proposed to extend the definition of probiotics in aquaculture to microbial "water additives". Simply probiotics are often defined as live intestinal bacteria that are added to promote the viability of the host (Skjermo and Vadstein 1999). Probiotics as live microbial feed supplements are known to improve human and terrestrial livestock health. They can be defined as microbial cells that are administered through the gastrointestinal tract and to be kept alive, with the aim of improving the health of the hosts (Gatesoupe 1999).

Last two decades, several definitions of probiotics have been proposed. Fuller (1989) gave a precise definition of probiotics which is still widely referred to, as a live microbial feed supplement, which beneficially affects the host by improving its intestinal balance. In aquaculture, the intestinal microbiota does not exist as entidy by itself, but there is a constant interaction with the environment and the host functions (Verschuere *et al.* 2000). The relationship of the intestinal microbiota to the aquatic habitat or food has been investigated (Cahill 1990). The host-microbe interactions are often qualitatively and quantitatively different for aquatic and terrestrial species as in aquatic environment, the hosts and microorganisms live in the same medium (Verschuere *et al.* 2000). Aquatic animals are surrounded by an environment that supports their pathogens independently of the host animals, and opportunistic pathogens can reach high densities around the animals (Moriarty 1998).

Generally, probiotics in aquaculture are administrated at early stages of animals, because, aquatic species usually prawns axenic eggs in the water, without further contact with the parents. This allows ambient bacteria to colonise the egg surface. In addition, freshly hatched larvae or newborn animals do not have a complete intestinal tract and have no microbial community in the intestinal tract, on the gills or on the skin (Verschuere *et al.* 2000). The early stages of aquatic larvae depend for their primary microbiota on the rearing water (Cahill 1990; Ringø and Birkbeck 1999). Therefore, the following modified definition is proposed and allows a broader application of the term "probiotic" and addresses to the objections in the study. A

probiotic is defined as a live microbial adjunct which has a beneficial effect on the host i) by modifying the host-associated or ambient microbial community, ii) by ensuring the use of feed or enhancing its nutritional value, iii) by enhancing the host response towards disease or iv) by improving the quality of its culture environment (Verschuere *et al.* 2000). Similarly, probiotics are defined as "live micro-organisms which when administered in adequate amounts confer a health benefit to the host" (FAO/WHO 2002). According to Kesarcodi-Watson et al. (2008), peptidoglycan and lipopolysaccharides derived from bacteria are probiotics, because the non-requirement of being a live culture would allow for a specific suggested immunostimulants (Itami *et al.* 1998; Smith *et al.* 2003). For example, rather than testing bacterial derivatives such as glucans or lipopolysaccharides, live *Bacillus* sp. and *V. alginolyticus* are probiotics (Gullian *et al.* 2004).

2.4.2 Components

Probiotics are obtained from various sources such as bacteria, brown and red algae and terrestrial fungi (Bricknell and Dalmo 2005) or bacteria from habitats (Rengpipat *et al.* 1998a) or marine yeast (Sajeevan *et al.* 2006). Probiotics can be divided into several groups, depending on their original sources such as bacteria, algae-derived, animal-derived, nutritional factors and hormones or cytokines (Sakai 1999). Many gram-positive obligate or facultative anaerobes are found in the gastrointestinal tracts of human and terrestrial animals, whereas gram-negative facultative anaerobes prevail in fish and shellfish, and symbiotic anaerobes may be dominant in the posterior intestine of some herbivorous tropical fish (Clements 1997). Bacteria genera also differ between fresh water and marine water species. *Vibrio* and *Pseudomonas* are the most common genera in crustaceans (Moriarty 1990), marine fish (Sakata 1990) and bivalves (Prieur *et al.* 1990), whereas *Aeromonas, Plesiomonas* and Enterobacteriaceae are dominant in freshwater fish (Sakata 1990). Microbial populations may be influenced by temperature and salinity changes (Hamid *et al.* 1978; Ringø and Strøm 1994). Due to adaptation in the marine environment, intestinal microbial populations of aquatic animals may change rapidly with the intrusion of microbes coming from water and food (Gatesoupe 1999).

Probiotics isolated from aquatic environments include vibrionaceae, pseudomonads, lactic acid bacteria, *Bacillus* spp. and yeast (Gatesoupe 1999). Some probiotic bacteria and vibrios are the most common genera associated with crustaceans (Moriarty 1997) and are common inhabitants of aquatic environments including prawn culture ponds (Otta *et al.* 1999). For example, probiotic *Pseudomonas* spp. in the marine environment produce a wide range of secondary metabolites (Raaijmakers *et al.* 1997; Vijayan *et al.* 2006). *Pseudomonas* sp. PM11 and *Vibrio fluvialis* PM17 isolated from gut of farmed sub-adult *P. monodon* are putative probiotics for prawn culture (Alavandi *et al.* 2004). *Pseudomonas* PS-102 isolated from brackish water showed suitability as probiotics for management and control of bacterial injections in *P. monodon* (Vijayan *et al.* 2006). Four probiotic strains isolated from the gastrointestinal tract of adult *L. vannamei* showed antagonism towards prawn-pathogenic bacterium, *V. parahaemolyticus* and reduced FCR in these prawns (Balcázar *et al.* 2007). In addition, a number of chemical agents, polysaccharides, plant extracts or some nutritional additives have been included in aquatic animal diets called immunostimulants (Gannam and Schrock 2001; Sakai 1999) as adjuncts to vaccination and as a potential route to the reduction of the widespread use of antibiotics (Burrells *et al.* 2001). Herbal immunostimulants namely methnolic extracted from five different herbal medicinal plants increased *P. monodon* resistance against viral pathogenesis caused by WSSV (Citarasu *et al.* 2006).

In addition, some bacterial strains of *Vibrio, Aeromonas, Pseudomonas* and *Alteromonas* can be used as probiotics in aquaculture (Irianto and Austin 2002a; Moriarty 2003). In contrast, other *Vibrio*, *Flavobacterium Pseudomonas*, *Alteromonas* and *Aeromonas* species are known pathogens and cause severe prawn mortalities (Moriarty 2003; Moriarty *et al.* 2005). Therefore, some bacterial strains are harmful to one species, but beneficial to other species. Non-pathogenic bacterial strains that are known to have antagonistic properties to pathogenic bacterial strains, can be referred as probiotic strains (Li *et al.* 2006; Rengpipat *et al.* 1998a; Vaseeharan and Ramasamy 2003). Gatesoupe (1999) listed a number of probiotics used in the aquaculture industry as most probiotics products contain nitrifying bacteria and/or *Bacillus* spp. The former and latter are quite different on direct and indirect actions on the water quality, respectively (Gomes *et al.* 2009). Probiotic products are primarily composed of highly concentrated bacteria, vitamins and

nutrients (Gatesoupe 1999; Verschuere *et al.* 2000). For instance, Efinol®L is a formulated probiotic product, which is enriched with some nutrients as a formulated combination of bacteria *Bacillus subtilis*, *B. licheniformes, Lactobacillus acidophillus* and *Saccharomyces cerevisiae*, along with selected amino acids, vitamins, minerals, free-flow and anti-caking agents (Gomes *et al.* 2009).

Among the large number of probiotic products in use today are bacteria spore formers, mostly of the genus *Bacillus* (Hong *et al.* 2005). The introduction of *Bacillus* spp. in close proximity to pond aerators reduces chemical oxygen demand, and increases prawn harvest (Porubcan 1991b). Among 80 bacterial strains isolated from healthy wild *L. vannamei*, *Vibrio* P62, *V.* P63 and *Bacillus* P64 show inhibitory effects against *V. harveyi* (S2) at 54, 19 and 34%, respectively. Moreover, *Bacillus* P64 shows both probiotic and immunostimulatory features, while *Vibrio* P62 only shows good probiotic properties (Gullian *et al.* 2004). *Bacillus* spores have been used as biocontrol agents to reduce vibrios in prawn culture facilities (Rengpipat *et al.* 2000; Skjermo and Vadstein 1999). *Bacillus* spp. are often antagonistic against other micro-organisms, including fish and shellfish pathogen bacteria (Gatesoupe 1999; Rengpipat *et al.* 2000). *Bacillus fusiformis* improved the survival and accelerated the metamorphosis of *P. monodon* and *L. vannamei* (Guo *et al.* 2009).

Generally, bacteria that have been successfully used as probiotics belong to *Vibrio* spp. (Garriques and Arevalo 1995; Gatesoupe 1989; Griffith 1995), *Bacillus* spp. (Gatesoupe *et al.* 1989; Guo *et al.* 2009; Moriarty 1998; Rengpipat *et al.* 1998a), *Pseudomonas* spp. lactic acid bacteria and yeast (Gatesoupe 1999; Gatesoupe *et al.* 1989; Gildberg and Mikkelsen 1995, 1998; Rengpipat *et al.* 1998a) and *Thalassobacter utilis* (Maeda and Liao 1992a). Probiotics are isolated from prawn culture water (Direkbusarakom *et al.* 1997; Nogami and Maeda 1992; Tanasomwang *et al.* 1998) from the intestine of different penaeid species (Rengpipat *et al.* 2000) and from the hepatopancreas of healthy wild prawns (Gullian *et al.* 2004).

2.4.3 Application methods

The effective use of probiotics, the timing, dosages, methods of administration and physiological conditions need to be considered (Sakai 1999). Probiotics should be

administered into the culture systems before disease outbreaks occur, to reduce disease-related losses (Anderson 1992). Theoretically, probiotics can elevate the innate defence mechanisms prior to exposure to a pathogen or improve survival following challenge to a specific pathogen when treated with probiotics (Bricknell and Dalmo 2005). Short-duration feeding of probiotics, followed by a period of control diet feeding, produces optimal effects on immune response and disease resistance (Bagni *et al.* 2000; Bridle *et al.* 2005; Couso *et al.* 2003). An appropriate probiotic density is at a concentration of 10^5 CFU/mL (Guo *et al.* 2009; Moriarty 1998; Zhou *et al.* 2009). In addition, over-dosages or prolonged administrations of probiotics can induce immunosuppression of continuous responses (Sakai 1999). However, *P. monodon* defied this theory by growing faster with glucan immersion at 0.5, 1 and 2 mg/mL than at 0.25 mg/mL (Sung *et al.* 1994).

There are various ways to introduce probiotics into prawns such as a supplement with feed, immersion, submersion, oral administration and intramuscular or intra-arterial/intraperitoneal injections, in which supplement with feed or in-feed route is more productive and practical than others (Azad *et al.* 2005; Hai *et al.* 2009a) as most probiotics are designed to be mixed with food (Gomes *et al.* 2009). In contrast, oral administration is considered a most practical method for prawn probiotics (Huang *et al.* 2006). Oral administration has advantages for prawns regardless of prawn size (Sakai 1999), as prawns can be treated at any stage of the culture period. A water-soluble commercial probiotic product, Efinol®L, as an anti-stress formula for aquaculture hatcheries can be dissolved in water during transport operations (Gomes *et al.* 2009). Immersion method is useful although it is limited to the juvenile stage or to the stage before releasing to grow-out ponds (Itami *et al.* 1998). *P. monodon* (Sung *et al.* 1994) and *P. latisulcatus* (Hai *et al.* 2009a) immersed in a suspension of probiotics showed higher disease resistance than those in the control, respectively. Probiotics can be applied directly into the rearing water (water additive) or supplemented with feed (feed additive) or through live feeds (Guo *et al.* 2009; Hai *et al.* 2009a; Hai and Fotedar 2009; Skjermo and Vadstein 1999; Zhou *et al.* 2009). During the culture period, a daily addition of probiotics into the larval prawn rearing systems gave better results for improving prawn survival than application every other day in (Guo *et al.* 2009).

Intramuscular immunisation of the WSSV enveloped protein VP19 and VP28 increased the *P. monodon* survival, and this result offers new strategies to control viral diseases in prawns and other crustaceans (Witteveldt *et al.* 2004b). An intramuscular injection of inactivated WSSV vaccines, then an intramuscular challenge with WSSV resulted in mixed protection of *P. japonicus*, while heat-inactivated WSSV did not induce resistance in the prawns, and recombinant rVP28 induced resistance. These results suggested that it is possible to vaccinate prawns with recombinant proteins against WSSV (Namikoshi *et al.* 2004). *P. japonicus* injected or immersed with the formalin-killed *Vibrio* bacterin reduced mortalities when they were challenged by *Vibrio* injection (Itami *et al.* 1989).

Feed additives are used to enhance the feed value for species-specific requirements. Probiotics can be used singly or as a combination (Gatesoupe 2002; Kesarcodi-Watson *et al.* 2008) or even as a mixture of probiotics and prebiotics. Probiotics based on a single strain are less effective than those based on mixed strains (Hai *et al.* 2009a; Hai and Fotedar 2009). Multistrain and multispecies probiotics have positively provided synergistic bacteria with complementary modes of action to enhance protection (Timmermans *et al.* 2004). It has been argued that in aquaculture the microbial habitat changes continuously. Therefore, a single bacterial species will need to remain dominant in a continuously changing environment. The beneficial bacterium must dominate the other microbiota, when administered (Verschuere *et al.* 2000).

2.4.4 Modes of action

Current probiotic applications and scientific data on mechanisms of action indicate that the non-viable microbial components act in a beneficial manner and this benefit is not limited just to the intestinal tract (Salmien *et al.* 1999). The several beneficiary effects of probiotics are through i) improvement in water quality, ii) production of inhibitory compounds or antibiotics, iii) competition for chemicals or energy/nutrient and for adhesion sites, iv) enhancement of the immune responses, v) interaction with phytoplankton, vi) supplement of macro- and micro-nutrients and enzymatic contribution to digestion, vii) modulation of interactions with the environment and viii) development of beneficial immune responses (Abidi 2003; Balcázar *et al.*

2006a; Gatesoupe 1999; Gomes *et al.* 2009; Irianto and Austin 2002a; Verschuere *et al.* 2000).

Probiotics have proved their effectiveness on improvement of rearing water quality in aquaculture (Borges *et al.* 2008; Moriarty 1998; Verschuere *et al.* 2000). Probiotics enhance decomposition of organic matter, reduce nitrogen and phosphorus concentrations and control ammonia, nitrite and hydrogen sulfide (Boyd and Massaut 1999). The application of commercial probiotics reduced organic matter accumulation, and enhanced environmental conditions for prawn farms (Suhendra *et al.* 1997), and mitigated the nitrogen (Wang *et al.* 2005) and phosphate pollution in the sediment of prawn *L. vannamei* ponds (Wang and He 2009). Probiotic *Bacillus* spp. were recently employed in an attempt to improve the aquatic environment in prawn aquaculture (Farzanfar 2006). Probiotic Efinol®L helped maintain the water quality possibly by lowering metabolic wastes with a significantly lower ammonia level than the control, during transportation of cardinal tetra (*Paracheirodon aexlrodi*) (Gomes *et al.* 2009). Several commercial probiotic products have sought to exploit the same idea that probiotic bacteria improve water quality leading to be beneficial to animal health (Gatesoupe 1999). *Bacillus* spp. degraded organic accumulation in prawn culture systems (Rengpipat *et al.* 1998a; Verschuere *et al.* 2000). Probiotics *Lactobacillus* spp. JK-8 and JK-11 facilitated the simultaneous removal of pathogenic bacteria and nitrogens from contaminated prawn farms (Ma *et al.* 2009). Actually, probiotics are created to stimulate the immune systems of the host through an improvement process of water quality or environmental conditions. The use of *Bacillus* spp. improves water quality while reducing pathogenic *Vibrio* spp. (Dalmin *et al.* 2001). The prawn tanks receiving probiotic inoculation showed no presence of pathogenic bacteria *V. parahaemolyticus* in any samples taken from the prawn's microbiota, while the control tanks had 10% of *V. parahaemolyticus* (Garriques and Arevalo 1995).

Inhibitory compounds excreted by probiotics, provide a hostile environment for pathogens include bacteriocins, lysozymes, proteases and hydro-peroxide (Balcázar *et al.* 2007). *Bacillus foraminis* and *B. cereus biovar toyoi* exhibit antagonisms against *Streptococcus iniae* and *Photobacterium damselae* (Guo *et al.* 2009). Generally, microbial populations release chemical substances, which have

bactericidal and bacteriostatic effects on other microbial populations. Probiotic *Lactobacillus* JK-11 produce sufficient organic acid, which associated with drop in pH, to antagonise many pathogenic bacteria (Ma *et al.* 2009). Probiotics promote the defence of the gut flora against pathogens (Skjermo and Vadstein 1999). Therefore, probiotics can alter inter-population relationships by influencing the outcome of competition for chemicals or available energy (Pybus *et al.* 1994).

Competition for chemicals or available energy may determine how different microbial populations coexist in the same ecosystem (Fredrickson and Stephnopoulos 1981). Competition for nutrients can theoretically play an important role in the composition of the microbiota of the intestinal tract or ambient environment of cultured aquatic animals, but there have been no comprehensive studies on this subject (Ringø and Gatesoupe 1998). A successful application of the principles of competitive exclusion of natural situations is not easy and remains a major task for microbial ecologists (Verschuere *et al.* 2000).

Competition for adhesion sites on guts or other tissue surfaces is one possible mechanism for preventing colonisation by pathogens (Verschuere *et al.* 2000). The ability to adhere to enteric mucus and wall surfaces is necessary for bacteria to establish in fish intestines (Olsson *et al.* 1992; Westerdahl *et al.* 1991). Bacterial adhesion to tissue surfaces is important during the initial stages of pathogenic infection (Krovacek *et al.* 1987). Competition for adhesion receptors with pathogens may be the first probiotic effect (Montes and Pugh 1993).

The application of probiotics improves the survival, growth rates and FCR of prawns (Balcázar *et al.* 2007; Hai *et al.* 2009a). Bacterial strain, PM-4, as a food source promoted the growth of *P. monodon* nauplii (Maeda and Liao 1992b). Administration of *Bacillus fusiformis* increased the *L. vannamei* survival when added daily (87.9±1.7%) or every other day (74.7±1.2%) (Guo *et al.* 2009). Photosynthetic bacteria and *Bacillus* spp. improved the growth performance of *L. vannamei* with high levels of lipase and cellulase activity (Wang 2007). The specific activities of amylase, total protease and lipase were higher in *Fenepenaeus indicus* to which probiotic *Bacillus* was applied, therefore, these prawns grew better than those in the control (Ziaei-Nejad *et al.* 2006). Probiotic *Bacillus coagulans* as a water additive

increased survival and digestive enzyme activities of *L. vannamei* larvae (Zhou *et al.* 2009). The survival of the probiotic-fed prawns challenged with a pathogenic *V. harveyi* was 100%; whereas, prawns reared without probiotics showed only 26% survival (Rengpipat *et al.* 1998a). The survival and wet weight of *L. vannamei* fed probiotics were higher than those that received oxytetracycline and the control (Garriques and Arevalo 1995). Probiotics effectively participate in the digestive processes by producing extracellular enzymes such as proteases, carbohydrolases and lipases and by providing growth factors (Arllano and Olmos 2002; Ochoa and Olmos 2006).

The application of probiotics in prawn culture can negate the use of antibiotics and chemotherapeutics (Chotigeat *et al.* 2004; Chythanya *et al.* 2002; Decamp *et al.* 2008; Sánchez-Martínez *et al.* 2007; Supamattaya *et al.* 2005). The application of antibiotics is expensive and detrimental on drug-resistant or more virulent, prevalence of drug residues in reared animals (Boyd and Massaut 1999; Esiobu *et al.* 2002; Moriarty 1999). The use of antibiotics should be disapproved not only for prophylactic but also for therapeutical treatment (Lavens and Sorgeloos 2000). Probiotics produce biochemical compounds such as bacteriocins, lysozymes, proteases and hydro peroxide (Balcázar *et al.* 2007). *Lactobacillus* spp. produce various compounds during lactic fermentation such as organic acids, diacetyl, hydro peroxide and bacteriocidal proteins (Farzanfar 2006; Rengpipat *et al.* 1998a; Verschuere *et al.* 2000). These compounds may activate the immune systems of animals and render them more resistant to infections by viruses, bacteria, fungi and parasites (Raa 1996) or inhibit the bacterial pathogens in aquaculture systems (Gram *et al.* 1999; Rengpipat *et al.* 1998a). These effects lead to better and more effective alternatives to manage the prawn health than administering antibiotics (Moriarty 1997; Verschuere *et al.* 2000). In a comparison with chloramphenicol on protecting *P. chinensis* PL from pathogenic vibrios, *Arthrobacter* XE-7 is regarded as a probiotic for the culture of *P. chinensis* larvae (Li *et al.* 2006).

In addition, several probiotic products have been experimentally tested for the control of diseases due to their potential to stimulate the non-specific immune system of prawns. Probiotics are promoted in aquaculture as a means of overcoming the immunosuppressive effects of stress occurring in intensive culture systems (Welker

et al. 2007). These products enhance immune responses, resistance to diseases and are excellent preventive tools against pathogens (Anderson 1992). Probiotics confer a health benefit for the host (Reid *et al.* 2003) such as *P. monodon* (Rengpipat *et al.* 1998a), *P. latisulcatus* (Hai *et al.* 2009a) and *L. vannamei* (Chiu *et al.* 2007), these probiotic-fed prawns can resist prawn pathogens *V. harveyi* and *V. alginolyticus*. Moreover, disease resistance to *V. penaeicida* (Itami *et al.* 1998) and *V. alginolyticus* (Yeh and Chen 2008) was enhanced by peptidoglycan and carrageenans supplemented with feed for *P. japonicus* and *L. vannamei*, respectively. As probiotics are an effective addition to disease control strategies in aquaculture (Balcázar *et al.* 2006b; Irianto and Austin 2002a), a study conducted by Moriarty (1998) has reinforced this achievement in the application of probiotic *Bacillus* spp. in penaeid prawn culture ponds and permitted continuous culture for over 160 days, while farms that did not use these probiotics experienced complete failure in all ponds within 80 days due to luminescent *Vibrio* spp.

Probiotics contribute to an improved stress resistance in prawn fry (Lavens and Sorgeloos 2000). Probiotics compete with bacterial pathogens for nutrients and/or inhibit the growth of pathogens, and can alter the prophylactic use of chemicals, antibiotics and biocides (Decamp *et al.* 2008; Moriarty 1999). *Pseudomonas* I-2 is a probiotic for control of prawn pathogenic vibrios as its chloroform extract can be used for the control of luminous *Vibrio* spp. in aquaculture systems (Chythanya *et al.* 2002). Prawns *L. stylirostris* fed probiotic *Pediococcus acidilactici* showed resistance to vibriosis under pond conditions (Castex *et al.* 2008). *L. vannamei* (Balcázar *et al.* 2007) and *P. latisulcatus* (Hai *et al.* 2009a) fed probiotics for 28 days, where immersion challenge was effective in reducing diseases caused by *V. parahaemolyticus* and *V. harveyi*, respectively. PG derived from *Bifidobacterium thermophilum* enhanced the phagocytic activity of granulocytes and increased resistance of *P. japonicus* when challenged by *Vibrio penaeicida* (Itami *et al.* 1998).

2.4.5 Strategies for use of probiotic in aquaculture

Probiotics should be tested for their inhibitory activity against different species of *Vibrio* and *Aeromonas* (Hai *et al.* 2007; Vijayan *et al.* 2006) that have been considered as major pathogens in aquaculture systems (Singermann 1990). Currently,

four methods are commonly employed to screen for inhibitory substances *in vitro* such as double layer, well-diffusion, cross-streak and disc-diffusion (Kesarcodi-Watson *et al.* 2008). For instance, Hai et al. (2007) have used bacteriocin-like inhibitory substance (BLIS), modified BLIS, disc-diffusion, well-diffusion and co-culture methods to select the most suitable probiotics for use in the cultivation of *P. latisulcatus*. Probiotics have to be evaluated for safety to the hosts (Verschuere *et al.* 2000). Initial *in vitro* screening is following by small-scale tests either for pathogenicity to the host (Chythanya *et al.* 2002) or host protection when challenged with pathogens (Gram *et al.* 2001; Irianto and Austin 2002b; Rengpipat *et al.* 1998a; Vaseeharan *et al.* 2004). Probiotics *Pseudomonas* spp. did not cause any harmful effects to *P. monodon* larvae (Vijayan *et al.* 2006) and *P. latisulcatus* juvenile (Hai *et al.* 2009a) upon challenge a dose of 10^7 CFU/mL. In agreement with these studies, immersion challenge indicated effectiveness at reducing disease caused by *V. parahaemolyticus* in probiotic-fed *L vannamei* (Balcázar *et al.* 2007). There are two major limitations to the screening approach that other modes of probiotic activities do not display on agar plates and positive results *in vitro* fail to determine the real *in vivo* effect (Kesarcodi-Watson *et al.* 2008). The certain properties that aid in the correct establishment of new, effective and safe probiotic products include i) not harmful to the host, ii) accepted by the host through ingestion and colonization and proliferation within the host, iii) reach the target organs where they can have effect, iv) work both *in vivo* and *in vitro*, and v) lacking virulence resistance genes or antibacterial resistance genes (Kesarcodi-Watson *et al.* 2008; Verschuere *et al.* 2000).

The transferral process of resistant genes between bacteria (Schwarz *et al.* 2001) means that antibiotic-resistant bacteria originating from prawn farms could potentially transfer plasmids to bacteria involved in human health problems (Kesarcodi-Watson *et al.* 2008). Fortunately, there is insufficient data to show a linkage to resistant gene transfer to humans (Kesarcodi-Watson *et al.* 2008). Governments and organisations have introduced much tighter restrictions for antibiotic use in animal production. For example, The European Union put a ban on the use of avoparcin in 1997 and in 1999 on virginiamycin, spiramcin, tylosin and bacitracin as well as growth promoters and use of all non-therapeutic antimicrobials (Delsol *et al.* 2005; Turnidge 2004). Many Asian countries have less antibiotic

control despite foreign restrictions on antibiotic-contaminated products for export markets, and antibiotics such as chloramphenicol have been detected at trace levels in prawn products from Thailand, Myanmar, India, Pakistan and Vietnam (Heckman 2004; Kesarcodi-Watson *et al.* 2008). For this reason the application of probiotics in aquaculture has been increasing remarkably as a commercially viable and safe alternative to chemicals and antibiotics.

Similarities to the functions of probiotics, the use of prebiotics enhances immune responses, improves colonic integrity, decreases incidence and duration of intestinal infections, down-regulates allergic response, and improves digestion and elimination foreign articles (Douglas and Sanders 2008).

2.5 PREBIOTICS

2.5.1 Introduction

Prebiotics are defined as nondigestible food ingredients that beneficially affect the growth and health of the host (Douglas and Sanders 2008; Gibson and Roberfroid 1995). Prebiotics are a group of diverse carbohydrate ingredients that are poorly understood in regards to their origin, fermentation profiles, and dosages required for health effects (Douglas and Sanders 2008). As similar functions with probiotics in aquaculture, prebiotics are commonly used in aquaculture. The dosage and frequency of administration of prebiotics in prawn health management need to be considered like other immunostimulants (Sajeevan *et al.* 2009). Overdoses of immunostimulants lead to immunosuppression rendering less protection where animals succumb to infection (Sajeevan *et al.* 2009). Marine yeast (*Candida sake* S165) as an immunostimulant for *F. indicus* PL showed an optimal dietary concentration at 10% whereas higher doses resulted in a lower immune profile and post challenge survival (Sajeevan *et al.* 2006).

According to Reid (2008), prebiotics are considered as means of influencing the gut microbiota and allergy risk, and probiotics influence newborn health and the health function of the liver and pancreas of organisms. Regular consumption of prebiotics enhances immune function, improves colonic integrity, decreases incidence and

duration of intestinal injections, down-regulates allergic response and improves digestion (Douglas and Sanders 2008). Prebiotics mannan oligosaccharides (Bio-Mos®) and ß-1,3-glucan are two commonly available products and are derived from the cell wall of yeast *Saccharomyces cerevisiae* (Couso *et al.* 2003; Waldroup *et al.* 2003). ß-glucan and Bio-Mos® enhance the immune responses of prawns (Chang *et al.* 2003; Fritts and Waldroup 2003; Hai and Fotedar 2009).

2.5.2 Bio-Mos®

Bio-Mos® are commonly applied in the animal husbandry industry with promises in suppressing enteric pathogens and modulating the immune response of chickens and turkeys (Iji *et al.* 2001; Kocher *et al.* 2005; Valancony *et al.* 2001; Waldroup *et al.* 2003). Bio-Mos® enhanced the growth and FCR of early weaned pigs (Davis *et al.* 2000) and pig productivity (Maribo 2003). No reports of effectiveness of Bio-Mos® have been found in the cultivation of *P. latisulcatus*.

Similar to the effects with Bio-Mos® in aquaculture, ß-glucans have been used as immunostimulants to enhance the defence potential of fish and shellfish against bacterial and viral infection (2000; 2003; Chang *et al.* 1999). ß-1,3-glucan is used to prevent or reduce animal mortalities (Couso *et al.* 2003) as they can enhance resistance of the host to bacterial and viral infections (Chang *et al.* 2003; López *et al.* 2003; Sung *et al.* 1998). ß-glucan enhanced the non-specific immune system in resistance to bacterial injections (Burrells *et al.* 2001) and improved the survival of WSSV-infected *P. monodon* (Chang *et al.* 2003), although an increase in dosages of ß-1,3-glucan in diets for *L. vannamei* from 0.02 to 0.1 % did not affect the expressions of the genes related to immune proteins in haemocytes and hepatopancreas (Wang *et al.* 2008b). The survival of *F. indicus* PL challenged orally with WSSV was highest when glucan incorporated into the diet at 0.2% compared to other concentrations of 0.05, 0.1, 0.3 and 0.4% (Sajeevan *et al.* 2009). *P. monodon* brooders fed ß-1,3-glucan at 0.02% diets were healthier than those without ß-1,3-glucan (Chang *et al.* 2000).

2.5.3 ß-1,3-glucan

ß-1,3-glucan can modulate the activity of phagocytes and other components of the innate immune system in both fish and mammals (Di Luzio 1985; Robertsen *et al.* 1994). Supplementing ß-1,3-glucan into feed enhanced haemocyte phagocytic activity, cell adhesion, superoxide anion production in *P. monodon* (Chang *et al.* 2000; Song and Hsieh 1994) and synthesised cells and ProPO in *L. vannamei* (López *et al.* 2003). Oral administration of ß-1,3-glucan (Itami *et al.* 1994) or peptidoglycan (Itami *et al.* 1998) to prawns enhanced disease resistance. *P. japonicus* treated with peptidoglycan enhanced the phagocytic activity of granulocytes and increased prawn resistance to *V. penaeicida* (Itami *et al.* 1998). ß-1,3-glucan increases the resistance against vibriosis in *P. japonicus* (Itami *et al.* 1998) and against vibriosis and WSSV in *P. monodon* (Sung *et al.* 1998). ß-glucan and *Vibrio* bacterins do not change the haemolymph protein profile of *P. monodon*, but haemagglutination activity partly accounts for the immunodulatory activity of these immunostimulants (Pais *et al.* 2008). *P. monodon* brooders fed ß-1,3-glucan were healthier as shown with their haemocyte phagocyte activity, cell adhesion and superoxide anion production than those without ß-1,3-glucan (Chang *et al.* 2000). *L vannamei* fed ß-1,3-glucan or vitamin C showed significantly greater growth rate than those without any immunostimulants (López *et al.* 2003).

In addition, the frequency of administration of glucan may give various results. According to Sajeevan *et al*., (2009) the application of alkali insoluble glucan extracted from the marine yeast isolate *C. sake* S165 (Sajeevan *et al.* 2006), once every seven days gave maximum survival of *F. indicus* PL, compared to daily, once every two days, once every five days and once every ten days. Many studies on the effects of ß-1,3-glucan on animals can be accessed, while the effect of ß-1,3-D-glucan on the cultivation of juvenile *P. latisulcatus* has not been investigated.

2.6 MORPHOLOGICAL STUDY

Scanning electron microscope (SEM) technique is widely used in aquaculture to describe morphological structures of animals (Beraldo *et al.* 2003; Johnston *et al.* 2005; Routray *et al.* 2006). SEM observations of the organic matrix in the otolith of

the teleost fish *Fundulus heteroclitus* and *Tilapia nilotica* (Watabe *et al.* 1982), indicated that the breakage process impacts on both algal cells themselves (60% of broken structures) and siliceous structures connecting sibling cells **(**40% of broken structures) (Sauriau and Baud 1994). Study on the digestive system of juvenile blacklip abalone (*Haliotis rubra*), Johnston *et al.* (2005) have applied SEM technique to describe morphology and the presence or absence of different tooth types. SEM was used to describe morphology of abnormalities of the operculum in gilthead sea bream (*Sparus aurata*) (Beraldo *et al.* 2003). The morphology of spermatozoa cryopreservation of Indian major carp, *Labeo rohita* (Routray *et al.* 2006), the motility, fertility and ultrastructural changes of ocean pout (*Macrozoarces americanus* L.) sperm after cryopreservation (Yao *et al.* 2000) were studied under a SEM. SEM also confirmed fouling by filamentous of at least two morphological types and also fouling by rod-shaped microbial community dynamics in a larval tropical rock lobster, *Panulirus ornatus*, indicative of a diverse epibiont community (Bourne *et al.* 2004). The use of SEM for investigating of intestinal structures of *P. latisulcatus* fed probiotics has not been studied.

Besides comparison of customised probiotics with prebiotics Bio-Mos® and ß-1,3-glucan, the customised probiotics need to be tested their mode of action on improvement of immune systems by challenge the probiotic-fed prawns with harmful bacteria, *Vibrio harveyi*.

2.7 CHALLENGES WITH *VIBRIO HARVEYI*

2.7.1 Challenges

Infectious diseases especially caused by bacterial and viral pathogens create serious loss (Primavera 1998) and are well-known to be the major constraints in the aquaculture industry (Scholz *et al.* 1999). Amongst the bacterial pathogens, *Vibrio* species are reputed for causing vibriosis in penaeid prawns (Lavilla-Pitogo *et al.* 1990; Phuoc *et al.* 2009), and act as primary pathogens in the rearing waters with increased *Vibrio* populations (Vandenberghe *et al.* 1998), or as opportunistic agents in secondary infection (Saulnier *et al.* 2000). *Vibrio* spp. are well known to be one of the dominant species of bacteria in prawn ponds (Hisbi *et al.* 2000). Outbreaks of

prawn vibriosis mostly happen in either a combination with physical stressors or following primary infections with other pathogens (Sung *et al.* 2001). Many stressors used for challenging prawns to measure the susceptibilities to various infections have been studied such as ammonia (Liu and Chen 2004), *Vibrio* spp. (Alapide-Tendencia and Dureza 1997; Phuoc *et al.* 2009), WSSV (Phuoc *et al.* 2009) or multiple stressors of virus and bacteria (Phuoc *et al.* 2008). In our study, *V. harveyi* was used to challenge in various prawns such as probiotic-treated *P. monodon* (Rengpipat *et al.* 2000).

2.7.2 *Vibrio harveyi*

Vibrio harveyi is a sodium chloride dependent, curve-rod shaped, Gram-negative bacterium found in the marine environment (Farmer III *et al.* 2005). *V. harveyi* is found in a free-living state in aquatic environments and parts of the normal flora of marine animals (Ruby and Morin 1979). Numerous strains of *V. harveyi* have been recognised as significant pathogens in aquaculture of marine fish (Pujalte *et al.* 2003; Zhang *et al.* 2001; Zorrilla *et al.* 2003), penaeid prawns (Karunasagar *et al.* 1994; Lavilla-Pitogo *et al.* 1998), lobsters (Bourne *et al.* 2006), mollusc (Nishimori *et al.* 1998) and coral (Sutherland *et al.* 2004). *V. harveyi* was isolated as the dominant luminous species with 94% in all penaeid prawn hatchery components except eggs and UV treated water, and 97.3% in prawn intestines (Abraham and Palaniappan 2004). The main source of *V. harveyi* can be broodstock, maturation and spawning facilities (Chrisolite *et al.* 2008).

The disease caused by *V. harveyi* is called as penaeid vibriosis, penaeid bacterial septicaemia, bolitas negricans, luminous vibriosis or red-leg diseases (Cano-Gomez *et al.* 2009). Signs of vibriosis include lethargy, tissue and appendage necrosis, slow growth, slow larval metamorphosis and body malformations, bioluminescence, muscle opacity, melanisation, empty midgut and anorexia (Karunasagar *et al.* 1994; Robertson *et al.* 1998).

V. harveyi has been frequently associated with infectious diseases in prawn culture (Le Moullac *et al.* 1998a) as the commercial culture of prawns has been hampered by endemic and epidemic infections such as vibriosis, in which *V. harveyi* is considered

to be one of the most harmful bacteria for prawns (Austin and Austin 2005). *V. harveyi* causes massive mortalities in the prawn culture industry worldwide (Karunasagar *et al.* 1994; Liu *et al.* 1996a; Liu *et al.* 1996b; Saeed 1995), or even up to 100% in larval stages of *P. monodon* and *P. japonicus* (Liu *et al.* 1996a; 1996b) leading to high economic losses in large-scale prawn aquaculture in Asia (Jiravanichpaisal *et al.* 1994), Australia (Pizzuto and Hirst 1995) and South America (Alvarez *et al.* 1998), Mexico (Vandenberghe *et al.* 1999), China (Vandenberghe *et al.* 1998), India (Karunasagar *et al.* 1994), Indonesia (Sunaryanto and Mariam 1986), Thailand (Jiravanichpaisal *et al.* 1994), the Philippines (Baticados *et al.* 1990; Lavilla-Pitogo *et al.* 1990) and Taiwan (Liu *et al.* 1996a).

The virulent mechanisms of *V. harveyi* are still not fully understood (Cano-Gomez *et al.* 2009). The effect of *V. harveyi* on *P. monodon* larvae is related to the age of prawns (Baticados *et al.* 1990; Prayitno and Latchford 1995). It confirms that older prawns develop an increasing resistance to these pathogens (Prayitno and Latchford 1995). Environmental factors of temperature and salinity play a role in *V. harveyi*-medicated vibriosis (Alavandi *et al.* 2006). Pathogenicity of *V. harveyi* has been related to a number of factors of secretion of extracellular products containing substances such as protease, lipase, haemolysins (Teo *et al.* 2003; Zhang and Austin 2000), lipopolysaccharide (Montero and Austin 1999) and bacteriocin-like substance (Prasad *et al.* 2005). The virulence of *V. harveyi* has been associated with luminescence (Manefield *et al.* 2000), quorum sensing (Henke and Bassler 2004), ability to form biofilm conferring resistance to disinfectants and antibiotics (Karunasagar *et al.* 1994), sucrose fermentation (Oakey and Owens 2000) and capacity to bind iron (Owens *et al.* 1996).

A method for detection of *V. harveyi* contamination or infection could facilitate disease prevention in the prawn aquaculture industry due to the close phylogenetic relationship of this species to other *Vibrio* species viz. *V. parahaemolyticus*, *V. alginolyticus,* and *V. campbellii* (Kita-Tsukamoto *et al.* 1993; Pedersen *et al.* 1998). *V. harveyi* is commonly used for challenge in aquatic organisms fed potential or putative chemicals, antibiotics or probiotics (Rengpipat *et al.* 1998a) when testing new prophylactic management or prevention means for vibriosis. Many studies have been undertaken on the effects of *V. harveyi* on the culture of aquatic animals at their

different stages of life (Karunasagar *et al.* 1994; Liu *et al.* 1996a; Liu *et al.* 1996b; Saeed 1995; Thaithongnum *et al.* 2006). *V. harveyi* isolated from Mahimahi (*Coryphaena hippurus* Linnaeus) was firstly used in this study with the aim to investigate the effectiveness of the probiotics *P. synxantha* and *P. aeruginosa* on the cultivation of juvenile *P. latisulcatus*.

2.8 ENCAPASULATIONS OF *ARTEMIA*

Artemia are known as valuable and indispensable food organisms in the industrial larviculture of fish and crustaceans (Lee and Ostrowski 2001; Liao *et al.* 2001; Lubzens 1987; Makridis *et al.* 2000; Marte 2003; Shields 2001) due to their small size, slow swimming behaviour, rapid reproduction rate, ability to be cultured at high density (Lubzens 1987; Lubzens *et al.* 1989; 2001), and usefulness for transferring probiotic bacteria to aquatic animals (Gatesoupe 1994; Makridis *et al.* 2000; Rombaut *et al.* 1999; Ziaei-Nejad *et al.* 2006). The two major problems in the rearing of larval stages of aquatic organisms are their nutrition and the development of microbial diseases (Touraki *et al.* 1996). The *Artemia* nauplii constitutes a food source that is very accepted by both marine and freshwater fish (Sorgeloos *et al.* 1986). *Artemia* are still playing an important role in any fresh or marine hatcheries, and are considered to be an essential part of any commercial hatcheries (Hanaee *et al.* 2005; Kolkovski *et al.* 2004).

Unfortunately, freshly hatched *Artemia* nauplii contain very low of ω-3 highly unsaturated fatty acids (HUFA), being deficient in docosahexaenoic acid (DHA) (Han *et al.* 2000). The importance of dietary DHA for proper development of marine fish larvae has been documented (Watanabe 1993). To overcome the lack of ω-3 HUFA in *Artemia* nauplii, various enrichment techniques have been developed (Han *et al.* 2000). The supplements or enrichments may include essential fatty acids (Ando *et al.* 2004; Bransden *et al.* 2005; Czesny *et al.* 1999; Evjemo *et al.* 2001; Han *et al.* 2000; Hanaee *et al.* 2005; McEvoy *et al.* 1998; Monroig *et al.* 2006; Monroig *et al.* 2007; Payne and Rippingale 2000; Robin 1998; Tonheim *et al.* 2000; Woods 2003), steroid hormones (Stewart *et al.* 2001), enrofloxacin (Roque and Gomez-Gil 2003), vitamins (Monroig *et al.* 2007), the diatom *Chaetoceros muelleri* (Ritar *et al.* 2004), and probiotics including *Pediococcus acidilactici* and *Saccharomyces cerevisiae*

(Gatesoupe 2002), *Lactobacillus sporogenes* (Venkat *et al.* 2004), and *Bacillus* spp. (Ziaei-Nejad *et al.* 2006).

In addition, supplying live food in rearing systems may introduce opportunistic bacteria, which cause massive mortalities of fish and crustacean larvae (Keskin *et al.* 1994; Lightner 1983; Nicolas *et al.* 1989). A solution for this problem is to replace opportunistic bacteria with other less aggressive probiotic bacteria (Makridis *et al.* 2000). Other positive effects of probiotics is their ability to out-compete other unwanted bacteria (Austin *et al.* 1995). Developing techniques to reduce bacterial contamination on *Artemia* is an important step in the process of administering probiotics for the production of marine prawn larvae in hatcheries (Ritar *et al.* 2006). Although enriching live food with probiotics has a positive effect on the hosts by improving the properties of the indigenous microflora (Havenaar *et al.* 1992), overdoses of several probiotics induced immunosuppression in animals (Sakai 1999). To avoid the negative effects of an overload of probiotics, there needs to be an adjustment of the quantitative properties of added probiotics in live food (Keskin *et al.* 1994; Nicolas *et al.* 1989; Skjermo and Vadstein 1993). Therefore, encapsulation of *Artemia* nauplii is a strategy to convey customised probiotics to *P. latisulcatus* aquaculture. The encapsulation technique has proved to be applicable for the delivery of chemotherapeutics via live food stuffs with all the relevant advantages that this method implies for the treatment of fish larval diseases (Touraki *et al.* 1996).

Contamination of customised probiotics with other unwanted bacteria during an encapsulation of *Artemia*, needs to be prevented. Ozone is one of the disinfectants used for treating water in aquaculture (Arimoto *et al.* 1996; Coman and Sellars 2007; Coman *et al.* 2005). Another medium of tryptone soya broth (TSB) was also employed for encapsulating *Artemia* with customised probiotics in this study.

2.9 ENCAPSULATION MEDIA

2.9.1 Ozone

Ozone is a powerful oxidising agent and is used as an effective disinfectant against viral, bacterial and fungal pathogens in aquatic systems (Suantika *et al.* 2001;

Summerfelt and Hochheimer 1997), specially in crustacean aquaculture (Danald *et al.* 1979; Theisen *et al.* 1998). Ozone can act directly as a disinfectant to oxidize microbial pathogens in marine systems (Coman and Sellars 2007). Ozone can be used routinely for the disinfection of eggs from various marine finfish (Arimoto *et al.* 1996) and crustaceans (Coman *et al.* 2005). The reaction of ozone with the presence of halides of bromide ion generates more stable residual oxidant by-products of bromine and bromate which can provide longer lasting microbial disinfection (Grguric *et al.* 1994; Tango and Gagnon 2003). Among a wide range of disinfection agents that have been found to be effective for elimination of pathogens in marine culture systems viz., organic solvents, pH, salinity, heat, ultraviolet radiation, ozone and several chemicals (Liltved *et al.* 1995), ozone is considered the most powerful of these for eliminating virus (Liltved *et al.* 1995; Schuur 2003), bacterial and fungi pathogens (Matsumura *et al.* 1998; Wedemeyer 1996).

Ozone may be toxic to marine larvae at different levels (Tango and Gagnon 2003). Marine crustaceans tolerate low levels of ozonation (Blogoslawski *et al.* 1977; Jiang *et al.* 2001; Meunpol *et al.* 2003). Oxidation-reduction potential (ORP) of 400 to 500 mV was not harmful to rock lobster (*Panulirus cygnus*) larvae (Ritar *et al.* 2006). *P. japonicus* embryos were not affected by exposure to seawater at residual oxidant concentrations (ROC) of 1 or 2 mg/L for 8 or 1 min, respectively (Sellars *et al.* 2005). *P. monodon* embryos tolerated ozonated seawater up to 1 mg/L for 4 min (Coman and Sellars 2007). Moreover, excessive ozonation causes deformities and eventual death of prawns (Ritar *et al.* 2006). High ozonation doses significantly reduced the hatching rate of *P. japonicus* embryos (Sellars *et al.* 2005).

The toxicity of ozone depends not only on dosages but also on the duration of exposure. White spot syndrome virus infectivity was completely inactivated by exposure to ozone at ROC of 0.5 and 0.8 mg/L for 10 min, and partially reduced at 0.2, 0.5 and 0.8 mg/L for 2min (Chang *et al.* 1998). *P. monodon* PL reared in seawater at ROC between 0.34 and 0.50 mg/L for 8 h experienced no mortalities, whereas mortalities were recorded when exposed for longer than 10 h (Meunpol *et al.* 2003). Unfortunately, no data was found for the use of ozone can be found for the encapsulation of *Artemia* nauplii with customised probiotics for the cultivation of *P. latisulcatus*.

2.9.2 Tryptone soya broth (TSB)

Another medium used for encapsulation of *Artemia* nauplii with customised probiotics is TSB, which has a long used history in the microbiology area as a media for culture many bacteria. In aquaculture, TSB was commonly used as a means for studies on bactopathology in fish and prawns (Chand and Sahoo 2006; Chen *et al.* 2008; Cheng and Chen 2002; Cheng *et al.* 2003; Cheng *et al.* 2002; Suhalim *et al.* 2008). TSB was used as a medium for isolation of *V. harveyi* bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments (Vinod *et al.* 2006). TSB was used for screening microbial cultures for 4-deoxynivalenol transformation ability (Guan *et al.* 2009), used for storage of a stock culture of *Aeromonas hydrophila* for further use (Harikrishnan *et al.* 2003). TSB was also used for regrowth of probiotics *Shewanella* sp. Pdp11 and *Shewanella* sp. Pdp13 (Díaz-Rosales *et al.* 2009), *Carnobacterium maltaromaticum* B26 and *C. divergens* B33 (Kim and Austin 2006). There are no available references on the use of TSB as a medium for encapsulation of *Artemia* nauplii with customised probiotics.

CHAPTER 3

SELECTION OF PROBIOTICS[1]

3.1 INTRODUCTION

Western king prawns are widely distributed throughout the Indo-west Pacific region (Dore and Frimodt 1987). They are considered a suitable species for aquaculture (Kathirvel and Selvaraj 1987) due to their established markets in Asia and Australia (Andrews and Bowen 1992) and their ability to tolerate a wide range of environmental conditions of salinity and temperature (Penn 1980; Ramasamy and Pandian 1984).

Historically, intensive prawn culture practices involve an indiscriminate use of chemicals and antibiotics leading to environmental degradation (Bachère 2000). However, past research has shown that probiotics could replace these chemicals and antibiotics (Li *et al.* 2006; Rengpipat *et al.* 1998a) and assist in the protection of disease-free aquaculture species from known pathogens (Gullian *et al.* 2004; Moriarty 2003; Rengpipat *et al.* 2000). Non-pathogenic strains of *Vibrio, Aeromonas, Pseudomonas* and *Alteromonas* that have known antagonistic properties to pathogenic strains can be used as probiotic strains (Irianto and Austin 2002a; Moriarty 2003).

Although probiotics offer a promising alternative to chemicals and antibiotics in marine prawn culture (Li *et al.* 2006; Rengpipat *et al.* 1998a; Vaseeharan and Ramasamy 2003), the selection of probiotics for specific marine aquatic species has to be considered carefully in order to make them species specific. Unfortunately, many probiotic products being sold contain inappropriate species/strains of bacteria and/or inadequate population densities (Balcázar *et al.* 2006a; Moriarty *et al.* 2005) required for specific aquatic species. Some spore-forming bacterial species produce a wide range of antagonistic compounds that can be valuable as probiotics (Moriarty 2003). No research has been conducted using various inhibition test methods to

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establish a protocol for selecting probiotics suitable for western king prawn culture. The aim of this study is to compare five inhibition test methods so that the most suitable one can be used to develop a protocol to select probiotics that can significantly reduce the growth of *Vibrio* species known to be pathogenic for western king prawns.

3.2 MATERIALS AND METHODS

3.2.1 Experimental samples

Ten wild western king prawns (4.02 ± 0.86 g), which were active and showed no sign of disease, and samples of surrounding water were collected from Peel Inlet, Mandurah, Western Australia (32°55'S 115°43E) and transported to the Animal Health Laboratories (AHL), Department of Agriculture and Food, Western Australia.

Probiotic strains isolated from two commercial probiotic products, Enviro-Pro Liquid Pro-Biotic Bacteria from Enviroplus, Perth, Western Australian and Sanolife^{R} from INVE Ltd., Thailand were used to test their ability to inhibit growth of bacteria isolated from the western king prawns and water samples. In addition, eight pathogenic *Vibrio* species previously isolated from diseased aquatic animals and stored in the culture collection at the AHL were also tested for growth inhibition by the probiotics and were coded from 16 to 23. They were 2 strains of *Vibrio splendidus* (identification numbers AS-02-3465-2 and 3) in tiger prawns (*Penaeus monodon*), 2 strains of *V. alginolyticus* (AS-02-3465-6 and AS-02-3916-4) in *P. monodon* and 1 additional strain (AS-02-654-5) of *V. alginolyticus* in sea hare (*Aplysia californica*), 2 strains of *V. harveyi* (AS-02-354-8 and AS-01-1738-3) in pearl oyster (*Pinctada maxima*) and mahi mahi (*Coryphaena hippurus*) and 1 strain of *V. parahaemolyticus* (AS-01-1738-1) in *C. hippurus*.

3.2.2 Isolation, identification and storage of bacteria

Bacteria from western king prawns, water samples and commercial probiotic products were isolated and identified by traditional biochemical test methods (Buller 2004; Farmer III and Hickman-Brenner 1992; Sneath *et al.* 1986) (*Appendix 1*). The

prawn samples were washed with 70% ethanol, followed by a distilled water wash. Hepatopancreas, gills and intestinal tracts of the western king prawns were dissected out and homogenised. Bacteria were isolated on MSA (Buller 2004), Marine Sea Salt Agar (MSSA) (Buller, personal communication, 2006), Thiosulfate-Citrate-Bile Salt Sucrose Agar (TCBS) (OXOID Australia), Blood Agar (BA) and MacConkey Agar (MCA) (OXOID Australia). Culture plates were incubated at 25 ^{o}C for 24, 48 and 72 h. The bacteria isolated from western king prawns and water samples were coded 1 to 15. None of these species could be positively identified to a known species. The bacteria isolated from probiotic products were coded A to F. Bacteria isolated from the prawns and the probiotic solutions were suspended into individual 1 mL aliquots of GLL (Glycerol Lab Lemco broth) (Buller 2004) and stored at -80 ^{o}C.

3.2.3 Methods for detecting growth inhibition of *Vibrio* spp. by probiotic strains

All *Vibrio* spp. isolated from the western king prawns and the nine pathogenic *Vibrio* species from the AHL culture collection were regrown on MSA media. A single colony at log phase of growth was selected from the culture plates and inoculated into 10 mL normal saline (NS).

BLIS (Bacteriocin-like inhibitory substance) or cross-streaking method (Chythanya *et al.* 2002; Gibson *et al.* 1998)

A single streak (0.5 cm in width) of each probiotic strain was made on MSA plates and plates were incubated at 25 ^{o}C for 72 h. The resulting growth on the plates was removed by scraping, using a sterile glass slide covered with sticky tape so as to minimise excessive scratching on the surface of the agar. Any remaining growth was killed by chloroform exposure. Chloroform (70%) was poured onto mats (roar reinforce wipers, Kimberley Clarke), which were placed in the base of a metal tray. The agar plates were inverted over racks placed across the tray, and left for 30 min. The plates were then air-dried for 10 min to remove any residual chloroform vapour.

The *Vibrio* spp. were streaked (one to five strains per plate) at least 5.5 cm in length at right angles to the streak of the probiotic being tested. The plates were further incubated for 24 h at 25 ^{o}C. The width of inhibition zones of each *Vibrio* species was

measured in millimetres and recorded. The inhibition zones were considered significant if they were found to be twice the width of the streak of the probiotic strain, which was typically greater than 10 mm after incubation. A *Vibrio* species that grew across the streak of the probiotic strain with no growth inhibition was considered resistant, whereas no growth of a *Vibrio* sp. was recorded as sensitive to the probiotics.

The modified BLIS method

Optical density (OD) of the inoculum was measured so as to obtain a known number of bacterial cells. Neat suspensions of each bacterial strain were prepared from the log phase culture on MSA. The suspensions were serially diluted using NS to obtain concentrations of 10, 10^{-1}, 10^{-2} and 10^{-3} CFU/mL. The ODs of the suspensions were measured using a spectrophotometer at wavelength of 560 nm, and 10 mL of the suspension was lawn inoculated onto MSA plates. The total bacteria counts (TBC) were recorded using the dilution that produced approximately 30 to 300 colonies per plate. A standard curve for estimating bacterial cell density was constructed by plotting OD as a function of TBC.

Each *Vibrio* species and probiotic strain being tested was suspended in NS, then adjusted to obtain a recommended OD of 10^3 CFU/mL (Garriques and Arevalo 1995; Gomez-Gil *et al.* 2000; Vaseeharan and Ramasamy 2003). The plates streaked with the inoculated probiotic strains (10^3 CFU/mL) were incubated for 72 h at 25 ^{o}C. The rest of the procedure for this test was the same as the BLIS method and after the addition of the *Vibrio* species the plates were incubated for a further 24 h at 25 ^{o}C. The inhibition zones between the *Vibrio* spp. and probiotic strains were recorded after 24 h of incubation at 25 ^{o}C.

Well-diffusion method (Chythanya *et al.* 2002; Vaseeharan and Ramasamy 2003)

The cultures of the *Vibrio* strains were prepared by pouring 2 mL of the inoculum (10^3 CFU/mL in NS at log phase) onto MSA plates to completely cover the surface of the agar. Excess solution was removed and drained before air-drying for 15 min in an incubator set at 30 ^{o}C. Six-mm diameter wells were punched into the agar using

pipette tips, which were cut to obtain a 6-mm diameter bore and then sterilised. Twenty microliters of each probiotic inoculum (10^3 CFU/mL) was carefully pipetted into each well. Two probiotics were tested per plate in triplicate. The diameter of the inhibition zones around the wells were recorded in millimetres after incubating the plates for 24, 48 and 72 h at 25 ^oC.

Disc-diffusion method (Chythanya *et al.* 2002)

A cell-free supernatant of each probiotic strain (10^3-10^7 CFU/mL) was obtained by centrifuging (Eppendorf Centrifuge 5804R) at 5,000 rpm for 10 min followed by filtration through a 0.20 μm Millipore membrane (MFS 25 disposable syringe filter unit). Blank sterile discs of 6 mm diameter (BBL^{TM}, Becton, Dickinson and Company Sparks, MD 21152 USA) were dipped into the cell-free supernatant and dried in an incubator for 15 min at 37 ^oC. The impregnated discs were placed onto MSA plates, which had been lawn inoculated with 10 μL suspension of each *Vibrio* species (10^3 CFU/mL). Two probiotic strains were tested in triplicate per plate, and plates were incubated for 24, 48 and 72 h at 25 ^oC. The ability of each probiotic strain to inhibit the growth of individual *Vibrio* spp. was determined by measuring the diameter in millimetres of the clear inhibition zone formed around the discs.

Co-culture method (Vaseeharan and Ramasamy 2003)

Probiotic strains and 17 selected *Vibrio* spp., visibly different from each other in size, shape and colour of the colonies and presence or absence of haemolysis, were used in the co-culture method. The initial cell density of selected *Vibrio* strains was approximately 10^3 CFU/mL, whereas the initial concentration of probiotic strains was 10^3, 10^5 or 10^7 CFU/mL. All inoculums were made in tryptone soya broth (TSB) (OXOID, Australia) containing a final concentration of 2% NaCl (Buller 2004). Ten millilitres of each *Vibrio* inoculum and each probiotic inoculum was mixed together, and then incubated for three days at 25 ºC. Aliquots from each broth were analysed daily to determine the density of each probiotic and *Vibrio* species. The individual TBC for each probiotic and tested *Vibrio* species was estimated by preparing 10-fold serial dilutions in triplicate. The differences in TBC between initial

inoculation and final concentration of the probiotic and *Vibrio* species were used to determine if the probiotic inhibited the growth of the *Vibrio* species being tested.

Further identification of two probiotics that showed antagonism to Vibrio spp

Probiotic strain B and C were identified by biochemical tests as *Pseudomonas* species. Further identification was performed using the Biolog MicroLog3 4.20 system at the Plant Pathology Laboratories, Department of Agriculture and Food, Western Australia (*Appendix 2*). A 100% probability was achieved for accurate identification when a SIM was at least 0.500 at 24 h.

3.3 RESULTS

Eight, six, seven and seven of the 15 different *Vibrio* spp. were isolated and identified to genus level in the gills, hepatopancreas (HP), intestine and water samples, respectively. The *Vibrio* spp. did not identify to any known species. Five *Pseudomonas* spp. and three *Bacilli* spp. were isolated from the commercial probiotic products I and II, respectively.

Only two (probiotics B and C identified as *Pseudomonas* sp.) out of the nine probiotic species inhibited the growth of all the *Vibrio* tested. These two probiotics were different from each other by size, shape and colour of the colonies. They were selected for testing in the following inhibition tests. The two other isolated probiotic species, *Pseudomonas* sp. and *Bacillus* sp., produced inhibition to only one and three *Vibrio* spp., respectively.

Although probiotic C produced a greater inhibitory effect against the *Vibrio* spp. than probiotic B for the three incubation periods (24, 48 and 72 h), the inhibition levels between the probiotics and *Vibrio* spp were not significantly ($P>0.05$) different between probiotic B and C at the same incubation periods (Table 3.1). The longer the incubation period the greater the inhibition against the *Vibrio* spp. was found. The inhibitory levels of the probiotics against the *Vibrio* spp. were significantly ($P<0.05$) different for the three incubation periods, except probiotic B when incubated for 48 and 72 h ($P>0.05$) (Table 3.1).

Table 3.1
The mean ± SE of the growth inhibition zone (mm) for each probiotic when tested against *Vibrio* spp. at different incubation periods of probiotics in the modified BLIS, well-diffusion and disc-diffusion methods

Incubation periods	Modified BLIS method		Well-diffusion method		Disc-diffusion method			
	Probiotic B	Probiotic C	Probiotic B	Probiotic C	Probiotic B		Probiotic C	
	10^3 CFU/mL	10^3 CFU/mL	10^3 CFU/mL	10^3 CFU/mL	10^3 CFU/mL	10^7 CFU/mL	10^3 CFU/mL	10^7 CFU/mL
24 h	$_1$1.67±0.12^a	$_1$1.87±0.16^a	$_1$0.97±0.04^a	$_1$0.96±0.04^a	-	$_1$8.08±0.10^a	-	$_1$9.23±0.23^b
48 h	$_1$2.45±0.18^b	$_1$2.76±0.19^b	$_1$1.05±0.05^a	$_1$1.07±0.05^{ab}	-	$_1$8.23±0.12^a	-	$_1$9.46±0.23^b
72 h	$_1$3.36±0.26^b	$_1$3.67±0.16^c	$_1$1.07±0.05^a	$_1$1.10±0.06^b	-	$_1$8.31±0.16^a	-	$_1$9.46±0.24^b

Values (mean ± SE) in any one row preceded by different subscript numbers in each method are significantly different at $P<0.05$. Values (mean ± SE) in any one column with different superscript letters in each method are significantly different at $P<0.05$. The mark "_" is resistant

Table 3.2
Cell densities (mean ± SE) (CFU/mL) of probiotics and the *Vibrio* spp. at the different incubation periods when probiotics were administered at the initial densities of 10^3, 10^5 and 10^7 CFU/mL in the co-culture method

Probiotics	Incubation periods	10^3 CFU/mL		10^5 CFU/mL		10^7 CFU/mL	
		***Vibrio* spp.**	**Probiotic B**	***Vibrio* spp.**	**Probiotic B**	***Vibrio* spp.**	**Probiotic B**
B	24 h	$_1$1.9 ± 0.1x 10^{3a}	$_1$2.3 ± 0.1 x 10^{3a}	$_1$1.8 ± 0.1 x 10^{3a}	$_2$2.3 ± 0.1 x 10^{6a}	$_1$1.9 ± 0.1 x 10^{3a}	$_2$2.1 ± 0.1 x 10^{8a}
	48 h	$_1$2.1 ± 0.1x 10^{3a}	$_2$2.7 ± 0.1 x 10^{3b}	$_1$2.0 ± 0.1 x 10^{3a}	$_2$2.8 ± 0.1 x 10^{6b}	$_1$2.2 ± 0.1 x 10^{3a}	$_2$2.6 ± 0.9 x 10^{8b}
	72 h	$_1$2.5 ± 0.2 x 10^{3a}	$_2$3.3 ±0.1 x 10^{3c}	$_1$2.2 ± 0.1 x 10^{3a}	$_2$3.2 ± 0.1 x 10^{6c}	$_1$2.5 ± 0.3 x 10^{3a}	$_2$3.2 ± 0.2 x 10^{8c}
		Vibrio spp.	Probiotic C	*Vibrio* spp.	Probiotic C	*Vibrio* spp.	Probiotic C
C	24 h	$_1$1.9 ± 0.1 x 10^{3a}	$_1$2.1 ± 0.1 x 10^{3a}	$_1$1.9 ± 0.1 x 10^{3a}	$_2$2.3 ± 0.1 x 10^{6a}	$_1$2.0 ± 0.1 x 10^{3a}	$_2$2.1 ± 0.1 x 10^{8a}
	48 h	$_1$2.2 ± 0.1 x 10^{3a}	$_2$2.8 ± 0.1 x 10^{3b}	$_1$2.1 ± 0.1 x 10^{3a}	$_2$2.8 ± 0.1x 10^{6b}	$_1$2.2 ± 0.1 x 10^{3a}	$_2$2.7 ± 0.1 x 10^{8b}
	72 h	$_1$2.5 ± 0.2 x 10^{3a}	$_2$3.4 ± 0.1 x 10^{3c}	$_1$2.2 ± 0.1 x 10^{3a}	$_2$3.3 ± 0.1 x 10^{6c}	$_1$2.3 ± 0.1 x 10^{3a}	$_2$3.3 ± 0.1 x 10^{8c}

Values for each probiotics initial cell density in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column not followed by the same superscript letters are significantly different at $P<0.05$.

Probiotic C produced greater inhibitory zones than probiotic B over the longer incubation periods. No significant difference (P>0.05) was observed between the three incubation periods for probiotic B, but significant differences (P<0.05) occurred between 24 and 72 h incubation for probiotic C (Table 3.1). The inhibition zones of the probiotics and the *Vibrio* spp. were not significantly (P>0.05) different between probiotic B and C at the same incubation periods (Table 3.1).

No inhibition of the *Vibrio* spp by the probiotics was observed at different incubation periods using the cell-free supernatants extracted from the probiotic cell density of 10^3 CFU/mL (Table 3.1). At a probiotic cell density of 10^7 CFU/mL only weak inhibition was recorded. Thirteen of the 23 *Vibrio* spp. were sensitive to the probiotic at this concentration, but the inhibition zones were not significantly different between incubation periods in both probiotics B and C. The inhibition zones (including the 6mm diameter discs) for the *Vibrio* spp. ranked from 7 to 10 mm and 8 to 11 mm for probiotic B and C respectively. Probiotic C showed a significantly higher (P<0.05) inhibition to the *Vibrio* spp. than probiotic B at the same incubation periods.

Vibrio spp. grew (10^3 CFU/mL) in the presence of the probiotics (10^3-10^7 CFU/mL), but cell densities of the *Vibrio* spp. never reached above their inoculation level of 10^3 CFU/mL. Cell densities of the *Vibrio* sp did not differ significantly (P>0.05) for the three incubation periods in all the initial probiotic densities of 10^3-10^7 CFU/mL. The probiotic cell densities, however, were significantly (P<0.05) different between incubation periods at all the probiotic initial densities (Table 3.2).

There were significant (P<0.05) differences between the cell densities of the probiotics and *Vibrio* spp. at the three incubation periods in all the probiotic initial densities, except at the first 24 h of incubation at the probiotic initial density of 10^3 CFU/mL (P>0.05) (Table 3.2).

The initial cell density of 10^3 CFU/mL was suitable for the BLIS, modified BLIS and co-culture methods, but the well-diffusion and disc-diffusion methods required a higher cell density of the probiotics to obtain effective inhibition. The sensitivity/resistance between the probiotics and *Vibrio* spp. could not be recoded

when all the *Vibrio* spp. did not have an equal opportunity to be tested with the probiotics in the co-culture method. The sensitive levels could not be used to compare between the *Vibrio* spp. because of their unknown initial cell densities in the BLIS method. Confusion on the degree of sensitivity between the probiotics and *Vibrio* spp. occurred when the probiotic initial cell density was lower than that of the *Vibrio* spp. in the well-diffusion and disc-diffusion methods (Table 3.3).

Table 3.3
Summary characteristics of five inhibition test methods

Methods	**Initial density (10^3 CFU/mL)**	**Sensitivity/Resistance**	**Degree of Sensitive levels**
BLIS	+	++	-
Modified BLIS	+	+++	+
Well-diffusion	- to +	+	- to +
Disc-diffusion	-	+	- to +
Co-culture	+	- to +	+

The symbols +++, ++ and + indicate highest, moderate and low response, respectively, and – indicates no response.

The probiotics B and C were identified as *Pseudomonas synxantha* and *P. aeruginosa*, respectively. The probability of accurate identification was 100% with the correct SIM levels of 0.504 for *P. synxantha* and 0.702 for *P. aeruginosa*.

3.4 DISCUSSION

In Manglaralto-Ecuador *Vibrio* spp. make up only 25% of the bacterial population isolated from wild prawns (30 ± 5.0 g) (Gullian *et al.* 2004), whereas *Vibrio* was the dominant genus (94%) in our study. As western king prawns were active and showed no symptoms of disease, they were considered to have normal microflora. Therefore, virulent strains were obtained from the AHL in order to understand the inhibition impact of the selected probiotics on both normal and virulent microflora. Persistent communities of both normal and harmful microflora exist in the midgut and hepatopancreas of prawns (Moriarty 2003). Stress and other factors can cause an imbalance in the population structure of the harmful bacteria such as *Vibrio, Flavobacterium, Pseudomonas, Alteromonas* and *Aeromonas* species (Moriarty *et al.* 2005), resulting in disease which can lead to severe prawn mortalities (Moriarty 2003). Fermenting bacteria, which include *Vibrio*, can become active and release

organic acids used by sulphate-reducing bacteria in prawn ponds, some organic acids are toxic to prawns (Moriarty *et al.* 2005). *Vibrio* can also become resistant to antibiotics such as chloramphenicol, furazolidone, oxytetracycline and streptomycin in formulated feed (Moriarty 1999).

Many available probiotics used in prawn farming contain *Lactobacillus* species that are also produced for human and terrestrial animals, and may not be appropriate for marine animals (Moriarty 2003). In Asia, some probiotics are advertised to contain *Clostridium, Pseudomonas putida, P. aeruginosa*, however these are usually ineffective in reducing pathogenicity in aquatic species (Moriarty 2003). Although a large number of spore-forming probiotics containing *Bacillus* spp. are in use (Hong *et al.* 2005), only two genera *Bacillus* and *Pseudomonas* were isolated and identified from the commercial probiotic tested in this study. *Bacillus subtilis* and *B. licheniformis* are used commercially as probiotics in aquaculture, mainly for prawns (Moriarty 2003). Another bacterial species *Pseudomonas*, present in some probiotic solutions, acts as a potential probiotic for marine prawns and has caused growth inhibition of a number of pathogens such as *Salmonella, Staphylococcus aureus* and *Vibrio parahaemolyticus, V. harveyi, V. fluvialis, Photobacterium damselae* (previously *V. damsela*), *V. vulnificus* and *Aeromonas* spp. (Chythanya *et al.* 2002; Daly *et al.* 1973; Goatcher and Westhoff 1975; Oblinger and Kreft 1990; Vijayan *et al.* 2006).

Among the five methods employed in the current study, the modified BLIS method proved to be the most suitable for determining not only sensitivity/resistance but also the degree of sensitivity between different *Vibrio* strains (at a constant density of 10^3 CFU/mL) and probiotics. The modified BLIS method is also simple and relatively practical compared to other methods. It has the advantage of testing the cell density to determine an appropriate concentration for use in the field. This method also allows a comparison of inhibitory effectiveness for different probiotics tested on the same range of pathogenic bacteria.

Likewise, the well-diffusion method can also test the degree of inhibition of the *Vibrio* spp. by the different probiotics. In the well-diffusion method the probiotics

required a higher initial density (inoculum) than the *Vibrio* spp. to achieve effective inhibition of the *Vibrio* spp.

The BLIS method is easy to perform. This method provides a rapid means of eliminating inappropriate probiotics by testing the inhibitory capacity of the probiotics against the selected bacteria. However, the degree of sensitivity between the *Vibrio* spp. and probiotics cannot be compared because the BLIS method does not use a known cell density of the *Vibrio* spp.

The disc-diffusion method is less suitable as a test for determining probiotic effectiveness against certain bacteria. Incorrect data on sensitivity or resistance between probiotics and bacteria may result if cell-free supernatants from probiotics are at densities too low to be effective (Smith 2006). In this study we found that the initial inoculum density of the probiotic needs to be at a higher concentration than that of the bacteria to accurately determine the effectiveness of the probiotic against that bacterium.

The co-culture method failed to provide a complete picture of inhibition capacity of probiotics to all isolated bacterial species. The co-culture method may be suitable for testing specific probiotics against specific pathogenic strains (Vaseeharan and Ramasamy 2003). This method investigated the dominance of the selected probiotics B and C at different densities to the *Vibrio* spp. In fact, all the *Vibrio* spp. did not have an equal opportunity to be tested with the probiotics due to their characteristics. For example, some swarming *Vibrio* strains could not be tested in this method because it was impossible to obtain an accurate TBC because of the swarming nature of the *Vibrio* spp. Other *Vibrio* spp. could not be tested because their colonies were indistinguishable visually from the colonies of the probiotics. This method is only suitable for testing probiotics and bacteria if selective media is available for each bacterium tested (including the probiotic).

The probiotic inoculum density, growth time of probiotic prior to the addition of bacteria, and incubation periods are factors to be considered when testing the inhibition capacity of specific probiotics. *Vibrio parahaemolyticus* at a density of 10^3 CFU/mL causes 100% mortality of *Litopenaeus vannamei* in hatcheries (Garriques

and Arevalo 1995). Probiotics at this density were found to be effective in inhibiting bacteria when tested by the BLIS, modified BLIS and co-culture methods. When probiotics were tested by the well-diffusion and disc-diffusion methods, higher concentrations of probiotic were required to inhibit bacteria under test.

Sensitivity or resistance between the probiotics and *Vibrio* spp. can be recorded when the probiotics and *Vibrio* spp were applied at the initial cell density of 10^3 CFU/mL in the BLIS, modified BLIS and co-culture methods, whereas the initial densities of probiotics need to be higher than the *Vibrio* spp. in the well-diffusion method. This is due to the fact that probiotics and *Vibrio* spp. appeared at the same time.

In the disc-diffusion method, the cell-free supernatants from the probiotic suspensions may contain antimicrobial compounds, but the concentrations of these compounds may not be sufficient to affect the growth of the *Vibrio* spp. Inhibition zones were seen when the cell-free supernatants were prepared from probiotics at 10^7 CFU/mL, but only 56.52% of the *Vibrio* spp. were sensitive to the probiotics at that concentration. At 10^3 CFU/mL, the cell-free supernatants from the probiotics did not affect the growth of the *Vibrio* spp. as no inhibition zone was observed during the incubation periods. The results may lead to confusion due to the probiotics inhibiting the growth of *Vibrio* spp. in all other inhibition test methods. Chythanya et al. (2002) showed that *Pseudomonas* I-2 can produce some extracellular anti-vibrio component with wide inhibition zones against several *Vibrio* species, but low volumes of cell free supernatant of *Pseudomonas* I-2 added to *Vibrio harveyi* had no inhibitory effect.

In the co-culture method, if *Bacillus* strains (probiotics) produced antimicrobial compounds that can inhibit bacteria, then the mortality rates of bacteria may increase (Moriarty 1999). Probiotics at 10^5-10^7 CFU/mL showed their dominant capacities to the *Vibrio* spp. Shifts in the dominance of probiotic strains may occur, even if antimicrobial compounds are not produced at a concentration to kill the majority of bacteria strains. Dominant strains may also change (Moriarty 1999). Therefore, application of probiotics at suitable initial cell densities is needed to produce effective antimicrobial compounds. However, the quantitative properties of the probiotic must be adjusted to avoid negative effects of an overload of probiotic

(Keskin *et al.* 1994; Nicolas *et al.* 1989; Skjermo and Vadstein 1993) and accomplish successful colonization at the same time (Munro *et al.* 1999).

The initial probiotic densities need to be higher than the *Vibrio* spp., because an antagonist (probiotic) must be present at significantly higher densities than a pathogen, as the degree of inhibition increases with a higher density of antagonist (Vaseeharan and Ramasamy 2003). Probiotics must be applied in advance, because the longer incubation period of the probiotics, produced larger the inhibition zones between the probiotics and *Vibrio* spp. in the modified BLIS, well-diffusion and disc-diffusion methods. The probiotics B and C may change the existing microbial community structure through the competition exclusion principle described by Smith (1993). Thus competition could be as a result of secretion of antimicrobial compounds, which can increase the mortality rates of existing microbial flora (Moriarty 1999). In the disc-diffusion method, the *Vibrio* spp. may outgrow the probiotic or the probiotic might not have sufficient time to produce antimicrobial compounds at a concentration that would inhibit the *Vibrio* spp.

Each probiotic gives different results at different incubation periods. In the modified BLIS method, if growth of probiotics is rapid in a chosen media, an inhibition test might only require one day of incubation before addition of test bacteria. During the incubation, the probiotics can produce sufficient antimicrobial compound to inhibit the growth of the *Vibrio* spp. The well-diffusion method required a different incubation period, because probiotic C produced greater inhibition of the *Vibrio* spp. than probiotic B, although the probiotic and *Vibrio* spp. were inoculated at the same cell-densities. Probiotic C grew and significantly dominated ($P<0.05$) the *Vibrio* spp. during the incubation periods. Probiotic B did not cause as great an inhibition of the bacteria as probiotic C. In the disc-diffusion method, the effects of the antimicrobial compounds on the *Vibrio* spp. significantly differed from probiotic B to C. Probiotic C produced a stronger inhibition on the growth of the *Vibrio* spp. than probiotic B.

According to the BLIS method, only two *Pseudomonas* strains (B and C) out of the three *Bacillus* and six *Pseudomonas* probiotic strains were effective at inhibiting the *Vibrio* spp. *Bacillus* are shown to improve the survival and growth of rainbow trout, *Oncorhynchus mykiss*, larvae and increases food absorption by enhancing protease

levels (Irianto and Austin 2002a; Moriarty 1999). *Bacillus* spp. also produces inhibition of other pathogenic bacteria in fish and shellfish (Gatesoupe 1999; Rengpipat *et al.* 2000). *Bacillus* species colonise the gut of the animal and compete successfully against pathogenic *Vibrio* species (Irianto and Austin 2002a; Moriarty 2003). *Bacillus subtilis* BT23 produced a greater inhibitory effect against growth of *Vibrio harveyi, Listonella (Vibrio) anguillarum, V. vulnificus* and *Photobacterium (Vibrio) damselae* than other *Bacillus* spp. tested (Vaseeharan and Ramasamy 2003). *Pseudomonas* I-2 is antagonistic to the prawn pathogens *Vibrio harveyi, V. fluvialis, V. parahaemolyticus, V. vulnificus* and *Photobacterium (Vibrio) damselae* and is a potential probiotic for marine prawns (Chythanya *et al.* 2002).

Probiotics B and C inhibited the *Vibrio* spp. in the tests conducted in this study. They were identified as *Pseudomonas synxantha* and *P. aeruginosa*, respectively. Based on the inhibition tests, *P. aeruginosa* proved to be more inhibiting than *P. synxantha*. Pseudomonads are one of the commonly associated genera in water. *P. aeruginosa* strain I-2 isolated from estuarine water showed an inhibitory effect against the pathogens *Vibrio harveyi, V. fluvialis, V. parahaemolyticus, Photobacterium* (*Vibrio*) *damselae* and *V. vulnificus* (Chythanya *et al.* 2002). An important criterion for a candidate probiotic for use as a biocontrol is that it should be non-pathogenic to the host. *Pseudomonas aeruginosa* strain I-2 meets these requirements as prawn larvae mortality was not observed at high levels (10^6 CFU/mL) of this probiotic strain (Chythanya *et al.* 2002).

With the inhibition methods tested in this study it was noted that probiotics must be given enough time to produce the antimicrobial substances before the addition of the bacteria under test, and that a better result of inhibition can be obtained when the probiotic inoculum concentration is higher than that of the bacteria. The initial probiotic density of 10^3 CFU/mL is suitable for the modified BLIS and co-culture methods, but a higher inoculum concentration was required for effective inhibition when tested by the well-diffusion and disc-diffusion methods.

Based on the modified BLIS method, a recommended protocol for the selection of probiotics for aquatic animals is described below:

- Test bacteria: Choose bacteria for inhibition tests either isolated from aquatic animals, probiotics or available from laboratories.
- Use suspensions of probiotic strains at a concentration of 10^3 CFU/mL in log phase of growth, which is usually between 18-24 h of incubation.
- Streak one line (0.5 cm in width) of each suspension of probiotics through the diameter plates of chosen media and incubate at 25^o C for 72 h.
- Remove the lawn probiotics from the plates using sterile slides covered with sticky tape.
- Remove any remaining growth of probiotic on the plates by inverting racks across the trays which contain chloroform (70%) and leave the plates for 30 min.
- Remove the residual chloroform vapour by air-drying for 10 min.
- Produce suspensions of 10^3 CFU/mL each bacterial strain at the log phase of growth curve between 18-24 h.
- Streak one line (0.5 cm in width and at least 5.5 cm in length) of each suspension of bacterial strains (one to five bacterial strains per plates) at right angles to the streaks of the probiotics and incubate further at 25 ^{o}C for 24 h.
- Determine resistant or sensitive levels between the selected probiotics and bacterial strains by measuring inhibition zones between them. The typical sensitivity should be greater than 10 mm

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CHAPTER 4

EFFECTS OF CUSTOMISED PROBIOTICS ON THE CULTIVATION OF WESTERN KING PRAWNS [2]

4.1 INTRODUCTION

The western king prawn, *Penaeus latisulcatus*, is a popular species in Australia and Japan and is widely distributed throughout the Indo-West Pacific region (Dore and Frimodt 1987). Attempts have been made to culture this species in China (Wang *et al.* 2004), Japan (Shokita 1970, 1984), Thailand (Ling 1973), India (Kathirvel and Selvaraj 1987; Kathirvel *et al.* 1986) and Australia (Pownall 1973, 1974; Prangnell 2007; Prangnell and Fotedar 2005; 2006b; 2004a; Sang and Fotedar 2004b). Increasing demand for aquaculture production has led to an indiscriminate use of chemicals and antibiotics in aquaculture resulting in environmental degradation (Bachère 2000). As a result, there is a need to find alternatives to chemicals and antibiotics for use in aquaculture (Li *et al.* 2006; Meunpol *et al.* 2003; Vaseeharan and Ramasamy 2003). These alternatives include using probiotics during rearing of aquatic species either as water additives or as feed supplements (Moriarty 1998; Skjermo and Vadstein 1999).

Probiotics can be applied either as single or mixed strains (Skjermo and Vadstein 1999). The effectiveness of probiotics on the health status of the hosts can be assessed by measuring the various immune parameters of the prawn haemolymph (Fotedar *et al.* 2001; Jussila *et al.* 1997). Hai *et al.* (2007) tested various inhibition methods for detecting the most appropriate commercial probiotics for use against bacteria in *P. latisulcatus*. Further research is required to investigate the effective dose of probiotics and the application method that could lead to improved health of the cultured prawns.

Previous applications of probiotics have proved beneficial to the host by improving growth, survival and health (Moriarty 1998; Skjermo and Vadstein 1999). Probiotics

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used as immunostimulants stimulate cellular and humoral (Sakai 1999) components of the immune systems of crustaceans (Fotedar *et al.* 2001). Stimulation of non-specific defence mechanisms enhances disease resistance and growth of the hosts (Skjermo *et al.* 2006). Hai *et al.* (2007) recently established a protocol for selecting probiotics that produced a higher level of inhibition of bacteria and pathogens commonly associated with disease in western king prawns. No information is available on the effect of these probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the health and immune system of western king prawns.

The aim of this study was to examine the effect of the previously tested *Pseudomonas synxantha* and *P. aeruginosa* (Hai *et al.* 2007) on the specific growth rate (SGR), survival, probiotic loads in rearing medium and immune parameters such as the total haemocyte count (THC), hyalinocytes (HC), semi-granulocytes (SGC) and granulocytes (GC), haemolymph clotting time and intestinal bacterial load in juvenile western king prawns.

4.2 MATERIALS AND METHODS

4.2.1 Probiotic culture

The two probiotics *Pseudomonas synxantha* and *P. aeruginosa* isolated from commercially available probiotic products of Enviro-Pro Liquid Pro-Biotic Bacteria (Enviroplus, Perth, Western Australia), and then identified by biochemical tests and Biolog MicroLog3 4.20 system were selected from our previous study (Hai *et al.* 2007). The probiotic solutions were suspended in 1 mL aliquots of GLL (Glycerol Lab Lemco broth) and stored at -80 ^{o}C (Buller 2004). They were regrown on Marine Salt Agar (MSA) and then inoculated into tryptone soya broth (TSB, OXOID, Australia) supplemented with 2% NaCl (Buller 2004), for 12 h at 25 ^{o}C and used as a stock probiotic broth. The stock culture was resuspended in a saline solution supplemented with 2% NaCl and serially diluted. Each dilution was lawn inoculated to an MSA plate. After overnight incubation at 25 ºC, the colonies were counted and a total bacterial count (TBC) was obtained for the original inoculum. A standard curve for estimating bacterial density was plotted using optical density (Spectrophotometer, BOECO S-20, Germany) as a function of TBC (Hai *et al.*

2007). An appropriate inoculum was calculated and added to each treatment to obtain the desired initial densities at the commencement of the experiment and immediately after every water exchange.

4.2.2 Experimental animals and setup

Western king prawn juveniles (4.54 ± 0.59 g in weight and 7.40 ± 0.37 cm in total length) were collected from the Mandurah Creek, Western Australia (32°32'57 S; 115°42'58 E) and the Canning River, Western Australia (32°00'39 S; 115°51'15 E). They were kept in the Curtin Aquatic Research Laboratory and fed a prawn feed ST#1 (43% protein, 6% fat and 2% fibre) (Ridley Aqua-Feed, Ridley AgriProducts Pty. Ltd, Queensland, Australia) until the commencement of the experiment.

Twenty one 70-L plastic tanks were filled with 15 L of ozonised water at 35 mg/L. Seawater was ozonised at 700 mV by an ozone generator (ZOX, model AQ-2KP, Environplus, Western Australia). All the tanks were connected to independent filtration units allowing the water to circulate. During the experimental period, aeration was continuously supplied and the prawns were stocked at twelve per tank (Plate 4.1).

Two application methods were tested to investigate the efficacy of the probiotics. In method one the probiotics were applied directly to the rearing medium, and in method two the probiotics were supplemented with the feed. The initial cell density of 10^5 CFU/mL (Moriarty 1998; Vijayan *et al.* 2006) was used at the commencement of the experiment and at every water exchange. Three treatments of the probiotics were tested; a treatment for each probiotic applied individually, and one treatment as a combination of both probiotics at equal concentrations (10^5 CFU/mL) and volumes.

Plate 4.1 Lay out of experimental tanks

An 84-day experiment was set up. Each treatment and control (without probiotic) were performed in triplicate and each replicate represented by one tank. The probiotics were applied daily to the rearing systems as the water exchange was undertaken daily at 10% with ozonised water. To ensure that the probiotics were applied equally amongst the treatments in the two application methods, the prawns were fed the formulated feed supplemented with the probiotics daily. The probiotic inoculum was sprayed onto the formulated feed and air-dried under a laboratory hood for 5 min before feeding. All prawns were fed twice a day at a rate of 3-5% body weight per day. The amount of food was adjusted in relation to the survival and body weight of the prawns. The uneaten feed was siphoned and dried in an oven at 120 ^{o}C for 12 h and used to estimate the food conversion ratio. The water circulation was stopped in all tanks for 2 h after every feeding to allow the prawns to feed.

4.2.3 Data collection

Water temperature was maintained between 24 and 25 ^{o}C using automatic heaters (Sonpar®, Model: HA-100, China). NO_2-N, NO_3-N, NH_3/NH_4^+, PO_4-P and pH were measured every two days using test kits (Aquarium pharmaceuticals Ltd, United Kingdom) and pH meter (CyberScan pH 300, Eutech Instruments, Singapore). Salinity was measured by a portable refractometer (RHS-10ATC) every three days

and maintained between 32-35 mg/L throughout the experiment by the addition of ozonised - deionised water.

The SGRs and survival of the prawns was measured by counting the number of juveniles at sampling time, measuring the individual weight (g) and calculated using the following equations:

$$SGR\ (\%\ g/day) = 100 \times (\ln W_t - \ln W_o)/t$$

Where SGR is the specific growth rate in weight (% g/day), W_t and W_o are the weight of the prawns at current time (t) and at the commencement of the experiment (o), respectively, and t is the number of rearing days (day).

$$Survival\ (\%) = (N_t - N_o) \times 100$$

Where N_t and N_o are the number of prawns at the end of the experiment (t) and at the commencement (o), respectively.

Food Conversion Ratio (FCR) was calculated based on the quantity of food consumed and the increase in biomass as follows:

$$FCR = Fc / \Delta W$$

Where FCR is food conversion ratio, Fc is the total food consumed (g) and ΔW is the total wet weight gained (g).

The bacterial loads in the rearing medium and intestine of the prawns were determined at the commencement of the experiment and fortnightly thereafter. One prawn was selected and rinsed in distilled water, quickly washed with 70% alcohol, and then rinsed again in distilled water to remove the external bacteria. The intestinal tract of the prawn was removed and then homogenised in a 1.5 mL microfuge tube using a micropestle. The homogenic samples were weighed, diluted serially with sterilised normal saline solution supplemented with 2% NaCl, and lawn inoculated to MSA plates (The dilutions were continued until a total plate count of 30 - 300 was achieved). The plates were incubated for 24 h at 25 ^{o}C. It was assumed that the probiotic loads in the intestines of the prawns resulted from the differences in bacterial loads between the treatments and control.

The immune parameters of the prawns such as THC, DHC, bacterial loads in the haemolymph (bacteraemia) and clotting time were investigated at the commencement of the experiment and fortnightly thereafter. The haemolymph was taken from the pericardial cavity of individual prawns (Plate 4.2) using a 1-mL syringe containing 0.2 mL of anticoagulant (1% glutaraldehyde in 0.2M sodium cacodylate, pH 7.0) and a 23-gauge needle to puncture the intersegmental membrane between the cephalothorax and the first abdominal segment. A 0.2 mL aliquot of haemolymph was dispensed into a 1.5 mL microfuge tube kept on ice.

Plate 4.2 Withdrawal of the prawn haemolymph at the pericardial cavity

The THCs were determined using a haemocytometer at 100-fold magnification. Cells were counted on both sides of the grids. THCs were calculated using the following equation.

$$\text{THC} = (\text{cells counted} \times \text{dilution factor} \times 1000) / \text{volume of grid } (0.1\ \text{mm}^3)$$

One drop of the mixture of anticoagulant and haemolymph was smeared onto a glass microscope slide. The smear was air dried and fixed in 70% methanol for 10 minutes. The fixed smears were stained in May-Grunwald and Giemsa stains for 10 minutes each (Bancroft and Stevens 1977). Three major different haemocyte groups were identified as recommended by Bauchau (1981), Söderhäll and Smith (1983),

Hose *et al.* (1990), Sequeira *et al.* (1995), Jussila *et al.* (1997), Johansson et al. (2000) and Fotedar (personal communication, 2007) such as the HCs, SGCs and GCs. A total of 200 cells were counted on each slide. The DHCs were calculated using the following equation.

$$\text{DHC (\%)} = \frac{\text{Number of different haemocyte cell types}}{\text{Total haemocyte cells counted}} x\,100$$

A 30 µL aliquot of haemolymph was quickly transferred and drawn into a capillary tube (Chase, Scientific Glass Inc. Rockwood, TN 33748, USA). The time that the haemolymph stopped moving back and forth was recorded. The haemolymph clotting time was ranked from 0 (0 - 10 s) to 9 (>90 s). If the clotting time lasted for longer than 90 s, 'no clot' was recorded for the haemolymph.

The bacterial loads in the haemolymph were assessed on MSA. Individual drops of the haemolymph aliquot were placed onto separate MSA plates and lawn inoculated (5 drops of haemolymph were tested from each animal). The plates were incubated for 24 h at 25 ^{o}C and CFUs were counted for each drop. CFU/mL for each sample was calculated on the basis of a total volume of 20 µL for each drop. The bacterial loads were ranked from 1 (0 - 250 CFU/mL) to 12 (2751 - 3000 CFU/mL) and the rank 13 was used for "too numerous for an accurate count".

All collected data was stored in Excel. The results were presented as means ± SE (Standard error). SPSS statistical package version 14.0 was used to conduct one way ANOVA (Analysis of variance). LSD (Least significant difference) post hoc tests were used to determine any significant differences between the tested variables of the probiotic populations in the rearing medium and in the juvenile prawn, the immune parameters, the specific growth rates and survival of juveniles. Games-Howell post hoc test was used, when variances were not homogeneous. All significant tests were performed at P=0.05.

4.3 RESULTS

The NO_2-N concentrations were significantly higher ($P<0.05$) than the control in those treatments where only *P. synxantha* was applied directly to the rearing medium, *P. aeruginosa* and the combined probiotics were supplemented with the formulated feed. The water temperature and salinity were maintained at 24 - 25 ^{o}C and 30 - 35 mg/L, respectively, during the experimental period. The water quality parameters such as pH, NO_3-N, NH_3-N and PO_4-P were not significantly different ($P>0.05$) amongst the treatments.

The SGRs of the prawns were not significantly ($P>0.05$) different among the treatments over the experimental period (Table 4.1). However, at 42 days of culture, the use of only *P. aeruginosa* resulted in significantly higher SGRs ($P<0.05$) compared to both methods of probiotic application (1.04 - 1.06% g/day), the control and the other treatments using only *P. synxantha* or the mixture of two probiotics (0.76 - 0.99% g/day). Application of the probiotics decreased the FCRs ($P<0.05$) without influencing ($P>0.05$) the prawn survival (Table 4.1).

Over the experimental duration, the probiotic loads in the rearing medium increased in all treatments. However, after 42 days of culture, the use of only *P. synxantha* in both application methods and the use of the combined probiotics added into the rearing medium did not significantly change the probiotic loads. At 28 or 42 days of culture, the probiotic loads in the prawn intestines were higher when the probiotics were supplemented with the formulated feed than the direct application into the rearing medium (Table 4.2).

Table 4.1
The mean ± SE of SGR, survival and FCR (n = 7) over the experimental period

Parameters	Time (day)	Without probiotic	Directly applied into the rearing medium			Indirectly supplemented with the formulated feed		
			P. synxantha	*P. aeruginosa*	*P. synxantha* and *P. aeruginosa*	*P. synxantha*	*P. aeruginosa*	*P. synxantha* and *P. aeruginosa*
SGR (% g/day)	14	$_{1}1.04 \pm 0.10^{a}$	$_{12}1.05 \pm 0.09^{a}$	$_{1}1.03 \pm 0.18^{a}$	$_{12}1.20 \pm 0.11^{a}$	$_{12}1.12 \pm 0.22^{a}$	$_{12}1.08 \pm 0.05^{a}$	$_{2}1.32 \pm 0.16^{a}$
	28	$_{1}0.95 \pm 0.06^{ab}$	$_{1}0.93 \pm 0.06^{ab}$	$_{1}1.02 \pm 0.12^{a}$	$_{1}1.08 \pm 0.15^{ab}$	$_{1}1.02 \pm 0.07^{ab}$	$_{1}1.01 \pm 0.17^{a}$	$_{1}1.14 \pm 0.03^{ab}$
	42	$_{12}0.87 \pm 0.06^{ab}$	$_{1}0.76 \pm 0.06^{b}$	$_{2}1.04 \pm 0.18^{a}$	$_{12}0.98 \pm 0.06^{abc}$	$_{12}0.90 \pm 0.02^{ab}$	$_{2}1.06 \pm 0.19^{a}$	$_{12}0.99 \pm 0.03^{bc}$
	56	$_{1}0.85 \pm 0.09^{ab}$	$_{1}0.78 \pm 0.02^{b}$	$_{1}0.94 \pm 0.08^{a}$	$_{1}0.83 \pm 0.04^{bc}$	$_{1}0.81 \pm 0.08^{b}$	$_{1}0.98 \pm 0.14^{a}$	$_{1}0.99 \pm 0.07^{bc}$
	70	$_{1}0.81 \pm 0.04^{ab}$	$_{1}0.75 \pm 0.00^{b}$	$_{1}0.93 \pm 0.08^{a}$	$_{1}0.81 \pm 0.04^{c}$	$_{1}0.82 \pm 0.05^{b}$	$_{1}0.94 \pm 0.10^{a}$	$_{1}0.92 \pm 0.07^{bc}$
	84	$_{1}0.76 \pm 0.05^{b}$	$_{1}0.74 \pm 0.02^{b}$	$_{1}0.85 \pm 0.07^{a}$	$_{1}0.76 \pm 0.03^{c}$	$_{1}0.80 \pm 0.02^{b}$	$_{1}0.86 \pm 0.07^{a}$	$_{1}0.84 \pm 0.07^{c}$
Survival (%)		$_{1}60.00 \pm 11.55$	$_{1}66.67 \pm 13.33$	$_{1}66.67 \pm 6.67$	$_{1}66.67 \pm 17.64$	$_{1}73.33 \pm 13.33$	$_{1}73.33 \pm 6.67$	$_{1}80.00 \pm 11.55$
FCR		$_{1}2.96 \pm 0.02$	$_{2}2.69 \pm 0.01$	$_{2}2.66 \pm 0.02$	$_{2}2.67 \pm 0.02$	$_{2}2.65 \pm 0.02$	$_{2}2.65 \pm 0.01$	$_{2}2.64 \pm 0.02$

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column in the same rearing medium or intestines not followed by the same superscript letters are significantly different at $P<0.05$.

Table 4.2
The mean ± SE (x 10^3) of the probiotic loads in the rearing medium (CFU/mL) and the intestines of prawns (CFU/g)

Probiotic population	Time (day)	Directly applied into the rearing medium			Indirectly supplemented with the formulated feed		
		P. synxantha	*P. aeruginosa*	*P. synxantha* and *P. aeruginosa*	*P. synxantha*	*P. aeruginosa*	*P. synxantha* and *P. aeruginosa*
Rearing medium (CFU/mL)	14	$_{1}13.5 \pm 1.7^{a}$	$_{1}13.8 \pm 1.2^{a}$	$_{1}11.2 \pm 2.6^{a}$	$_{1}14.2 \pm 1.5^{a}$	$_{1}10.8 \pm 1.5^{a}$	$_{1}10.5 \pm 1.0^{a}$
	28	$_{1}21.9 \pm 4.7^{a}$	$_{1}24.2 \pm 1.0^{ab}$	$_{1}21.6 \pm 6.0^{a}$	$_{1}14.9 \pm 1.7^{a}$	$_{1}20.6 \pm 5.5^{ab}$	$_{1}18.9 \pm 3.0^{ab}$
	42	$_{1}37.8 \pm 3.3^{b}$	$_{12}31.8 \pm 3.7^{bc}$	$_{12}33.8 \pm 2.9^{b}$	$_{12}30.1 \pm 3.0^{b}$	$_{12}28.5 \pm 5.5^{bc}$	$_{2}25.1 \pm 5.2^{bc}$
	56	$_{1}39.3 \pm 6.3^{b}$	$_{12}35.0 \pm 0.7^{cd}$	$_{12}36.0 \pm 3.3^{b}$	$_{12}31.3 \pm 3.5^{b}$	$_{12}30.0 \pm 1.5^{bc}$	$_{2}27.0 \pm 3.5^{bc}$
	70	$_{1}43.0 \pm 4.5^{b}$	$_{12}42.0 \pm 3.2^{cd}$	$_{12}40.0 \pm 4.0^{b}$	$_{12}36.3 \pm 6.2^{b}$	$_{2}32.0 \pm 4.0^{cd}$	$_{2}32.3 \pm 0.9^{c}$
	84	$_{1}46.4 \pm 5.8^{b}$	$_{12}42.8 \pm 2.0^{d}$	$_{12}42.1 \pm 5.9^{b}$	$_{12}38.8 \pm 2.9^{b}$	$_{12}41.8 \pm 2.9^{d}$	$_{2}32.8 \pm 1.9^{c}$
Intestine of prawns (CFU/g)	14	$_{1}1.1 \pm 0.2^{a}$	$_{1}0.9 \pm 0.09^{a}$	$_{1}0.9 \pm 0.04^{a}$	$_{1}0.9 \pm 0.05^{a}$	$_{1}1.6 \pm 0.2^{a}$	$_{1}1.3 \pm 0.1^{a}$
	28	$_{12}2.3 \pm 0.5^{b}$	$_{1}2.2 \pm 0.3^{b}$	$_{12}2.5 \pm 0.3^{b}$	$_{12}2.7 \pm 0.3^{b}$	$_{2}3.3 \pm 0.4^{b}$	$_{2}3.2 \pm 0.4^{b}$
	42	$_{1}2.8 \pm 0.3^{bc}$	$_{1}3.0 \pm 0.4^{bc}$	$_{1}2.8 \pm 0.4^{bc}$	$_{2}4.0 \pm 0.4^{c}$	$_{2}4.2 \pm 0.3^{bc}$	$_{1}3.7 \pm 0.6^{b}$
	56	$_{1}3.1 \pm 0.2^{bc}$	$_{1}3.0 \pm 0.3^{bc}$	$_{1}2.6 \pm 0.5^{b}$	$_{2}4.2 \pm 0.3^{cd}$	$_{2}4.4 \pm 0.6^{c}$	$_{2}4.6 \pm 0.3^{cd}$
	70	$_{1}3.3 \pm 0.3^{c}$	$_{1}3.6 \pm 0.3^{c}$	$_{1}3.6 \pm 0.3^{cd}$	$_{2}5.1 \pm 0.3^{de}$	$_{2}5.6 \pm 0.1^{d}$	$_{2}5.4 \pm 0.3^{d}$
	84	$_{1}3.5 \pm 0.3^{c}$	$_{1}3.8 \pm 0.2^{c}$	$_{1}3.8 \pm 0.4^{d}$	$_{2}5.5 \pm 0.6^{e}$	$_{2}5.5 \pm 0.2^{d}$	$_{2}5.5 \pm 0.4^{d}$

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column in the same rearing medium or intestines not followed by the same superscript letters are significantly different at $P<0.05$.

The probiotic load in the rearing medium showed a strong positive correlation with the probiotic load in the prawn intestines. Applying the probiotics into the rearing medium resulted in a higher correlation between the probiotic load in the rearing medium and in the prawn intestines when the single probiotic was applied (R^2 = 0.9346 and 0.9791) compared to the combined applications of the probiotics (R^2 = 0.8906). When the probiotics were supplemented in the feed, the correlation between the probiotic loads was lowered for the individual probiotics (R^2 = 0.8921 and 0.9) compared to the combined probiotic application (R^2 = 0.9753). The relationship between the probiotic loads in the rearing medium and prawn intestines was higher when the probiotics were applied into the rearing medium (R^2 = 0.9023) than when the probiotic were supplemented with the formulated feed (R^2 = 0.8737) (Table 4.3).

Table 4.3
The relationship between the probiotic loads in the rearing medium and the intestines of the prawns

Application method	**Probiotic**	**Equation**	**R^2**
Directly applied into rearing medium	*P. synxantha*	y = 0.067x + 457.18	0.9346
	P. aeruginosa	y = 0.0958x - 281.5	0.9791
	P. synxantha and *P. aeruginosa*	y = 0.0818x + 217.47	0.8906
Indirectly supplemented with the formulated feed	*P. synxantha*	y = 0.151x - 412.28	0.9
	P. aeruginosa	y = 0.135x + 410.19	0.8921
	P. synxantha and *P. aeruginosa*	y = 0.1856x - 585.06	0.9753
Direct method(*)		*y = 0.0789x + 204.26*	*0.9023*
Indirect method(*)		*y = 0.1494x - 16.782*	*0.8737*

(*): A combination of three treatments in the same application methods.

The application of the probiotics gradually increased the THC of the prawn haemolymph in all treatments, but the difference was not of a significant value (Table 4.4). The rearing of the prawns without probiotic resulted in a strong positive correlation between the THCs and proportion of HCs (R^2 = 0.9852); whereas, a strong negative correlation between the THCs and proportion of HCs was recorded when treated with the probiotics (Table 4.5)

Table 4.4
The mean ± SE of immune parameters of the prawns over the experimental period

Immune parameters	Time (h)	Without probiotic	Directly applied into the rearing medium			Indirectly supplemented with the formulated feed		
			P. synxantha	*P. aeruginosa*	*P. synxantha* and *P. aeruginosa*	*P. synxantha*	*P. aeruginosa*	*P. synxantha* and *P. aeruginosa*
THC (x 10^6 Cells/mL)	0	$_1$4.70 ± 0.10^{a}	$_1$4.70 ± 0.10^{a}	$_1$4.70 ± 0.10^{a}	$_1$4.70 ± 0.10^{a}	$_1$4.70 ± 0.10^{a}	$_1$4.70 ± 0.10^{a}	$_1$4.70 ± 0.10^{a}
	14	$_1$4.77 ± 0.11^{ab}	$_1$4.81 ± 0.12^{ab}	$_1$4.80 ± 0.11^{ab}	$_1$4.84 ± 0.10^{ab}	$_1$4.77 ± 0.11^{ab}	$_1$4.81 ± 0.10^{ab}	$_1$4.85 ± 0.11^{ab}
	28	$_1$4.83 ± 0.13^{abc}	$_1$4.86 ± 0.12^{ab}	$_1$4.87 ± 0.13^{abc}	$_1$4.89 ± 0.09^{abc}	$_1$4.85 ± 0.11^{abc}	$_1$4.89 ± 0.09^{abc}	$_1$4.93 ± 0.09^{abc}
	42	$_1$4.90 ± 0.12^{abc}	$_1$4.94 ± 0.11^{ab}	$_1$4.95 ± 0.12^{abcd}	$_1$4.97 ± 0.09^{abcd}	$_1$4.93 ± 0.10^{abcd}	$_1$4.99 ± 0.07^{abcd}	$_1$5.01 ± 0.09^{bcd}
	56	$_1$4.98 ± 0.12^{abc}	$_1$5.01 ± 0.13^{b}	$_1$5.05 ± 0.13^{bcd}	$_1$5.09 ± 0.08^{bcd}	$_1$5.01 ± 0.10^{bcd}	$_1$5.06 ± 0.05^{bcd}	$_1$5.16 ± 0.10^{bcd}
	70	$_1$5.03 ± 0.13^{bc}	$_1$5.06 ± 0.14^{b}	$_1$5.11 ± 0.12^{cd}	$_1$5.16 ± 0.07^{cd}	$_1$5.07 ± 0.09^{cd}	$_1$5.13 ± 0.06^{cd}	$_1$5.23 ± 0.09^{cd}
	84	$_1$5.09 ± 0.13^{c}	$_1$5.11 ± 0.15^{b}	$_1$5.18 ± 0.13^{d}	$_1$5.22 ± 0.08^{d}	$_1$5.13 ± 0.09^{d}	$_1$5.21 ± 0.06^{d}	$_1$5.29 ± 0.09^{d}
HC (%)	0	$_1$55.67 ± 2.60^{a}	$_1$55.67 ± 2.60^{a}	$_1$55.67 ± 2.60^{a}	$_1$55.67 ± 2.60^{a}	$_1$55.67 ± 2.60^{a}	$_1$55.67 ± 2.60^{a}	$_1$55.67 ± 2.60^{a}
	14	$_1$57.33 ± 1.76^{ab}	$_1$55.17 ± 2.74^{ab}	$_1$54.33 ± 2.91^{ab}	$_1$54.50 ± 3.33^{ab}	$_1$55.00 ± 2.89^{a}	$_1$54.33 ± 2.91^{ab}	$_1$54.50 ± 3.33^{ab}
	28	$_1$59.67 ± 0.88^{abc}	$_1$54.17 ± 2.62^{ab}	$_1$53.00 ± 3.04^{abc}	$_1$53.50 ± 3.18^{ab}	$_1$53.50 ± 3.18^{ab}	$_1$52.67 ± 3.06^{abc}	$_1$53.00 ± 3.62^{abc}
	42	$_1$61.83 ± 0.33^{abc}	$_2$52.67 ± 2.62^{ab}	$_2$52.00 ± 3.18^{abc}	$_2$52.17 ± 3.03^{ab}	$_2$52.00 ± 3.18^{ab}	$_2$49.00 ± 2.08^{abc}	$_2$50.50 ± 4.54^{abc}
	56	$_1$65.33 ± 1.20^{bcd}	$_2$51.33 ± 2.03^{ab}	$_2$50.33 ± 3.77^{abc}	$_2$50.33 ± 3.91^{ab}	$_2$49.33 ± 3.84^{ab}	$_2$47.33 ± 2.73^{abc}	$_2$48.67 ± 4.48^{abc}
	70	$_1$67.33 ± 2.03^{cd}	$_2$49.00 ± 1.53^{ab}	$_2$46.67 ± 5.18^{bc}	$_2$48.00 ± 3.52^{ab}	$_2$47.00 ± 3.51^{ab}	$_2$46.00 ± 3.06^{bc}	$_2$46.17 ± 4.34^{bc}
	84	$_1$70.85 ± 3.06^{d}	$_2$46.67 ± 2.59^{b}	$_2$45.17 ± 4.60^{c}	$_2$46.50 ± 3.75^{b}	$_2$44.17 ± 3.92^{b}	$_2$44.17 ± 3.66^{c}	$_2$44.83 ± 4.42^{c}
SGC (%)	0	$_1$18.17 ± 1.74^{a}	$_1$18.17 ± 1.74^{a}	$_1$18.17 ± 1.74^{a}	$_1$18.17 ± 1.74^{a}	$_1$18.17 ± 1.74^{a}	$_1$18.17 ± 1.74^{a}	$_1$18.17 ± 1.74^{a}
	14	$_1$17.17 ± 1.59^{ab}	$_1$18.83 ± 1.59^{ab}	$_1$18.83 ± 1.60^{ab}	$_1$19.00 ± 1.61^{ab}	$_1$19.00 ± 1.61^{ab}	$_1$19.00 ± 1.44^{ab}	$_1$19.17 ± 1.45^{ab}
	28	$_1$16.17 ± 1.48^{abc}	$_1$20.17 ± 1.76^{abc}	$_1$20.17 ± 1.75^{abc}	$_1$20.83 ± 1.76^{abc}	$_1$20.67 ± 1.74^{abc}	$_1$20.50 ± 1.53^{abc}	$_1$20.67 ± 1.74^{abcd}
	42	$_1$15.33 ± 1.17^{abc}	$_2$21.83 ± 1.45^{abcd}	$_2$21.83 ± 1.45^{abcd}	$_2$22.33 ± 1.48^{abcd}	$_2$22.17 ± 1.45^{abcd}	$_2$22.17 ± 1.45^{abcd}	$_2$22.33 ± 1.30^{abcd}
	56	$_1$14.17 ± 1.20^{abc}	$_2$23.00 ± 1.61^{bcd}	$_2$23.00 ± 1.62^{bcd}	$_2$23.50 ± 1.76^{bcd}	$_2$23.17 ± 1.74^{bcd}	$_2$23.50 ± 1.73^{bcd}	$_2$23.50 ± 1.15^{bcd}
	70	$_1$13.17 ± 1.01^{bc}	$_2$24.33 ± 2.05^{cd}	$_2$24.33 ± 2.05^{cd}	$_2$24.83 ± 2.19^{cd}	$_2$24.67 ± 2.03^{cd}	$_2$24.83 ± 1.76^{cd}	$_2$25.17 ± 1.45^{cd}
	84	$_1$12.33 ± 1.17^{c}	$_2$25.33 ± 2.24^{d}	$_2$25.33 ± 2.04^{d}	$_2$26.17 ± 2.59^{d}	$_2$25.67 ± 1.92^{d}	$_2$25.83 ± 1.76^{d}	$_2$26.50 ± 1.15^{d}
GC (%)	0	$_1$8.17 ± 0.60^{a}	$_1$8.17 ± 0.60^{a}	$_1$8.17 ± 0.60^{a}	$_1$8.17 ± 0.60^{a}	$_1$8.17 ± 0.60^{a}	$_1$8.17 ± 0.60^{a}	$_1$8.17 ± 0.60^{a}
	14	$_1$7.33 ± 0.44^{ab}	$_{12}$8.33 ± 0.67^{a}	$_2$8.67 ± 0.33^{ab}	$_1$8.83 ± 0.44^{ab}	$_2$8.50 ± 0.29^{a}	$_2$8.33 ± 0.44^{a}	$_2$8.50 ± 0.29^{ab}
	28	$_1$6.33 ± 0.44^{bc}	$_2$9.00 ± 0.50^{ab}	$_2$9.33 ± 0.17^{bc}	$_1$9.50 ± 0.29^{bc}	$_2$9.17 ± 0.17^{ab}	$_2$8.83 ± 0.44^{ab}	$_2$9.33 ± 0.17^{bc}
	42	$_1$5.67 ± 0.33^{cd}	$_2$9.67 ± 0.60^{bc}	$_2$10.33 ± 0.17^{cd}	$_2$10.50 ± 0.00^{cd}	$_2$10.00 ± 0.29^{bc}	$_2$9.67 ± 0.33^{bc}	$_2$10.33 ± 0.17^{cd}
	56	$_1$5.00 ± 0.29^{d}	$_2$10.33 ± 0.73^{cd}	$_2$11.17 ± 0.33^{de}	$_2$11.33 ± 0.17^{de}	$_2$10.67 ± 0.44^{cd}	$_2$10.17 ± 0.33^{cd}	$_2$11.17 ± 0.33^{de}
	70	$_1$4.83 ± 0.33^{de}	$_2$10.83 ± 0.73^{de}	$_2$11.50 ± 0.29^{e}	$_2$11.67 ± 0.17^{e}	$_2$11.00 ± 0.29^{cd}	$_2$10.67 ± 0.33^{cd}	$_2$11.50 ± 0.29^{e}

	84	$_{1}4.50 \pm 0.29^{e}$	$_{2}11.50 \pm 0.58^{e}$	$_{2}12.00 \pm 0.29^{e}$	$_{2}12.17 \pm 0.17^{e}$	$_{2}11.50 \pm 0.29^{d}$	$_{2}11.17 \pm 0.33^{d}$	$_{2}12.00 \pm 0.29^{e}$
Clotting time (rank)	0	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$
	14	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$
	28	$_{1}6.67 \pm 0.33^{ab}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}5.67 \pm 0.33^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}6.00 \pm 0.58^{a}$	$_{1}5.67 \pm 0.88^{a}$
	42	$_{1}7.00 \pm 0.58^{ab}$	$_{12}6.00 \pm 0.58^{a}$	$_{12}6.00 \pm 0.58^{a}$	$_{2}5.33 \pm 0.33^{a}$	$_{12}6.00 \pm 0.58^{a}$	$_{12}5.67 \pm 0.67^{a}$	$_{12}5.67 \pm 0.88^{a}$
	56	$_{1}7.00 \pm 0.58^{ab}$	$_{2}5.33 \pm 0.67^{a}$	$_{12}5.67 \pm 0.67^{a}$	$_{2}5.33 \pm 0.33^{a}$	$_{12}5.67 \pm 0.33^{a}$	$_{2}5.33 \pm 0.33^{a}$	$_{2}5.33 \pm 0.88^{a}$
	70	$_{1}7.67 \pm 0.33^{b}$	$_{2}5.00 \pm 0.58^{a}$	$_{2}5.00 \pm 0.58^{a}$	$_{2}4.67 \pm 0.33^{a}$	$_{2}5.00 \pm 0.58^{a}$	$_{2}5.33 \pm 0.33^{a}$	$_{2}5.00 \pm 0.88^{a}$
	84	$_{1}7.67 \pm 0.33^{b}$	$_{2}5.00 \pm 0.58^{a}$	$_{2}5.00 \pm 0.58^{a}$	$_{2}4.67 \pm 0.33^{a}$	$_{2}5.00 \pm 0.58^{a}$	$_{2}5.00 \pm 0.33^{a}$	$_{2}4.67 \pm 0.88^{a}$
Bacterial loads (rank)	0	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.00 \pm 0.00^{a}$
	14	$_{1}3.33 \pm 0.33^{a}$	$_{1}3.33 \pm 0.33^{a}$	$_{1}3.33 \pm 0.33^{a}$	$_{1}3.33 \pm 0.33^{a}$	$_{1}3.00 \pm 0.00^{a}$	$_{1}3.00 \pm 0.00^{a}$	$_{1}2.67 \pm 0.33^{ab}$
	28	$_{1}3.67 \pm 0.33^{ab}$	$_{12}3.33 \pm 0.33^{a}$	$_{1}3.33 \pm 0.33^{a}$	$_{12}2.67 \pm 0.33^{a}$	$_{2}2.33 \pm 0.33^{ab}$	$_{12}2.67 \pm 0.33^{a}$	$_{2}2.33 \pm 0.33^{ab}$
	42	$_{1}4.33 \pm 0.33^{bc}$	$_{2}3.00 \pm 0.58^{ab}$	$_{2}3.33 \pm 0.33^{a}$	$_{2}3.00 \pm 0.00^{a}$	$_{3}2.33 \pm 0.33^{ab}$	$_{3}2.33 \pm 0.33^{ab}$	$_{3}2.00 \pm 0.00^{bc}$
	56	$_{1}4.67 \pm 0.33^{c}$	$_{2}2.33 \pm 0.33^{b}$	$_{2}2.67 \pm 0.33^{a}$	$_{2}2.33 \pm 0.33^{ab}$	$_{3}1.67 \pm 0.33^{bc}$	$_{3}1.67 \pm 0.33^{b}$	$_{3}1.33 \pm 0.33^{cd}$
	70	$_{1}4.33 \pm 0.33^{bc}$	$_{2}2.33 \pm 0.33^{b}$	$_{2}1.67 \pm 0.33^{b}$	$_{2}2.00 \pm 0.00^{ab}$	$_{3}1.33 \pm 0.33^{cd}$	$_{3}0.67 \pm 0.33^{c}$	$_{3}0.67 \pm 0.33^{d}$
	84	$_{1}5.00 \pm 0.00^{c}$	$_{2}2.33 \pm 0.33^{b}$	$_{2}1.67 \pm 0.33^{b}$	$_{2}1.67 \pm 0.33^{b}$	$_{3}0.67 \pm 0.33^{d}$	$_{3}0.33 \pm 0.33^{c}$	$_{3}0.33 \pm 0.33^{d}$

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column in the same rearing medium or intestines not followed by the same superscript letters are significantly different at $P<0.05$.

Between 70 and 84 days of culture, the application of the probiotics significantly decreased (P<0.05) the HC proportion; whereas, the HC proportion in the control significantly increased (P<0.05) after 56 days of culture. The significant difference of these opposite trends was observed after 42 days onwards (Table 4.4). The HC proportion showed a strong positive correlation with the SGC and GC proportions. The relationship between the HC and SGC proportions was higher when the probiotics were supplemented with the formulated feed (R^2 = 0.9692) compared to the probiotics applied directly into the rearing medium (R^2 = 0.9296); whereas, the relationship between the HC and GC proportions was higher when the probiotics were applied into the rearing medium (R^2 = 0.9193) compared to the probiotics supplemented with the formulated feed (R^2 = 0.8897) (Table 4.5). The use of the probiotics significantly increased (P<0.05) the SGC and GC proportions of the prawn haemolymph; whereas, the SGC and GC proportions of the prawns decreased significantly (P<0.05) in the control. The significant difference (P<0.05) of these opposite trends was observed after 42 days of culture for the SGC proportion and either 14 or 42 days of culture for the GC proportion (Table 4.4). The SGC proportion showed a strong correlation with the GC proportion for both application methods (Table 4.5).

Increases in the clotting time and bacterial load in the haemolymph were observed in the control; whereas, the clotting time and bacterial load in the haemolymph gradually increased when the probiotics were applied. No significant difference in clotting time ranks (P>0.05) was recorded between the single and combined probiotics and between the two application methods. The bacterial load in the prawn haemolymph was significantly (P<0.05) greater when the probiotics were supplemented with the formulated feed compared to direct application into the rearing medium after 42 days of culture, but no significant difference (P>0.05) was observed between the single and combined probiotics. In both application methods, the bacterial load in the haemolymph were lower when only *P. aeruginosa* was applied compared to only *P. synxantha* application (Table 4.4).

Table 4.5
The relationship between the proportions of THC and HC, HC and SGC, HC and GC, SGC and GC

Relationship	**Application method**	**Probiotic**	**Equation**	**R^2**
THC and HC	Without probiotic		y = 38.526x -126.21	0.9852
	Directly applied into rearing medium	*P. synxantha*	y = -21.687x + 158.96	0.8957
		P. aeruginosa	y = -21.962x + 159.77	0.9403
		P. synxantha and *P. aeruginosa*	y = -17.889x + 140.66	0.9606
	Indirectly supplemented with the formulated feed	*P. synxantha*	y = -24.817x + 173.31	0.9695
		P. aeruginosa	y = -23.961x + 169	0.9841
		P. synxantha and *P. aeruginosa*	y = -19.059x + 146.23	0.9687
	Directly applied into rearing medium(*)		*y = -20.007x +150.66*	*0.9068*
	Indirectly supplemented with the formulated feed(*)		*y = -21.167x + 155.75*	*0.9228*
HC and SGC	Without probiotic		y = -0.3828x + 39.164	0.9896
	Directly applied into rearing medium	*P. synxantha*	y = -0.8036x + 63.531	0.9558
		P. aeruginosa	y = -0.6852x + 56.628	0.9532
		P. synxantha and *P. aeruginosa*	y = -0.8587x + 66.361	0.971
	Indirectly supplemented with the formulated feed	*P. synxantha*	y = -0.6928x + 57.345	0.9707
		P. aeruginosa	y = -0.66x + 54.92	0.9921
		P. synxantha and *P. aeruginosa*	y = -0.7405x + 59.59	0.9958
	Directly applied into rearing medium(*)		*y = -0.7598x + 60.982*	*0.9296*
	Indirectly supplemented with the formulated feed(*)		*y = -0.6857x + 56.671*	*0.9692*
HC and GC	Without probiotic		y = -0.2375x + 20.835	0.9115
	Directly applied into rearing medium	*P. synxantha*	y = -0.3749x + 29.219	0.969
		P. aeruginosa	y = -0.3618x + 28.627	0.9099
		P. synxantha and *P. aeruginosa*	y = -0.4333x + 32.637	0.9444
	Indirectly supplemented with the formulated feed	*P. synxantha*	y = -0.3145x + 25.932	0.9612
		P. aeruginosa	y = -0.2642x + 22.752	0.9931
		P. synxantha and *P. aeruginosa*	y = -0.3594x + 28.284	0.9776
	Directly applied into rearing medium(*)		*y = -0.3888x + 30.095*	*0.9183*
	Indirectly supplemented with the formulated feed(*)		*y = -0.3018x + 25.094*	*0.8897*
SGC and GC	Without probiotic		y = 0.63x – 3.6092	0.9497
	Directly applied into rearing medium	*P. synxantha*	y = 0.4623x – 0.325	0.9955
		P. aeruginosa	y = 0.5377x – 1.4845	0.99
		P. synxantha and *P. aeruginosa*	y = 0.5079x – 0.9249	0.9851
	Indirectly supplemented with the formulated feed	*P. synxantha*	y = 0.454x – 0.0989	0.9908
		P. aeruginosa	y = 0.3991x + 0.7906	0.9947
		P. synxantha and *P. aeruginosa*	y = 0.4851x + 0.6338	0.9807
	Directly applied into rearing medium(*)		*y = 0.5052x + 0.9662*	*0.9629*
	Indirectly supplemented with the formulated feed(*)		*y = 0.4493x + 0.0478*	*0.9567*

(*): A combination of three treatments in the same application methods.

4.4 DISCUSSION

The application of probiotics enhances the health of animals by minimising the pathogenic effects via the improvement of the water quality (Borges *et al.* 2008; Moriarty 1998; Verschuere *et al.* 2000). In our study, all water quality parameters were within a suitable range for the culture of western king prawns and for other penaeid prawns (Chen *et al.* 1990; Prangnell and Fotedar 2005; 2006b; Ray and Chien 1992). The water quality parameters except NO_2-N were not significantly different between the control and other treatments in the current study. Although the NO_2-N concentrations were higher when the probiotics were applied compared to no probiotic applications, these concentrations (0.28 ± 0.05 – 0.33 ± 0.07 mg/L) were still under the maximum toleration limit of 4.5 mg/L (Boyd 1996). Therefore, in the current study, the water quality parameters did not affect the use of the probiotics on the rearing of the prawns. The rearing media in our current study and MSA in our previous study (Hai *et al.* 2007) both have provided the conducive culture media to promote the growth of the probiotics and bacteria in the prawns.

The SGRs of the prawns in our study are higher than in the study conducted by Prangnell and Fotedar (2005), however the higher SGRs were not a consequence of the probiotic applications as the control in current study showed the same SGRs. In contrast, Skjermo *et al.* (2006) and Maeda and Liao (1992b) found that the growth of Atlantic cod (*Gadus morhua*) and black tiger prawn nauplii (*Penaeus monodon*) improved when exposed to ß-1,3-glucan and bacterial strain PM-4, respectively. The health of the prawns improved after 28 days of the probiotic applications, as most of the mortalities occurred during the first 28 days of culture. No mortalities occurred after 42 days. The probiotics played an important role in increasing the survival of the prawns, and reducing the FCRs in the rearing of the prawns. When exposed to the probiotics, the prawns consumed less feed leading significantly lower FCRs, helped them to digest the feed effectively so that the prawns had the same SGRs but higher FCRs when the prawns were grown without probiotics.

THCs and DHCs play important roles in the crustacean immune response and are used to assess the health of the animals (Jussila *et al.* 1997). Other health assessors include cell recognition, phagocytosis, melanization, cytotoxicity and cell to cell

communication (Johansson *et al.* 2000). Other studies found that THCs decreased when western rock lobster (*Panulirus cygnus*) were exposed to air (Fotedar *et al.* 2001), when prawns (*Macrobrachium malcolmsonii*) were exposed to NO_2-N at 0.063 and 0.314 mg/L for 120 h (Chand and Sahoo 2006), and when white prawns (*Litopenaeus vannamei*) were exposed to 488 μg/L sulfide (Hsu and Chen 2007). THCs in the American lobsters (*Homarus americanus*) (Stewart *et al.* 1967) and in the blue crab (*Callinectes sapidus*) (Johnson 1976) declined in the presence of harmful bacteria and THCs were lower in moribund western rock lobsters than in the healthy ones (Jussila *et al.* 1997), but in the current study the THCs of the prawns were higher following application of the probiotics, indicating that probiotics have a positive effect on the health of prawns. The probiotics also stimulated the prawns to produce more THCs, again an indication that probiotics have improved the health of prawns. Similar to our study, when exposed to mitogenic stimulation, the haemocytes of the kuruma prawns (*Penaeus japonicus*) proliferated up to sixfold compared to haemocytes in non-stimulated individuals (Sequeira *et al.* 1996).

The immune system of crustaceans is non-specific and relies on phagocytosis, encapsulation and agglutination (Smith and Soderhall 1983). Probiotics enhance the immune response of aquatic animals (Verschuere *et al.* 2000). Our study has shown that when the prawns were exposed to customised probiotics altered DHC (relative proportions of HC, SGC and SGC in THC) compared to the prawns which were not exposed to any probiotic. HCs are chiefly involved in phagocytosis (Johansson *et al.* 2000), and in the current study the HC proportion decreased with the application of the probiotics. The prawns not exposed to the probiotics were more stressed and produced more HCs compared to those exposed to the probiotics. In addition, HCs were the most numerous of the haemocytes in kuruma prawns (Sequeira *et al.* 1995), western rock lobster (Jussila *et al.* 1997) and this was reflected in the current study where the HC proportion was 44.17 ± 3.66 to 70.83 ± 3.06% of the total haemocytes. These results are similar to those reported by Jussila et al. (1997) where the HC proportion of healthy white western rock lobsters were lower than those of moribund ones. Similarly, the HC proportion of healthy red western rock lobsters was also lower than those of the moribund ones (Jussila *et al.* 1997). The HC proportion of white prawns declined by 21 and 31% for 24 and 48 h exposure to 488 μg/L sulfide, respectively (Hsu and Chen 2007).

In our study, application of the probiotics resulted in significant differences between the proportion distributions of SGC and GC in the prawn haemolymph. The response of DHC to different stressors is not well understood as previous reports have produced inconsistent results while using DHC as stress indicators in crustacean (Johansson *et al.* 2000; Jussila *et al.* 1997). Further, DHC has been reported to be dependent on sex, moult cycle, histochemical procedures employed to investigate DHC and exposure time (Bauchau 1981; Sequeira *et al.* 1995). In our study, application of the probiotics resulted in decrease in HC and increase in SCG and CG, and both these changes are indication of healthier prawns. However, decrease in the proportion of SGC when the probiotic were applied is in contradiction a study reported by Jussila *et al.* (1997). This contradiction could be due to volatility in the proportional distribution of HC, SCG and GC as their proportional distribution can be time-dependent (Fotedar *et al.* 2006). Moreover, there is an overlap in the functionality of SGCs and GCs as both of them are known for cytotoxicity and storage and release of prophenoloxidase system (Hose *et al.* 1990; Johansson *et al.* 2000; Martin *et al.* 1996). Animals with higher proportion of GC and lower proportion of SGC are considered to be healthier. For example, the proportion of GC was lower in moribund white and red western rock lobster than the healthy ones (Jussila *et al.* 1997), and the number of GCs in air-exposed western rock lobster was lower than those not exposed to air (Fotedar *et al.* 2001). Similarly, the proportion of SGC in healthy red western rock lobster was lower than moribund animals (Jussila *et al.* 1997).

The strong positive and negative correlations between the THC and proportion of HC in both application methods without and with the probiotics, respectively, indicates that the use of the probiotics decreased the HC proportion in the prawn haemolymph. Similarly, the strong negative correlation between the HC and SGC proportions, and between HC and GC proportions were reported for western rock lobster (Fotedar *et al.* 2006; Jussila *et al.* 1997). The SGC proportion has also shown a strong positive correlation with the GC proportion. Further, the HC proportion was reversely related to the SGC and GC proportions.

Aquatic animals come into contact with a great number of bacteria in their habitat. Uninjured healthy blue crabs (*Callinectes sapidus*) do not have sterile haemolymph (Welsh and Sizemore 1985). Elevated bacterial loads suggest a decline in immune capacity and probably increased susceptibility to infection (Fotedar *et al.* 2001). Bacterial load in haemolymph increased when western rock lobster were exposed to air (Fotedar *et al.* 2001), and when blue crabs (*Callinectes sapidus*) were subjected to the stresses of commercial capture, handling and transport (Welsh and Sizemore 1985). In our study, the use of the probiotics reduced the bacterial load in the haemolymph resulting in healthier prawns. Therefore, these probiotics act as immunostimulants to enhance the defence potential of prawns against bacterial infection (Chang *et al.* 2000; Song and Hsieh 1994; Song *et al.* 1997).

Foreign particles invading the haemolymph impair the clotting time of the haemolymph (Durliat and Vranckx 1983). High clotting times indicate a decline in resistance against pathogens, pointing to lower immune status (Fotedar *et al.* 2001). The clotting time of haemolymph from western rock lobster held for 1 day in storage tanks was 3.5 ± 0.2 (Fotedar *et al.* 2006). Western rock lobster exposed to air for 12 h and 18 h caused a significant increase in the clotting time as compared to those not exposed to air (Fotedar *et al.* 2001). High clotting times also were found in air-exposed western rock lobster groups (Fotedar *et al.* 2001) compared to the lobsters not exposed to the air. The clotting times of the prawns in the current study were higher in the control than those in other treatments indicating the probiotics improved the immune status of the prawns.

It is important to consider effective methods for probiotic administration in order to get the best results (Sakai 1999). Both application methods used in this study for *P. synxantha* and *P. aeruginosa* were effective in transferring the probiotics into the intestines of the prawns, as the probiotic loads in the rearing medium showed a closely correlated with those in the intestines of the prawns (R^2 from 0.8737 to 0.9023). Similar to the results of Skjermo and Vadstein (1999), Azad *et al.* (2005), the application of probiotics into the rearing medium in current study was less effective than supplementation with the feed. In addition, the survival and SGRs were higher when the probiotics were supplemented with the formulated feed compared to the probiotics applied into the rearing medium. The in-feed route for

administration of probiotics is advantageous in inducing growth of prawns and is more practical.

Dietary manipulation, using three different diets of fish meal, standard and bio-processed soybean meal, affected the gut microbiota loads of Atlantic cod (*Gadus morhua* L.) (Ringø *et al.* 2006). In the same way, application of probiotics with formulated feed can affect the bacterial loads (Moriarty 1998) in the prawn intestines. This was the case in this study where a higher probiotic load colonised the intestines when probiotics were supplemented with the formulated feed compared to applying the probiotics into the rearing medium. The prawns that received the probiotics supplemented with the formulated feed were healthier than those which received the probiotics via the rearing medium as indicated by higher THCs observed in the former. Although there was no significant difference in the SGC between the two application methods, the prawns fed the probiotics with the feed exhibited higher SGC proportion than those receiving the probiotics via the rearing medium.

A combination of two probiotics was shown to improve the growth of prawns (Gatesoupe 2002). Similarly, a mixture of two probiotics *Lactobacillus coagulans* and *S. cerevisiae* was more effective in increasing the growth of catla (*Catla catla*) larvae and mrigal (*Cirrhinus mrigala*) fry (Mohanty *et al.* 1996). On the contrary, in our study the combined probiotics did not significantly increase the SGRs of the prawns compared to the individual probiotics, but the combination of probiotics did show an improvement in the health of the prawns as reflected with higher THCs and SGC proportion. The prawns with higher THCs when exposed to the combined probiotics were less stressed than those exposed to the individual probiotics. *P. aeruginosa* was more effective for improving prawn health than *P. synxantha*.

The prawns exposed to the combined probiotics either through feed or media had lower HC proportion and higher GC and SGC proportions, and hence are healthier than those exposed to the individual probiotics. Probiotics have also been shown to control the spread of diseases caused by harmful bacteria and lead to a dramatic improvement in the health of prawns (Abidi 2003). In our study, the prawns receiving the combined probiotics were healthier than those receiving the individual probiotics as reflected by the lower bacterial loads in the haemolymph in the former.

The combination of the two probiotics was more effective for the prawns than individual use.

In this study, the improved health of the prawns receiving the probiotics was reflected in altered immune parameters such as THCs and HC, SGC and GC proportions, clotting times and bacterial load in the haemolymph. The benefit of probiotics was further indicated in the lower FCRs and higher survivals in the prawns. The in-feed route of administration of the probiotics was advantageous as well as practical. When tested individually, *P. aeruginosa*, as a probiotic, improved the health of the prawns more effectively than that of *P. synxantha*. Overall, the combination of both probiotics offered more advantages for improving health than the individual probiotics, as reflected in the results from testing the immune status of the prawns. The use of the probiotics (*P. synxantha* and *P. aeruginosa*) enhanced the health, survival and specific growth rates of western king prawn juveniles and, consequently, these probiotics can be recommended for use in the culture of this species of prawns.

CHAPTER 5

PREBIOTICS AND PROBIOTICS ON THE CULTIVATION OF WESTERN KING PRAWNS [3]

5.1 INTRODUCTION

The western king prawn, *Penaeus latisulcatus*, is one of the most popular species in the Indo-West Pacific region (Dore and Frimodt 1987). Recent studies on the cultivation of this species have been undertaken in Australia by Sang and Fotedar (2004a; 2004b), Prangnell and Fotedar (2005; 2006b), Prangnell (2007), Hai *et al.* (2007). As the regular use of antibiotics and chemicals as preventative and curative measures for disease leads to drug-resistant bacteria and harmful effects on the environment (Bachère 2000, 2003)., alternatives to antibiotics and chemicals to improve the quality and sustainability of aquaculture production have been seen as desirable (Li *et al.* 2006; Meunpol *et al.* 2003; Rengpipat *et al.* 1998a; Vaseeharan and Ramasamy 2003). The use of immunostimulants such as prebiotics and probiotics have been considered (Bachère 2003).

Both prebiotics Bio-Mos® and ß-1,3-glucan are two commercially available products, derived from the cell wall of yeast *Saccharomyces cerevisiae* (Couso *et al.* 2003; Waldroup *et al.* 2003), in which Bio-Mos® were recently applied in the animal husbandry industry and have shown promise in suppressing enteric pathogens and modulating the immune response of chickens and turkeys (Iji *et al.* 2001; Kocher *et al.* 2005; Valancony *et al.* 2001; Waldroup *et al.* 2003). Bio-Mos® also enhanced the growth and FCR of early weaned pigs (Davis *et al.* 2000) and pig productivity (Maribo 2003). There are a few publications on the use of Bio-Mos® on chickens and pigs, but no reports of its effectiveness have been found on the culture of western king prawns. In addition, ß-1,3-glucan has successfully enhanced resistance against bacterial and viral infections (Chang *et al.* 2003; López *et al.* 2003; Sung *et al.* 1998), used for preventing or reducing animal mortalities (Couso *et al.* 2003), ß-1,3-glucan has been proven to modulate the activity of phagocytes and other components

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of the innate immune system in both fish and mammals (Di Luzio 1985; Robertsen *et al.* 1994). The influence of glucans on the immune function and disease resistance in fish has been reviewed (Sakai 1999), but the effect of ß-1,3-D-glucan on western king prawns has not been investigated.

Probiotics are used as water additives or as feed supplements (Moriarty 1998; Skjermo and Vadstein 1999). Previous applications of probiotics have proved beneficial to the host by improving growth, survival and health (Moriarty 1998; Skjermo and Vadstein 1999). Our previous studies demonstrated that *P. synxantha* and *P. aeruginosa* are potential probiotics for use in the culture of western king prawns (Hai *et al.* 2007).

This study was conducted on juvenile western king prawns to compare the effects of the two commercially available prebiotics Bio-Mos®, ß-1,3-glucan and the previously customised probiotics *Pseudomonas synxantha* and *P. aeruginosa* (Hai *et al.* 2007) using the physiological response (SGR, survival, probiotic load in the intestine, haemolymph clotting time, surface structures of the intestines) and the immune response (the THC, hyalinocytes (HC), semi-granulocytes (SGC) and granulocytes (GC), and bacterial load in the haemolymph) of the prawns.

5.2 MATERIALS AND METHODS

5.2.1 Experimental materials and animals

Two prebiotics namely Bio-Mos® (Alltech inc., Nicholasvill, Ky, USA) and ß-1,3-D-glucan (Beta-Mune™, Germany), and two customised probiotics *Pseudomonas synxantha* and *P. aeruginosa* were employed in this study. The method of isolation and identification of these probiotics is described in our earlier publication (Hai *et al.* 2007). The probiotic solutions were prepared in marine using salt agar (MSA) plates and normal saline as in the previous work (Hai *et al.* 2009a). An appropriate inoculum at the desired density of 10^5 CFU/mL was prepared from an optical density reading at 560 nm (S-20 spectrophotometer Boeco, Germany) and compared to previously prepared standards (Hai *et al* 2007).

Western king prawn juveniles (4.63 ± 0.39 g weight and 7.65 ± 0.36 cm in total length) were collected from the Canning River, Western Australia (32°00'39 S; 115°51'15 E). They were kept in the Curtin Aquatic Research Laboratory and fed a formulated prawn feed ST#1 (43% protein, 6% fat and 2% Fibre) (Ridley Aqua-Feed, Ridley AgriProducts Pty. Ltd, Queensland, Australia) until the commencement of the experiment.

5.2.2 Experimental setup

Sixteen 70-L plastic tanks were filled with 15 L of ozonised water at 35 ppt. Seawater was ozonised at 700 mV by an ozone generator (ZOX, model AQ-2KP, Environplus, Western Australia). All the tanks were connected to independent filtration units allowing the water to circulate. During the experimental period, aeration was continuously supplied, and the prawns were stocked at 18 per tank and cultured for 84 days.

Four feed treatments consisting of two prebiotics (Bio-Mos® and ß-1,3-glucan), the two customised probiotics and the control without immunostimulants were set up in triplicate. Treatment one consisted of the formulated feed mixed with Bio-Mos® at a concentration of 0.5% obtained by dissolving in distilled water at 0.1 g/mL. Treatment two contained ß-1,3-D-glucan mixed with the feed at 0.2% (Chang *et al.* 2003) and dissolved in distilled water at 0.04 g/mL. The third treatment contained both probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) at equal concentrations of 10^5 CFU/mL (Moriarty 1998; Vijayan *et al.* 2006; Ziaei-Nejad *et al.* 2006) and sprayed into the formulated feed at 20 mL of inoculum per kg. For all treatments, the feed was coated with fish oil blend (Bait mate®, Western Australia) and air-dried under a laminar flow hood for 30 min. The feed mixed with the immunostimulants was kept in the refrigerator at 4 °C until use. The feed was given twice at 3-5% of the prawn body weight per day. The uneaten feed was siphoned from the tanks and dried in an oven at 120 °C for 12 h and used in the estimation of the food conversion ratio. The water circulation was stopped in all tanks for 2 h after every application of feed to allow the prawns to ingest the feed without disturbance.

5.2.3 Data collection

The water temperature was maintained between 24 and 25 ^{o}C using automatic heaters (Sonpar®, Model: HA-100, China). NO_2-N, NO_3-N, $NH_3/NH_4^{+,}$ PO_4-P and pH were measured every two days using chemical test kits (Aquarium pharmaceuticals Ltd, UK) and a pH meter (CyberScan pH 300, Eutech Instruments, Singapore). The salinity was measured by a portable refractometer (RHS-10ATC) every three days and maintained between 32 - 34 ppt throughout the experiment by the addition of ozonised deionised water.

The SGRs and survival of the prawns was measured by counting the number of juveniles at sampling time, measuring the weight (g), and calculated using the following equations.

$$SGR\ (\%\ g/day) = 100 \times (\ln W_t - \ln W_o)/t$$

$$Survival\ (\%) = (N_t - N_o) \times 100$$

Where:

SGR is the specific growth rate in weight (% g/day), W_t and W_o are the weight of the prawns at current time (t) and at the commencement of the experiment (o), respectively, and t is the number of rearing days.

N_t and N_o are the number of prawns at the end of the experiment (t) and at the commencement (o), respectively.

The consumed food was estimated as the difference between the given food and uneaten food. Food conversion ratio (FCR) was calculated based on the quantity of food consumed and the increase in biomass as follows:

$$FCR = Fc / \Delta W$$

Where FCR is food conversion ratio, Fc is the total food consumed (g) and ΔW is the total wet weight gained (g).

The bacterial load in the prawn intestine was determined at the commencement of the experiment and every three weeks thereafter. Three prawns from one experimental tank were selected and rinsed in distilled water, quickly washed with 70% alcohol, and then rinsed again in distilled water to remove the external bacteria. The intestinal tract of the prawn was removed and homogenised in a 1.5 mL microfuge tube using a

micropestle. The homogenic samples were weighed, diluted serially with sterilised 2% saline solution, and lawn inoculated to MSA plates. The plates were incubated for 24 h at 25 ^{o}C.

The immune parameters of the prawns such as THC, differential haemocyte counts (DHC) and bacterial loads in the haemolymph (bacteraemia rank), and clotting time rank were investigated at the commencement of the experiment and every three weeks thereafter. The haemolymph was taken from the pericardial cavity of individual prawns using a 1-mL syringe containing 0.2 mL of anticoagulant (1% glutaraldehyde in 0.2M sodium cacodylate, pH 7.0) and a 23-gauge needle to puncture the intersegmental membrane between the cephalothorax and the first abdominal segment. A 0.2-mL aliquot of haemolymph was dispensed into a 1.5-mL microfuge tube kept on ice.

The THCs were determined using a haemocytometer at 100-fold magnification. Cells were counted on both sides of the grids. THCs were calculated using the following equation.

THC = (cells counted x dilution factor x 1000) / volume of grid (0.1 mm^3)

One drop of the mixture of anticoagulant and haemolymph was smeared onto a glass microscope slide. The smear was air dried and fixed in 70% methanol for 10 minutes. The fixed smears were stained in May-Grunwald and Giemsa stains for 10 minutes each (Bancroft and Stevens 1977). Three major different haemocyte groups were identified as recommended by Bauchau (1981), Söderhäll and Smith (1983), Hose *et al*. (1990), Sequeira et al. (1995), Jussila et al. (1997), Johansson et al. (2000) and Fotedar (personal communication, 2007) such as the HCs, SGCs and GCs. A total of 200 cells were counted on each slide. The DHCs were calculated using the following equation.

$$\text{DHC (\%)} = \frac{\text{Number of different haemocyte cell types}}{\text{Total haemocyte cells counted}} x 100$$

A 30-µL aliquot of haemolymph was quickly transferred and drawn into a capillary tube (Chase, Scientific Glass Inc. Rockwood, TN 33748, USA). The time that the haemolymph stopped moving back and forth was recorded. The haemolymph

clotting time was ranked from 0 (0 - 10 s) to 9 (>90 s). If the clotting time lasted for longer than 90 s, 'no clot' was recorded for the haemolymph.

The bacterial load in the haemolymph was assessed using total bacterial counts on MSA plates. Individual drops of the haemolymph aliquot were placed onto separate MSA plates and lawn inoculated (5 drops of haemolymph were tested from each animal). The plates were incubated for 24 h at 25 ^{o}C and CFUs were counted for each drop. CFU/mL for each sample was calculated on the basis of a total volume of 20 μL for each drop. The bacterial loads were ranked from 1 (0 - 250 CFU/mL) to 12 (2751 - 3000 CFU/mL) and the rank 13 was used for "too numerous for an accurate count".

Prawn intestine samples were collected at the commencement, 6th week and at the terminal of the experiment. The samples were immersed in 3% buffered glutaraldehyde (3% glutaraldehyde in 0.1M cacodylate buffer) for 2-3 h. All traces of glutaraldehyde were removed from the samples by washing 2-3 times in buffer (10-15 min for each time). The post-fix specimens were immersed in 2% osmium tetroxide in buffer for 1-2 h. The specimens were then dehydrated through graded ethanol at 50, 70, 80, 90, 100 and 100% for 15-30, 15-30, 15-30, 30, 30 and 30 min, respectively. The specimens were dried by chemical transitional ratios of hexamethyldisilazane and ethanol at 50:50, 75:25 and 100:0% at twice for each ratio and 5 min for each time. The specimens were air-dried at room temperature. They were mounted with carbon tape and carbon paint and then coated with gold by sputter coater. They were examined under a scanning electron microscope (SEM) (Philips XL30, Netherlands) at 10 kV (Mishra and Prasad 2005).

5.2.4 Data analysis

All collected data were stored in Excel. The results were presented as means ± SE (Standard error). The SPSS statistical package version 14.0 was used to conduct one way ANOVA (Analysis of variance). LSD (least significant difference) post hoc tests were used to determine any significant differences between the tested variables of the probiotic populations in the rearing medium and in the juvenile prawn, the immune parameters, the specific growth and survival of the prawn. The Games-Howell post

hoc test was used, when variances were not homogeneous. All significant tests were performed at the P=0.05 level.

5.3 RESULTS

5.3.1 SGR, survival and FCR

Supplementation of three immunostimulants significantly increased (P<0.05) the SGR and survival and decreased (P>0.05) the FCR of the prawns. There was no significant difference in the SGR, survival and FCR of the prawns fed either prebiotics or probiotics. The highest SGR (0.88 ± 0.02 % g/day) and highest survival (77.95 ± 0.86%) was found in the prawns fed ß-1,3-glucan, and the probiotic combination, respectively. The FCR was the highest (2.97 ± 0.01) in the prawns fed without immunostimulants and the lowest (2.64 ± 0.02) in the prawns fed ß-1,3-glucan (Table 5.1).

Table 5.1
The mean ± SE of SGR, survival and FCR (n = 5) of the prawns fed the three immunostimulants over the experimental period

Parameters	Time (day)	Control	Bio-Mos®	ß-1,3-glucan	Customised probiotics
SGR (% g/day)	21	$_{1}1.06 \pm 0.01^{a}$	$_{1}1.05 \pm 0.02^{a}$	$_{1}1.05 \pm 0.01^{a}$	$_{2}1.16 \pm 0.04^{a}$
	42	$_{1}0.91 \pm 0.03^{b}$	$_{1}0.93 \pm 0.01^{b}$	$_{2}1.08 \pm 0.01^{a}$	$_{2}1.04 \pm 0.02^{b}$
	63	$_{1}0.83 \pm 0.02^{c}$	$_{1}0.84 \pm 0.02^{c}$	$_{2}0.96 \pm 0.02^{b}$	$_{1}0.84 \pm 0.01^{c}$
	84	$_{1}0.77 \pm 0.01^{d}$	$_{2}0.83 \pm 0.01^{c}$	$_{2}0.88 \pm 0.02^{c}$	$_{2}0.83 \pm 0.02^{c}$
Survival (%)		$_{1}70.36 \pm 1.49$	$_{12}73.69 \pm 1.43$	$_{12}73.75 \pm 1.76$	$_{2}77.95 \pm 0.86$
FCR		$_{1}2.97 \pm 0.01$	$_{2}2.69 \pm 0.01$	$_{2}2.64 \pm 0.02$	$_{2}2.66 \pm 0.01$

Values in any one row not preceded by the same subscript numbers are significantly different at P<0.05. Values in any one column not followed by the same superscript letters are significantly different at P<0.05.

5.3.2 Bacterial loads in the prawn intestines

Over the experimental period, the bacterial load in the prawn intestines significantly increased (P<0.05) with the exception of prawns fed Bio-Mos®. The prawns fed the prebiotic immunostimulants, Bio-Mos® and ß-1,3-glucan, and the control showed no significant differences (P>0.05) on bacterial load in the haemolymph. From the 42nd day, the bacterial load in the prawns fed the probiotics were significantly higher (P<0.05) than those fed the prebiotics or the control (Table 5.2).

Table 5.2
The mean ± SE (x 10^3) of the bacterial load in the intestines of the prawns fed the three immunostimulants over the experimental period

Time (day)	Control	Bio-Mos®	ß-1,3-glucan	Customised probiotics
0	$_1$0.34 ± 0.007^a	$_1$0.34 ± 0.007^a	$_1$0.34 ± 0.007^a	$_1$0.34 ± 0.007^a
21	$_1$1.29 ± 0.08^b	$_1$1.31 ± 0.17^b	$_1$1.27 ± 0.08^b	$_1$1.50 ± 0.15^b
42	$_1$1.44 ± 0.12^{bc}	$_1$1.59 ± 0.07^b	$_1$1.82 ± 0.19^{bc}	$_2$2.28 ± 0.19^c
63	$_1$1.43 ± 0.20^{bc}	$_1$1.39 ± 0.11^b	$_1$1.59 ± 0.22^{bc}	$_2$3.05 ± 0.31^d
84	$_1$1.86 ± 0.12^c	$_1$1.70 ± 0.34^b	$_1$1.96 ± 0.22^c	$_2$2.86 ± 0.30^d

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column not followed by the same superscript letters are significantly different at $P<0.05$.

5.3.3 Immune responses

Over the experimental period, the feeding with any immunostimulants did not significantly change ($P>0.05$) the THC of the prawns. The highest THC of 5.50 ± 0.50 x 10^6 CFU/mL was in the prawns fed the probiotics at 84 days of culture. After 42 days of culture, the prawns fed the three immunostimulants showed a significantly lower ($P<0.05$) percentages of hyaline cells, and higher ($P<0.05$) percentages of granular and semi-granular cells than those fed no immunostimulants. The percentages of hyaline cells significantly decreased ($P<0.05$) when the prawns were fed the three immunostimulants on either the 42nd or 63rd day onward, while they did not significantly increase ($P>0.05$) when the prawns were not supplied any immunostimulants over the experimental period. The percentages of granular and semi-granular cells significantly increased ($P<0.05$) on either the 21st or 42nd and either the 21st or 63rd day onward, respectively. After 21 days of culture, the clotting time ranks of the prawns fed the immunostimulants did not decrease significantly ($P>0.05$), while they significantly increased ($P<0.05$) in the prawns fed without any immunostimulants. The bacterial load in haemolymph (bacteraemia ranks) was significantly higher ($P<0.05$) in the prawns fed without the immunostimulants than in those fed the three immunostimulants. The lowest bacterial load in haemolymph was in the prawns fed the probiotics (Table 5.3).

Table 5.3

The immune responses (mean ± SE) of the prawns fed the three immunostimulants and without any immunostimulants over the experimental period

Immune responses	Time (day)	Control	Bio-Mos®	ß-1,3-glucan	Customised probiotics
THC (x10^6 cells/ml)	0	$_1$4.70 ± 0.26^a	$_1$4.70 ± 0.26^a	$_1$4.70 ± 0.26^a	$_1$4.70 ± 0.26^a
	21	$_1$4.73 ± 0.29^a	$_1$4.93 ± 0.20^a	$_1$4.97 ± 0.23^a	$_1$4.90 ± 0.20^a
	42	$_1$4.80 ± 0.15^a	$_1$4.93 ± 0.23^a	$_1$4.93 ± 0.24^a	$_1$5.13 ± 0.29^a
	63	$_1$4.77 ± 0.26^a	$_1$4.93 ± 0.45^a	$_1$5.00 ± 0.46^a	$_1$5.17 ± 0.37^a
	84	$_1$4.93 ± 0.26^a	$_1$5.03 ± 0.38^a	$_1$5.37 ± 0.28^a	$_1$5.50 ± 0.50^a
Hyaline (%)	0	$_1$55.67 ± 1.52^a	$_1$55.67 ± 1.52^a	$_1$55.67 ± 1.52^a	$_1$55.67 ± 1.52^a
	21	$_1$58.02 ± 1.21^a	$_1$54.75 ± 1.42^a	$_1$55.73 ± 2.69^a	$_1$54.19 ± 2.14^a
	42	$_1$62.01 ± 1.73^a	$_2$53.40 ± 2.55^{ab}	$_2$51.60 ± 3.53^{ab}	$_2$52.88 ± 2.54^a
	63	$_1$61.91 ± 1.16^a	$_2$48.07 ± 2.00^{bc}	$_2$48.12 ± 1.97^b	$_2$49.61 ± 1.56^{ab}
	84	$_1$63.89 ± 2.55^a	$_2$47.35 ± 1.18^c	$_2$47.06 ± 1.25^b	$_2$45.19 ± 1.85^b
Granular (%)	0	$_1$8.18 ±1.55^a	$_1$8.18 ± 1.55^a	$_1$8.18 ± 1.55^a	$_1$8.18 ± 1.55^a
	21	$_1$7.33 ± 0.33^a	$_1$7.93 ± 0.07^a	$_1$7.67 ± 0.33^a	$_1$8.19 ± 0.19^a
	42	$_1$6.71 ± 1.73^a	$_2$8.75 ± 0.38^a	$_2$9.72 ± 0.36^b	$_2$10.71 ± 0.42^b
	63	$_1$4.95 ± 0.16^b	$_2$10.58 ± 0.42^b	$_2$11.59 ± 0.34^c	$_2$11.71 ± 0.38^b
	84	$_1$4.53 ± 0.29^b	$_2$10.75 ± 0.25^b	$_2$11.19 ± 0.43^c	$_2$11.17 ± 0.59^b
Semi-Granular (%)	0	$_1$18.19 ± 0.81^a	$_1$18.19 ± 0.81^a	$_1$18.19 ± 0.81^a	$_1$18.19 ± 0.81^a
	21	$_1$17.18 ± 0.51^b	$_1$19.73 ± 0.80^b	$_1$20.08 ± 1.10^b	$_1$21.42 ± 1.25^b
	42	$_1$16.48 ± 1.73^{bc}	$_2$21.22 ± 1.61^{bc}	$_2$24.34 ± 0.86^c	$_2$23.86 ± 2.58^{bc}
	63	$_1$14.00 ± 0.58^{bc}	$_2$23.33 ± 1.45^{bc}	$_2$24.15 ± 0.71^c	$_2$24.72 ± 0.82^{bc}
	84	$_1$13.33 ± 1.40^c	$_2$23.41 ± 2.11^c	$_2$25.80 ± 1.17^c	$_2$26.10 ± 0.72^c
Clotting time (rank)	0	$_1$6.33 ± 0.56^a	$_1$6.33 ± 0.56^a	$_1$6.33 ± 0.56^a	$_1$6.33 ± 0.56^a
	21	$_1$5.67 ± 0.33^{ab}	$_1$5.67 ± 0.33^b	$_1$5.33 ± 0.33^b	$_1$5.33 ± 0.33^b
	42	$_1$5.33 ± 1.73^b	$_1$5.33 ± 0.33^b	$_1$5.60 ± 0.29^b	$_1$5.53 ± 0.22^b
	63	$_1$6.33 ± 0.33^a	$_{12}$5.80 ± 0.07^b	$_{12}$5.56 ± 0.28^b	$_2$5.20 ± 0.24^b
	84	$_1$7.75 ± 0.25^c	$_2$5.21 ± 0.24^b	$_2$5.29 ± 0.34^b	$_2$4.75 ± 0.10^b
Bacterial loads in haemolymph (rank)	0	$_1$3.00 ± 0.00^a	$_1$3.00 ± 0.00^a	$_1$3.00 ± 0.00^a	$_1$3.00 ± 0.00^a
	21	$_1$3.33 ± 0.33^a	$_1$3.33 ± 0.33^a	$_1$3.33 ± 0.33^a	$_1$3.33 ± 0.33^a
	42	$_1$3.67 ± 0.33^a	$_{12}$3.33 ± 0.33^a	$_{12}$3.33 ± 0.33^a	$_2$2.67 ± 0.33^{ab}
	63	$_1$3.33 ± 0.33^a	$_{12}$2.67 ± 0.33^{ab}	$_{12}$2.67 ± 0.33^{ab}	$_2$2.33 ± 0.33^b
	84	$_1$3.00 ± 0.58^a	$_{12}$2.33 ± 0.33^b	$_{12}$2.33 ± 0.33^b	$_2$2.00 ± 0.00^b

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column not followed by the same superscript letters are significantly different at $P<0.05$.

5.3.4 Surface structure of the prawn intestines

Over the experimental period, the surface structure of the prawn intestines changed remarkably among treatments. The crypts in the intestines of the prawns fed the immunostimulants were deeper than those in prawns not fed with the immunostimulants (Plate 5.1 and 5.2). Supplementary feed with Bio-Mos® resulted

in changes in the rotational cluster of upper and lower folds, which were larger and deeper as the upper folds were clearly separated from the lower folds (Plate 5.1B and 5.2B). The upper folds under the effect of ß-1,3-glucan tended to be larger, so consequently the lower folds appeared as streaks (Plate 5.1C and 5.2C). Under the effect of probiotics, the surface of the intestines was larger with an increase in the small perpendicular folds in the upper folds. The lower and upper folds were separated less clearly than those in the ß-1,3-glucan-fed prawns (Plate 5.1D and 5.2D).

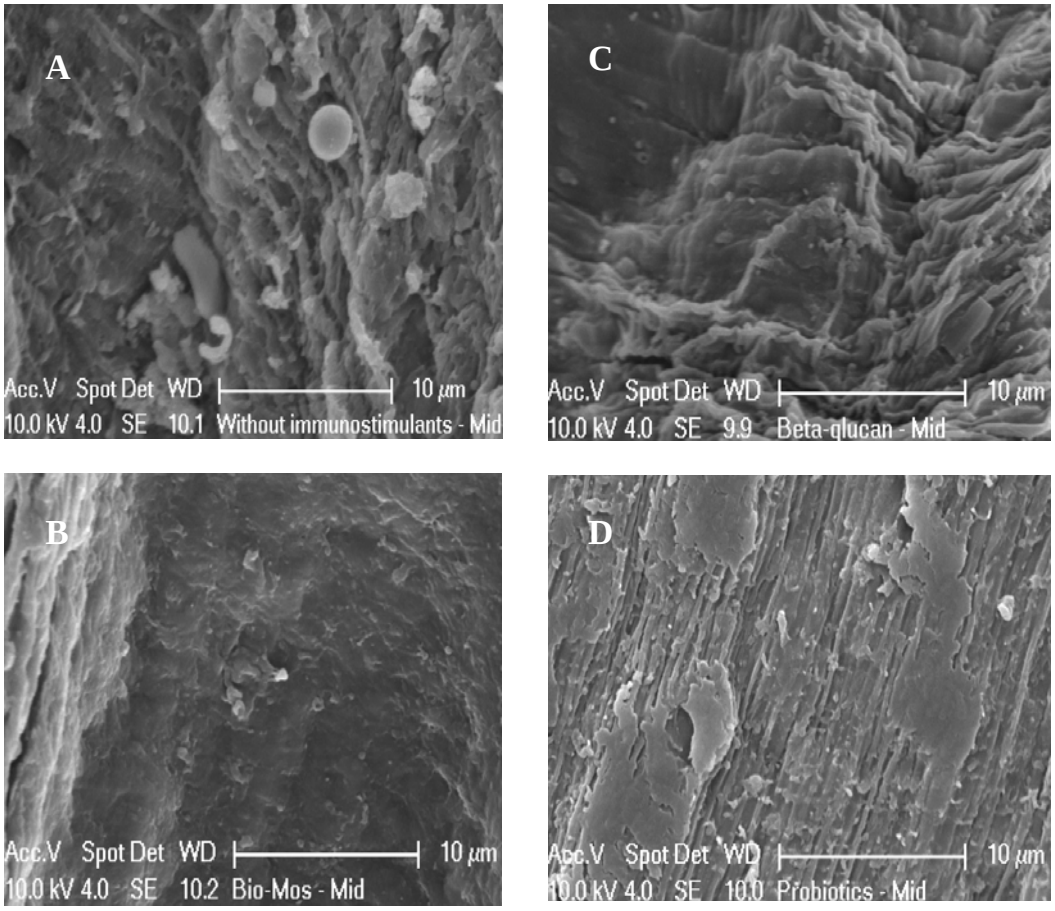

Plate 5.1 The surface structure of the prawn intestine samples at the 6th week without immunostimulants (A), supplemented with Bio-Mos® (B), ß-1,3-D-glucan (C) and the customised probiotics (D).

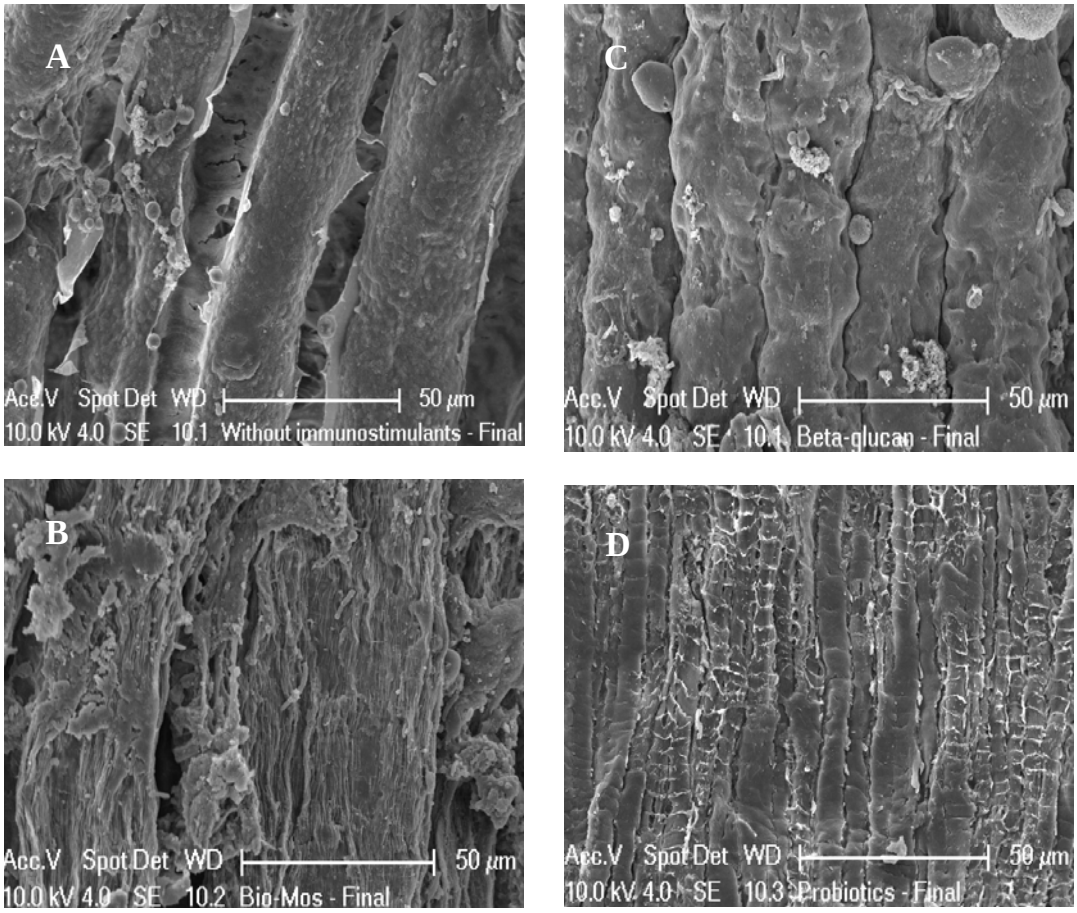

Plate 5.2 The surface structure of the prawn intestine samples at the 12th week without immunostimulants (A), supplemented with Bio-Mos® (B), ß-1,3-D-glucan (C) and the customised probiotics (D).

5.4 DISCUSSION

Stimulation of the non-specific defence mechanisms by using specific biological compounds, called immunostimulants, enhances the disease resistance and growth of the hosts (Skjermo *et al.* 2006). Invertebrates are not equipped with cells that are analogous to antibody producing lymphocytes in vertebrates (Sakai 1999). The defence weapon of invertebrates is the innate immune system such as physical barriers, cellular and humoral components (Magnadóttir 2006). Live bacteria in probiotics and prebiotics are known as immunostimulants (Smith *et al.* 2003) and act as alternative treatments to antibiotics and chemicals, and play the role of alarm molecules to activate the immune system (López *et al.* 2003). The benefits of the use of prebiotics and probiotics to the host have been mentioned by several authors (Douglas and Sanders 2008; Reid 2008; Verschuere *et al.* 2000; Wang *et al.* 2008a).

In this study, the immunostimulants supplemented either prebiotics (Bio-Mos® and ß-1,3-D-glucan) or customised probiotics with the feed improved the growth, survival and immune response of the prawns. However, this study could not detect the relative advantages of one immunostimulant over the others.

Although both prebiotics and probiotics are additives and adjuvants, prebiotics are non-digestible substances, while probiotics are live organisms and digestible substances (Douglas and Sanders 2008; Reid 2006). Both Bio-Mos® and ß-1,3-glucan are derived from yeast (*Saccharomyces cerevisiae*) cell wall (Couso *et al.* 2003; Waldroup *et al.* 2003). Several strains of baker's yeast are excellent sources of ß-glucan (Chang *et al.* 2003), were found to be good immune enhancers in crustaceans because of its effect on crustacean immune systems (Ai *et al.* 2007). Recently, Bio-Mos® has been tested and promoted as a potential alternative to antibiotics and chemicals used in the pig, broiler and poultry (chickens and turkey) industries (Iji *et al.* 2001; Kocher *et al.* 2005; Staykov *et al.* 2007; Valancony *et al.* 2001; Waldroup *et al.* 2003), ß-1,3-glucan is used in small dosages in humans, while probiotics are used not only in humans (Douglas and Sanders 2008; Mombelli and Gismondo 2000; Sullivan and Nord 2002) but also in aquatic animals (Irianto and Austin 2002a).

In this study, Bio-Mos® at 0.5%, ß-1,3-D-glucan at 0.2% and a combination of two probiotics (10^5 CFU/mL) at 20 mL/kg supplemented with the feed to the prawn culture for 84 days has improved the SGR, survival and FCR and immune response of the prawns. It is essential that correct dosages of immunostimulants are used through appropriate application methods in order to achieve the desired results. Overdose or prolonged administration of immunostimulants can induce immunosuppression of continuous responses (Sakai 1999). Researchers have used different concentrations of Bio-Mos® (Eren *et al.* 1999; Fisher *et al.* 2001; Fritts and Waldroup 2003; Iji *et al.* 2001; Kocher *et al.* 2005; Kumprecht and Zobac 1997; Maribo 2002; Shafey *et al.* 2001; Waldroup *et al.* 2003), ß-1,3-glucan (Ai *et al.* 2007; Chang *et al.* 2003; Couso *et al.* 2003; López *et al.* 2003; Wang *et al.* 2008b) and probiotics (Bricknell and Dalmo 2005; Moriarty 1998; Rengpipat *et al.* 2000; Vijayan *et al.* 2006; Yoshida *et al.* 1995) with varying outcomes in terms of SGR, FCR and immune response of various target animals. In addition, continuous

exposure to immunostimulants can induce tolerance of the hosts due to desensitisation of the immune system to immunostimulants, therefore, the immune response can also be lost, immunostimulants boost and maintain the immune system to heightened levels until immunostimulants are withdrawn (Bricknell and Dalmo 2005). For example, the number of nitroblue tetrazolium-positive cells in African catfish (*Clarias gariepinus*) fed glucan or oligosaccharide increased only for 30 days, but not for 45 days of culture (Yoshida *et al.* 1995).

The three immunostimulants have shown similar effects on increase in the SGR, survival and decrease in the FCR of the prawns. Similarly, supplementing diets with Bio-Mos® reduced the FCRs of broilers (Waldroup *et al.* 2003), poultry (Fritts and Waldroup 2003) and hens (Kocher *et al.* 2005) and ß-glucan enhanced the growth of yellow croaker (*Pseudosciena crocea*) (Ai *et al.* 2007), pacific white prawns (*Litopenaeus vannamei*) (López *et al.* 2003) and improved the survival of white spot syndrome virus (WSSV)-infected black tiger prawns (*Penaeus monodon*) (Chang *et al.* 2003). Studies on the effects of probiotics on aquatic animals have suggested that microorganisms have a beneficial effect on the digestive processes too (Reid 2008). In this study, the three immunostimulants have increased the surface structure of the prawn intestines compared to those without any immunostimulants. Changing intestinal morphology of smaller crypts with a larger number leads to an increase in the surface structure of the intestines. The nutrient absorption was better when the prawns were fed either Bio-Mos® or ß-1,3-D-glucan or probiotics than those fed without any immunostimulants. Consequently, lower FCRs were observed in the prawns fed the three immunostimulants rather than those without immunostimulants. The surface intestines of the prawns fed ß-1,3-D-glucan seemed to be largest with larger upper folds so that the prawns absorbed the feed better than the those fed without ß-1,3-D-glucan leading to a lower FCR.

Diets supplemented with immunostimulants are of considerable benefit by boosting the prawns' innate defences. Prebiotics and probiotics can modulate immunity and promote immune boosting effects (Reid 2008). The perceived benefits of using immunostimulants in larviculture of aquatic animals are numerous, such as the improvement in welfare, health and production (Rautava *et al.* 2002; Viljanen *et al.* 2005). Prebiotics influenced the gut microbiota of pigs (Rekiel *et al.* 2007). The use

of probiotics also conferred immunomodulatory effects and reduced allergic tendency (Waldroup *et al.* 2003). Similar to our previous study (Hai *et al.* 2009a), the use of the three immunostimulants did not change the THC, but the probiotics decreased the HC proportion and increased the SCG and CG proportions of the prawns. In contrast, the proportion of SGC increased in a study conducted by Jussila *et al.* (1997). The decrease in the SGC proportion could be due to fluctuation of HC, SCG and GC proportions by time (Fotedar *et al.* 2006) as there is an overlap in the functionality of SGCs and GCs on cytotoxicity and the storage and release of the prophenoloxidase system (Hose *et al.* 1990; Johansson *et al.* 2000; Martin *et al.* 1996). The lower GC and higher SGC proportions of moribund white and red western rock lobster than the healthy ones (Jussila *et al.* 1997), and the number of GCs in air-exposed western rock lobster was lower than those not exposed to air (Fotedar *et al.* 2001); whereas, no beneficial effects were found on the use of immunostimulants (Anderson 1997; deBaulny *et al.* 1996; Kawakami *et al.* 1998), and some preparations of immunostimulants have been toxic to animals (Bullock *et al.* 2000; Salinas *et al.* 2004).

Immunostimulants can be used as a suitable alternative to antibiotics or growth promoters (Rekiel *et al.* 2007). Probiotics *Bacillus* spp. can be a suitable alternative to the prophylactic use of antibiotics and chemicals (Decamp *et al.* 2008) as many antibiotic compounds are naturally produced by a huge range of probiotics (*Bacillus* species), which secrete many enzymes to compete for nutrients and sites and inhibit other bacteria if probiotics are present in high numbers (Moriarty 1998). There was a similar effect on broilers of the antibiotic of copper, the FCR of broilers fed Bio-Mos® was lower than that fed without a combination of antibiotic and Bio-Mos® (Waldroup *et al.* 2003). Bio-Mos® improved the intestinal morphological characteristics of broiler chickens (Loddi *et al.* 2004). Gilthead seabream (*Sparus aurata*) that were fed glucan showed enhanced protection against *Photobacterium damselae* (Couso *et al.* 2003). ß-1,3-glucan enhanced the haemocyte phagocytic activity, cell adhesion ans superoxide anion production of black tiger prawn brooders (Chang *et al.* 2000), produced a positive effect on the immune system of WSSV-infected black tiger prawns (Chang *et al.* 2003), and on the innate immunity of large yellow croaker (Ai *et al.* 2007). In this study, the prawns that received either the prebiotics or the probiotics resulted in healthier prawns indicated by higher THC,

lower clotting time and bacterial load in the haemolymph compared to those that did not receive any immunostimulants.

There were some different effects on the physiological and immune response of the prawns fed the three immunostimulants. The use of the probiotics maintained a higher bacterial load in the prawn intestines and a lower level of bacterial load in the prawn haemolymph compared to those fed the prebiotics or without any immunostimulants. The direct use of probiotic bacteria contributed to an increase in the total bacterial counts in the prawn intestines, but the prawns can prevent bacterial contamination to their haemolymph. The higher survival also occurred in the prawns fed the probiotics, while higher SGR and lower FCR occurred in the prawns fed ß-1,3-D-glucan. ß-1,3-D-glucan has the most positive effect on the culture of the prawns as the prawns converted the feed more effectively than those fed other immunostimulants. In addition, the prawns fed the probiotics with the higher bacterial load in the intestines may digest the feed better with significant lower FCR and be healthier with significant lower bacterial load in haemolymph than those without probiotics.

Overall, the decision to use either prebiotics or probiotics should be governed by the costs of these products and ease of their applications. Currently, commercial Bio-Mos® and ß-1,3-glucan are easily available in the market at AU$5-10/kg and $9.95/200 mg (YoungAgain™ 2008), respectively. Prebiotics are commonly used at small dosages; whereas, customised probiotics need long procedures to isolate, identify, purify and test, and their true costs may vary.

CHAPTER 6

PROBIOTIC-FED WESTERN KING PRAWNS CHALLENGED WITH *VIBRIO HARVEYI* [4]

6.1 INTRODUCTION

The western king prawn (*Penaeus latisulcatus*) is one of the popular species in the Asian Pacific region for farming purposes. Several studies on the cultivation of this species have recently been undertaken in Australia by Sang and Fotedar (2004a; 2004b), Prangnell and Fotedar (2005; 2006b), Prangnell (2007), Hai et al (2007), Hai et al. (2009a) and Hai and Fotedar (2009). In recent decades, the intensification of prawn culture has developed rapidly and the industry has experienced serious losses because of diseases and environmental deterioration (Bondad-Reantaso *et al*. 2005; Rodríguez et al. 2007). The commercial culture of prawns has been hampered by endemic and epidemic infections caused by *V. harveyi*, which is considered to be one of the most harmful bacteria for prawns (Austin and Austin 2005; Le Moullac *et al.* 1998a), and causes high mortalities in the prawn culture industry worldwide (Karunasagar *et al.* 1994; Lavilla-Pitogo *et al.* 1998; Liu *et al.* 1996a; Liu *et al.* 1996b; Saeed 1995).

Substantial increases in the regular use of chemical additives and veterinary medicines as preventative and curative means against diseases has led to antimicrobial-resistant strains of pathogenic bacteria (Bachère 2000, 2003; Nomoto 2005). Therefore, alternatives to antibiotics and chemicals are desirable to improve the quality and sustainability for prawn production (Li *et al.* 2006; Meunpol *et al.* 2003; Rengpipat *et al.* 1998a; Vaseeharan and Ramasamy 2003). One such alternative is the use of probiotics, which is seen to have potential and is favoured in the aquaculture industry.

Previous applications of probiotics through water additives or as feed supplements (Moriarty 1998; Skjermo and Vadstein 1999), have proved beneficial to the host by

[4] Paper submitted for publication (*Appendix 7*)

improving growth, survival and health (Moriarty 1998; Skjermo and Vadstein 1999). Our previous studies have demonstrated that customised probiotics *P. synxantha* and *P. aeruginosa* are appropriate probiotics for the use in the cultivation of *P. latisulcatus* (Hai *et al.* 2009a; Hai and Fotedar 2009; Hai *et al.* 2007). However, a rational evaluation of the effectiveness of probiotics in aquaculture needs to be undertaken (Wang *et al.* 2008a). Therefore, an immersion challenge of *P. latisulcatus* with *V. harveyi* was investigated in this study, in which *V. harveyi* isolated from Mahimahi (*Coryphaena hippurus*) was used to infect *P. latisulcatus* and then to investigate the effectiveness of the customised probiotics on *P. latisulcatus* by examining the physiological and immune responses of the prawns.

6.2 MATERIALS AND METHODS

6.2.1 Animals and experimental culture procedures

Juvenile western king prawns (4.57±0.42 g) were collected from the Canning River, Western Australia (32°00'39 S; 115°51'15 E) and transferred to the Curtin Aquatic Research Laboratory. They were kept in 200 L composite tanks and fed twice a day with prawn formulated feed ST#1 (43% protein, 6% fat and 2% fibre) (Ridley Aqua-Feed, Ridley AgriProducts Pty. Ltd, Queensland, Australia) until the experiment commenced.

The previously tested probiotics, *P. synxantha* and *P. aeruginosa* (Hai *et al.* 2007) were employed in this study. The method of isolation and identification of these probiotics is described by Hai et al. (2007). The probiotic solutions were prepared in normal saline after subculturing on marine salt agar (MSA) (Buller 2004) as in the previous work (Hai *et al.* 2009a; Hai and Fotedar 2009). A known pathogenic bacterial strain of *V. harveyi* isolated from diseased Mahimahi and stored at -80 °C at the Animal Health Laboratory, Department of Agriculture and Food, Western Australia, was used for this study. Both probiotics and *V. harveyi* solutions were cultured at 25 °C for 24 h in tryptic soy broth (OXOID, Adelaide, SA, Australia) containing a final concentration of 2% NaCl (Buller 2004). The bacterial inoculum was prepared in 2% saline using a probiotic concentration of 10^5 CFU/mL (Moriarty 1998; Vijayan *et al.* 2006; Ziaei-Nejad *et al.* 2006) and *V. harveyi* concentration of

10^3, 10^5 and 10^7 CFU/mL. The concentrations were obtained using an optical density reading of 560 nm (Spectrophotometer BOECO S-20, Hamburg, Germany) against a standard curve as determined previously (Hai *et al.* 2007).

6.2.2 Experimental setup

The entire experiment consisted of two phases. In phase 1, the juvenile western king prawns were cultured for 28 days and then subjected to *V. harveyi* exposures for another 7 days, which was termed as phase 2.

During phase 1, a group of 100 juvenile western king prawns (4.12± 0.36 g) was fed the formulated diet supplemented with customised probiotics. The second group of 100 juvenile prawns (4.13± 0.21g) was fed the formulated diet without any supplementation of probiotics, as a control group. Seawater ozonated at 700 mV by an ozone generator (ZOX, model AQ-2KP, Environplus, WA, Australia) was used as a cultured medium for this study. Each prawn group was equally stocked into four 70-L plastic tanks. Each tank was filled with 15 L of ozonated water and connected to independent filtration units allowing the water to circulate. Aeration was continuously supplied and the water temperature was maintained at 25 ^oC using an automatic heater (Sonpar®, Model: HA-200, Zhongshan, Guangdong, China). Fifteen percent of water was exchanged weekly with ozonated seawater. The prawn formulated feed was sprayed with 20 mL of 2% saline solution with 20 mL of prepared inoculum of the two probiotics at equal 50:50 v/v per kg for probiotic-fed group. Some feed was sprayed with same volume of saline solution and prepared inoculum without probiotics for non-probiotic fed group (Hai *et al.* 2009a; Hai and Fotedar 2009). The prawns were fed twice a day at 3-5 % body weight.

During phase 2, both prawn groups were separately challenged with *V. harveyi* in a water bath placed in a laboratory hood (Biological safety cabinet class 1, BTR Environmental Pty Ltd., Australia) so as to prevent the spread of contamination of *V. harveyi*. Twelve 2-L glass beakers were filled with 1.5 L of ozonised seawater at 35 parts per thousand. The beakers were set up as a random design in a water bath, in which an automatic heater (Sonpar®, Model: HA-200, Zhongshan, Guangdong, China) and a pump (Accent pump WWA1000, Waterwerks Australia Pty Ltd,

Victoria, Australia) were installed on one side of the water bath to maintain water circulation and a temperature of 25 ^{o}C during the challenge periods (Phase 2). The beakers were also covered firmly by a net to prevent the prawns escaping from the beakers.

The juvenile prawns were stocked at 6 per beaker. The *V. harveyi* inoculum was added to the beakers as three treatments consisting of concentrations of 10^3, 10^5 and 10^7 CFU/mL against a control without *V. harveyi* and the challenge was run in triplicate.

During the challenge period, aeration was supplied continuously. The water was maintained at a level of 1.5 litres per beaker by syphoning uneaten feed and replacing with 2% saline solution containing the appropriate concentrations of *V. harveyi* suspension for each concentration. The dead prawns were removed from the beakers after every 12 h. During 7 days of challenge, the prawns were continuously supplemented with their respective formulated feeds as per their nominated two groups.

6.2.3 Data collection

The data were collected and recorded at day 0, day 14 and day 28 during phase 1. During phase 2, the observable disease symptoms of the prawns challenged with *V. harveyi* were recorded after every 12 h. The prawns were sampled at the commencement of the challenge and also when only one moribund prawn was left in each treatment. The 100% survival and survival-hours for the treatment were recorded when 100% of prawns were alive and when at least one prawn was able to survive in the treatment, respectively.

The prawn survival was measured by counting the number of prawns at the commencement and at the nominated sampling times, and calculated using the following equation:

$$\text{survival (\%)} = (N_t - N_o) \times 100$$

where N_t and N_o are the number of prawns at the end of the time (t) and at the commencement (o), respectively.

The lethal time 50% (LT_{50}) was obtained from the regression equations between the prawn mortalities (%) and time (h).

The total bacterial load in the intestine was determined by selecting one prawn per treatment, which was rinsed in distilled water, followed by a quick wash with 70% alcohol, and then rinsed again in distilled water to remove the external bacteria. The prawn intestines were then removed and homogenised in a 1.5 mL microfuge tube using a micropestle. The homogenic samples were weighed, diluted serially with sterile 2% saline solution, and lawn inoculated onto MSA plates as our previous study. The plates were incubated for 24 h at 25 ^{o}C and the colonies counted to obtain the total bacterial count per gram.

The prawn haemolymph was taken from the pericardial cavity of individual prawns using a 1-mL syringe containing 0.2 mL of anticoagulant (1% glutaraldehyde in 0.2M sodium cacodylate, pH 7.0) and a 23-gauge needle to puncture the intersegmental membrane between the cephalothorax and the first abdominal segment. A 0.2-mL aliquot of haemolymph was dispensed into a 1.5 mL microfuge tube and kept on ice.

The Total haemocyte count (THC) was determined using a haemocytometer at 100-fold magnification. Haemocyte cells were counted on both sides of the grids. THCs were calculated using the following equation.

$$\text{THC} = (\text{cells counted} \times \text{dilution factor} \times 1000) / \text{volume of grid } (0.1\ \text{mm}^3)$$

One drop of the mixture of anticoagulant and haemolymph was smeared onto a glass microscope slide. The smear was air dried and fixed in 70% methanol for 10 minutes. The fixed smears were stained in May-Grunwald and Giemsa stain for 10 minutes each (Bancroft and Stevens 1977). Three major haemocyte groups were identified as hyalinocyte (HC), semi-granulocyte (SGC) and granulocyte (GC) (Hose *et al.* 1990; Johansson *et al.* 2000; Jussila *et al.* 1997; Sequeira *et al.* 1995). A total of 200 cells were counted on each slide. The differential haemocyte count (DHC) was calculated using the following equation.

$$\text{DHC (\%)} = \frac{\text{Number of different haemocyte cell types}}{\text{Total haemocyte cells counted}} x\,100$$

To measure haemolymph clotting time a 30-μL aliquot of haemolymph was quickly transferred and drawn into a capillary tube (Chase, Scientific Glass Inc. Rockwood, TN 33748, USA). The time at which the haemolymph stopped moving back and forth was recorded. The haemolymph clotting time was ranked from 0 (0 - 10 s) to 9 (>90 s). If the clotting time lasted for longer than 90 s, 'no clot' was recorded for the haemolymph.

The bacterial load in the haemolymph of the prawns was assessed on MSA. Individual drops of the haemolymph aliquot were placed onto separate MSA plates and lawn inoculated (5 drops of haemolymph were tested from each animal). The plates were incubated for 24 h at 25 ^{o}C and CFUs were counted for each drop. CFU/mL for each sample was calculated on the basis of a total volume of 20 μL for each drop. The bacterial loads were ranked from 1 (0 - 250 CFU/mL) to 12 (2751 - 3000 CFU/mL) and the rank 13 was used for "too numerous for an accurate count".

The methods used to determine the survival, THC, DHC, clotting time, bacteraemia and bacterial load in the intestine of the prawns, were the same for both groups in both phases.

6.2.4 Data analyses

The data were presented as mean ± SE (standard error). Statistical Package for the Social Sciences (SPSS) version 17.0 was used to conduct one way ANOVA (analysis of variance). LSD (least significant difference) post hoc tests were used to determine any significant differences between the tested variables of the physiological and immune responses, and survival of the prawns. The Games-Howell post hoc test was used, when variances were not homogeneous. All significant tests were performed at P=0.05.

6.3 RESULTS

Till day 14, before the prawns were challenged with *V. harveyi* (phase 1), the survival of the probiotic-fed prawns was significantly higher ($P<0.05$) than those of the prawns not fed probiotic. At day 14, among all physiological and immune responses, only the bacterial load in the prawn intestines was significantly higher ($P<0.05$) when the prawns were fed a diet without the probiotics. At day 28, application of the probiotics resulted in significantly higher ($P<0.05$) SGC, GC proportions, and significantly lower ($P<0.05$) clotting time and bacterial load in the prawn intestines (Table 6.1).

Table 6.1
The physiological and immune responses (mean ± SE) of the prawns that were fed and not fed the customised probiotics (*in italic* in bracket) before challenging with *V. harveyi*

Parameter	**Day 0**	**Day 14**	**Day 28**
Survival (%)	$_1100.00 \pm 00^a$ *($_1100.00 \pm 00^a$)*	$_287.00 \pm 1.31^a$ *($_276 \pm 0.02^b$)*	$_285.00 \pm 1.11^a$ *($_273 \pm 0.00^b$)*
THC ($\times 10^6$ Cells/mL)	$_14.73 \pm 0.12^a$ *($_14.73 \pm 0.12^a$)*	$_14.88 \pm 0.11^a$ *($_14.73 \pm 0.01^a$)*	$_14.94 \pm 0.12^a$ *($_14.76 \pm 0.03^a$)*
Hyalinocyte (%)	$_156.67 \pm 3.48^a$ *($_156.67 \pm 3.48^a$)*	$_154.33 \pm 2.91^a$ *($_157.33 \pm 1.76^a$)*	$_152.67 \pm 3.06^a$ *($_157.67 \pm 0.65^a$)*
Semi-Granulocyte (%)	$_118.17 \pm 1.74^a$ *($_118.17 \pm 1.74^a$)*	$_120.00 \pm 1.76^a$ *($_115.16 \pm 1.75^a$)*	$_121.00 \pm 1.44^a$ *($_114.18 \pm 1.48^b$)*
Granulocyte (%)	$_18.17 \pm 0.60^a$ *($_18.17 \pm 0.60^a$)*	$_18.50 \pm 0.29^a$ *($_17.35 \pm 0.62^a$)*	$_19.50 \pm 0.44^a$ *($_17.26 \pm 0.45^b$)*
Clotting time (rank)	$_16.33 \pm 0.33^a$ *($_16.33 \pm 0.33^a$)*	$_16.67 \pm 0.33^a$ *($_16.00 \pm 0.58^a$)*	$_15.67 \pm 0.58^a$ *($_17.00 \pm 0.33^b$)*
Bacteraemia (rank)	$_13.00 \pm 0.00^a$ *($_13.00 \pm 0.00^a$)*	$_13.33 \pm 0.33^a$ *($_12.67 \pm 0.33^a$)*	$_13.67 \pm 0.33^a$ *($_12.67 \pm 0.33^a$)*
Bacterial load in intestine ($\times 10^3$ CFU/g)	$_10.34 \pm 0.009^a$ *($_10.34 \pm 0.009^a$)*	$_10.45 \pm 0.006^a$ *($_21.97 \pm 0.21^a$)*	$_10.56 \pm 0.004^a$ *($_33.74 \pm 0.41^b$)*

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Value in any column at the same parameters not followed by the same superscript letters are significantly different at $P<0.05$.

When the prawns were challenged with *V. harveyi* (phase 2), the LT_{50} estimates were shorter when *V. harveyi* concentrations were increased. When challenged with the similar *V. harveyi* concentrations, the LT_{50} values of the prawns fed probiotics was significantly longer ($P<0.05$) than those not fed probiotics (Table 6.2). The absence of the probiotics in the diets resulted in a reduction in the survival-hours of the prawns challenged with any *V. harveyi* concentrations. At *V. harveyi* concentration of 10^3 CFU/mL, the 100% survival-hours of the prawns fed probiotics were three-times higher than those of the prawns not fed probiotics (Figure 6.1).

Table 6.2
The lethal time 50% (LT_{50}) of the prawns that were fed and not fed the customised probiotics and the correlation between the prawn mortalities (%) and time (h)

	***V. harveyi* concentrations**	**Equation**	**R^2**	**LT_{50} (h)**
Prawns were not fed probiotics	10^3	y = 2.8241x – 5	R^2 = 0.9877	55
	10^5	y = 1.7063x + 5.291	R^2 = 0.9790	26
	10^7	y = 1.0101x - 5.6566	R^2 = 0.9857	20
Prawns were fed probiotics	10^3	y = 0.6465x - 11.576	R^2 = 0.9009	95
	10^5	y = 0.9097x -11.677	R^2 = 0.9607	68
	10^7	y = 1.6204x - 17.857	R^2 = 0.9088	42

y and x presented the prawn mortalities and time, respectively.

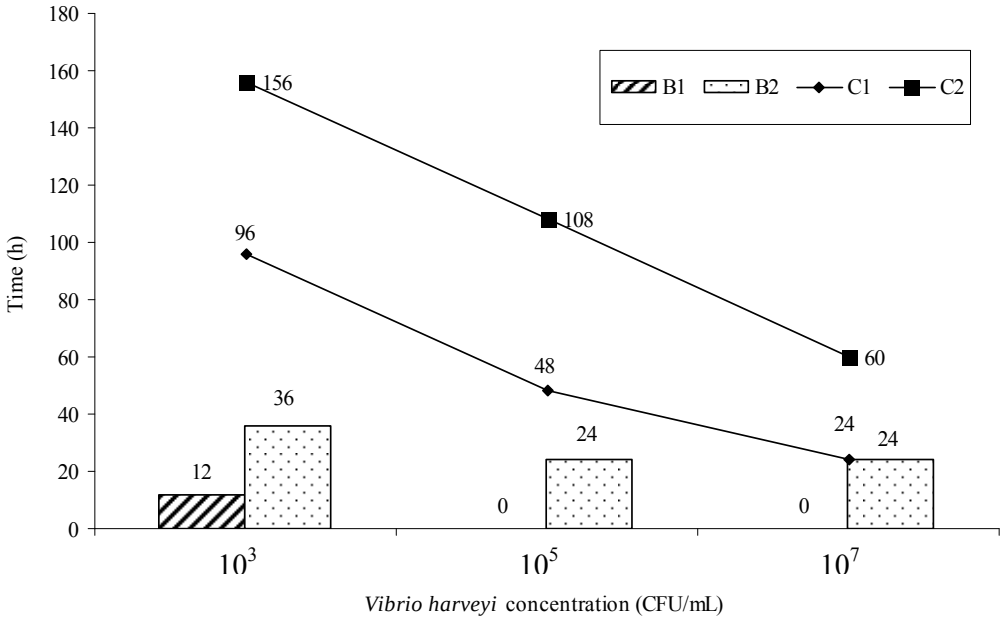

Figure 6.1 The 100% survival hours of the prawns that were not fed probiotics (B1) and probiotic-fed prawns (B2), the survival hours of the prawns that were not fed probiotics (C1) and probiotic-fed prawns (C2) challenged with *V. harveyi* at 0, 10^3, 10^5 and 10^7 CFU/mL.

In the probiotic-fed prawn group, at 24 h onward, all concentrations of *V. harveyi* significantly altered ($P<0.05$) the physiological and immune responses of the prawns compared to the control. A 24-h challenge with *V. harveyi* at 10^3 and 10^5 CFU/mL had no significant influence on the physiological and immune responses. However, these responses significantly changed ($P<0.05$), when the concentration of *V. harveyi* was increased to 10^7 CFU/mL. At 36 h of challenge, the GC proportions, bacteraemia and bacterial load in the prawn intestines showed significant differences ($P<0.05$) between the challenges with *V. harveyi* at 10^3 and 10^5 CFU/mL (Table 6.3).

In the prawn group not fed probiotic, at 60 h of challenge onward, challenge with *V. harveyi* significantly changed ($P<0.05$) the physiological and immune responses of the prawns. At 60 h of challenge, only *V. harveyi* at 10^7 CFU/mL significantly changed ($P<0.05$) the physiological and immune responses of the prawns. At 108 h of challenge, the prawns challenged with *V. harveyi* at 10^3 CFU/mL showed significantly higher ($P<0.05$) GC proportions and bacterial load in the prawn intestine than those challenged with *V. harveyi* at 10^5 CFU/mL (Table 6.4).

Most moribund prawns showed loss of appetite, movement and caseation in the 6th abdominal segment initially, followed by caseation in other parts of the body. The eroded appendages included the walking legs, antennae, antennal scales, from the uropods to abdominal segment, which were eroded progressively over time. Black gills and black spots along the abdominal segments appeared more frequently before the prawns appeared moribund (Plate 6.1 and 6.2).

Plate 6.1 The healthy prawns after 36 h of exposure to *V. harveyi*

Table 6.3
The physiological and immune responses (mean ± SE) of the probiotic-fed prawns during the challenge with *V. harveyi* at 0, 10^3, 10^5 and 10^7 CFU/mL

Time (h)	*V. harveyi* (CFU/mL)			
	0	**10^3**	**10^5**	**10^7**
THC (× 10^6 Cells/mL)				
0	$_1$4.89 ± 0.15^a	$_1$4.89 ± 0.15^a	$_1$4.89 ± 0.15^a	$_1$4.89 ± 0.15^a
24	$_1$4.73 ± 0.12^a	$_2$2.73 ± 0.13^b	$_2$2.77 ± 0.37^b	$_3$1.83 ± 0.15^b
36	$_1$4.7 ± 0.06^a	$_2$2.33 ± 0.09^b	$_2$2.00 ± 0.15^c	
96	$_1$4.6 ± 0.27^a	$_2$1.73 ± 0.07^b		
Hyalinocyte (%)				
0	$_1$52.00 ± 3.69^a	$_1$52.00 ± 3.69^a	$_1$52.00 ± 3.69^a	$_1$52.00 ± 3.69^a
24	$_1$52.33 ± 3.28^a	$_2$62.33 ± 2.33^b	$_2$61.67 ± 0.67^b	$_3$73.00 ± 2.31^b
36	$_1$51.67 ± 3.38^a	$_2$68.33 ± 1.76^b	$_2$66.00 ± 1.53^b	
96	$_1$51.67 ± 3.33^a	$_2$70.67 ± 2.96^b		
Semi-Granulocyte (%)				
0	$_1$21.00 ± 1.44^a	$_1$21.00 ± 1.44^a	$_1$21.00 ± 1.44^a	$_1$21.00 ± 1.44^a
24	$_1$21.67 ± 0.88^a	$_2$14.33 ± 0.33^b	$_2$13.33 ± 2.40^b	$_3$8.33 ± 0.88^b
36	$_1$21.33 ± 1.45^a	$_2$10.33 ± 1.20^c	$_2$8.67 ± 0.33^c	
96	$_1$21.33 ± 1.20^a	$_2$8.33 ± 0.88^c		
Granulocyte (%)				
0	$_1$8.50 ± 0.50^a	$_1$8.50 ± 0.50^a	$_1$8.50 ± 0.50^a	$_1$8.50 ± 0.50^a
24	$_1$8.33 ± 0.33^a	$_2$6.33 ± 0.33^b	$_2$6.00 ± 0.58^b	$_3$3.00 ± 0.58^b
36	$_1$8.83 ± 0.44^a	$_2$4.67 ± 0.33^c	$_3$3.33 ± 0.33^c	
96	$_1$8.67 ± 0.33^a	$_2$3.33 ± 0.33^d		
Clotting time (rank)				
0	$_1$5.67 ± 0.33^a	$_1$5.67 ± 0.33^a	$_1$5.67 ± 0.33^a	$_1$5.67 ± 0.33^a
24	$_1$6.00 ± 0.00^a	$_1$6.33 ± 0.33^{ab}	$_1$6.67 ± 0.67^{ab}	$_2$8.67 ± 0.33^b
36	$_1$5.67 ± 0.33^a	$_2$7.00 ± 0.58^{bc}	$_2$7.33 ± 0.33^b	
96	$_1$5.67 ± 0.33^a	$_2$7.67 ± 0.33^c		
Bacteraemia (rank)				
0	$_1$2.33 ± 0.33^a	$_1$2.33 ± 0.33^a	$_1$2.33 ± 0.33^a	$_1$2.33 ± 0.33^a
24	$_1$2.33 ± 0.33^a	$_2$5.33 ± 0.88^b	$_2$5.67 ± 0.33^b	$_3$8.33 ± 0.33^b
36	$_1$2.00 ± 0.00^a	$_2$6.33 ± 0.88^{bc}	$_3$8.00 ± 0.58^c	
96	$_1$2.00 ± 0.00^a	$_2$7.33 ± 0.33^c		
Bacterial load in intestine (× 10^3 CFU/g)				
0	$_1$3.68 ± 0.43^a	$_1$3.68 ± 0.43^a	$_1$3.68 ± 0.43^a	$_1$3.68 ± 0.43^a
24	$_1$3.43 ± 0.44^a	$_2$5.11± 0.25^b	$_2$5.53 ± 0.24^b	$_3$7.82 ± 0.23^b
36	$_1$3.35 ± 0.58^a	$_2$5.69 ± 0.30^b	$_3$7.36 ± 0.31^c	
96	$_1$3.33 ± 0.51^a	$_2$7.36 ± 0.32^c		

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column not followed by the same superscript letters are significantly different at $P<0.05$.

Table 6.4
The physiological and immune responses (mean ± SE) of the prawns that were not fed probiotics during the challenge with *V. harveyi* at 0, 10^3, 10^5 and 10^7 CFU/mL

Time (h)	***V. harveyi* (CFU/mL)**			
	0	**10^3**	**10^5**	**10^7**
THC (x 10^6 Cells/mL)				
0	$_1$4.92 ± 0.12^a	$_1$4.92 ± 0.12^a	$_1$4.92 ± 0.12^a	$_1$4.92 ± 0.12^a
60	$_1$4.93 ± 0.12^a	$_2$2.97 ± 0.27^b	$_2$2.83 ± 0.34^b	$_3$2.07 ± 0.15^b
108	$_1$4.69 ± 0.12^a	$_2$2.53 ± 0.12^{bc}	$_2$2.20 ± 0.27^c	
156	$_1$4.78 ± 0.12^a	$_2$2.03 ± 0.12^c		
Hyalinocyte (%)				
0	$_1$52.67 ± 3.06^a	$_1$52.67 ± 3.06^a	$_1$52.67 ± 3.06^a	$_1$52.67 ± 3.06^a
60	$_1$52.45 ± 3.06^a	$_2$61.67 ± 2.73^{bc}	$_2$60.67 ± 1.45^b	$_3$72.33 ± 1.76^b
108	$_1$52.08 ± 3.06^a	$_2$66.67 ± 2.33^c	$_2$66.00 ± 1.53^b	
156	$_1$51.87 ± 3.06^a	$_2$70.67 ± 2.96^{cd}		
Semi-Granulocyte (%)				
0	$_1$21.00 ± 1.44^a	$_1$21.00 ± 1.44^a	$_1$21.00 ± 1.44^a	$_1$21.00 ± 1.44^a
60	$_1$21.78 ± 0.25^a	$_2$15.00 ± 0.58^b	$_2$14.33 ± 1.86^b	$_3$8.67 ± 0.67^b
108	$_1$22.06 ± 0.58^a	$_2$10.67 ± 0.88^c	$_2$9.00 ± 0.58^b	
156	$_1$22.45 ± 0.64^a	$_2$9.33 ± 0.67^c		
Granulocyte (%)				
0	$_1$8.83 ± 0.44^a	$_1$8.83 ± 0.44^a	$_1$8.83 ± 0.44^a	$_1$8.83 ± 0.44^a
60	$_1$8.88 ± 0.56^a	$_1$6.33 ± 0.33^b	$_1$6.00 ± 0.58^b	$_2$3.33 ± 0.33^b
108	$_1$8.78 ± 0.25^a	$_2$5.00 ± 0.00^c	$_3$3.67 ± 0.33^c	
156	$_1$9.67 ± 0.04^a	$_2$3.67 ± 0.33^d		
Clotting time (rank)				
0	$_1$6.00 ± 0.58^a	$_1$6.00 ± 0.58^a	$_1$6.00 ± 0.58^a	$_1$6.00 ± 0.58^a
60	$_1$6.00 ± 0.58^a	$_1$6.33 ± 0.33^{ab}	$_1$6.67 ± 0.67^a	$_2$8.33 ± 0.33^b
108	$_1$5.67 ± 0.88^a	$_2$7.00 ± 0.58^{ab}	$_2$7.33 ± 0.33^a	
156	$_1$5.67 ± 0.88^a	$_2$7.67 ± 0.33^b		
Bacteraemia (rank)				
0	$_1$2.67 ± 0.33^a	$_1$2.67 ± 0.33^a	$_1$2.67 ± 0.33^a	$_1$2.67 ± 0.33^a
60	$_1$2.33 ± 0.33^a	$_2$5.00 ± 0.58^b	$_2$5.00 ± 0.58^b	$_3$8.33 ± 0.67^b
108	$_1$2.33 ± 0.33^a	$_2$6.00 ± 0.58^{bc}	$_2$7.33 ± 0.33^c	
156	$_1$2.00 ± 0.00^a	$_2$7.00 ± 0.58^c		
Bacterial load in intestine (x 10^3 CFU/g)				
0	$_1$3.74 ± 0.41^a	$_1$3.74 ± 0.41^a	$_1$3.74 ± 0.41^a	$_1$3.74 ± 0.41^a
60	$_1$3.81 ± 0.28^a	$_1$4.77 ± 0.13^b	$_1$4.87 ± 0.28^b	$_2$7.49 ± 0.41^b
108	$_1$4.02 ± 0.18^a	$_1$5.35 ± 0.10^b	$_2$7.02 ± 0.16^c	
156	$_1$4.25 ± 0.32^a	$_2$7.03 ± 0.12^c		

Values in any one row not preceded by the same subscript numbers are significantly different at $P<0.05$. Values in any one column not followed by the same superscript letters are significantly different at $P<0.05$.

Plate 6.2 The moribund prawns with the cross disease symptom after 36 h of challenge.

6.4 DISCUSSION

The application of probiotics improves the survival, growth and food conversion ratio of various prawns such as *L. vannamei* (Balcázar *et al.* 2007; Garriques and Arevalo 1995; Guo *et al.* 2009; Wang 2007; Zhou *et al.* 2009), *P. monodon* (Maeda and Liao 1992b) and *Fenenpenaeus indicus* (Ziaei-Nejad *et al.* 2006). The survival of probiotic-fed *P. monodon* challenged with *V. harveyi* was 100%; whereas, *P. monodon* reared without probiotics showed only 26% survival (Rengpipat *et al.* 1998a). In agreement with these studies, our previous studies (Hai *et al.* 2009a; Hai and Fotedar 2009) and this study showed that the probiotic-fed prawns had higher survival and growth rates than those not fed probiotics. Probiotics can increase the innate immune system of fish and prawns (Sakai 1999) and disease resistance of the host through stimulation of non specific defence mechanisms (Skjermo *et al*. 2006). In our studies, the probiotic-fed prawns were healthier, indicated by higher SGC and GC proportions, significantly lower clotting time and intestinal bacterial load. The administered probiotics not only improved the prawn physiological responses but also accelerated the immune responses.

A number of infection routes by bacteria can be involved where *V. harveyi* caused significant mortalities of *P. monodon* larvae and postlarvae in an immersion challenge (Lavilla-Pitogo *et al*. 1990). An immersion challenge with *V. carchariae* resulted in increase in mortality of brown-spotted grouper (*Epinephelus tauvina*) (Saeed 1995). In contrast, an immersion challenge with *V. campbellii* did not affect the *L. vannamei* survival (Phuoc *et al.* 2009). Most outbreaks of prawn vibriosis

happen either in combination with physiological stress factors or following primary infections with other pathogens (Sung *et al.* 2001). In our study, the probiotics helped prawns to facilitate in improvement of disease resistance against *V. harveyi* infection resulting in longer survival-hours. *V. harveyi* infection has caused the appendages eroded, black gills and black spots along the abdominal segments; whereas, bacterial infections in *P. monodon* displayed red disease syndrome (Alapide-Tendencia and Dureza 1997).

V. harveyi is regarded as an opportunistic pathogen (Saeed 1995), as *V. harveyi* from broodstock, water, *Artemia* or bacterial biofilms on the surface of plastic or cement tanks (Abraham and Palaniappan 2004; Karunasagar *et al.* 1996) may lead to a disease outbreak under stressful conditions (Thaithongnum et al. 2006). Therefore, the prior application of probiotics into a new environment and culture conditions is recommended for preventing the effect of infectious bacteria on the host. As the prawns in our study were fed probiotics for 28 days before they were challenged with *V. harveyi*, the effects of challenge were reduced. The probiotic-fed prawns were stronger with higher resistance against the pathogen *V. harveyi* due to longer survival-hours and LT_{50} values.

The bactericity of *V. harveyi* on the hosts depends on the concentration of *V. harveyi* used for the challenge. A challenge of *V. harveyi* at 10^2-10^4 CFU/mL caused significant mortalities of *P. monodon* larvae and postlarvae (Karunasagar *et al.* 1994; Lavilla-Pitogo *et al.* 1990; Prayitno and Latchford 1995). In our study, *V. harveyi* at 10^7 CFU/mL were more harmful than the lower concentrations shown by shorter survival-hours. The 5-day LC_{50} values for 164-g silvery black porgy (*Acanthopagrus cuvieri*) and 156-g *E. tauvina* were 10^7 and 10^9 CFU/fish, respectively (Saeed 1995). Other studies have also showed that the disease outbreaks in prawns can occur with concentrations of *V. harveyi* at 10^2-10^3 CFU/mL (Lavilla-Pitogo *et al.* 1990). In our study, the probiotic-fed prawns resisted *V. harveyi* at 10^3 CFU/mL as the 100% survival-hour of these prawns was three times longer than those of the prawns that were not fed probiotics. *V. harveyi* challenge at 10^3 CFU/mL for 48 h resulted in only 51.50% survival of *P. monodon* (Prayitno and Latchford 1995), while the LT_{50} value of *P. latisulcatus* fed probiotics were at 90 h in our study. Hence, the probiotics at 10^5 CFU/mL provided a positive effect on the *P. latisulcatus* survival.

Adding an artificial dominant bacterial strain is the main factor used to manipulate indigenous microbiota communities (Balcázar *et al.* 2006a). Competitive exclusion is one of the ecological processes so that the small changes affecting the growth and mortality of one species may lead to a change of species dominance in the community (Moriarty 1998). In our previous studies, the probiotics showed a high inhibition test against *V. harveyi* (Hai *et al.* 2007), and the probiotic-fed prawns showed healthier signs than those fed without probiotics (Hai *et al.* 2009a; Hai and Fotedar 2009). In our current study, the probiotic-fed prawns died after 36 h of challenge with *V. harveyi* at 10^3 CFU/mL. It is possible that 28 days of feeding with probiotics could not provided enough time for probiotics to be dominant over *V. harveyi* even at low concentration of 10^3 CFU/mL.

THC and DHC are the health assessors for aquatic animals (Jussila *et al.* 1997). The THC decreased when *Litopenaeus stylirostris* (Le Moullac and Haffner 2000), *L. vannamei* (Cheng et al. 2005), *P. monodon* (Wang and Chen 2006) were temperature stressed, when both bacterial-injected *L. vannamei* (Hsu and Chen 2007) and *Marsupenaeus japonicus* (Cheng *et al*. 2007) were exposed to sulfide, and *Panulirus cygnus* was exposed to the air (Fotedar *et al*. 2001). THC decreased in American lobster (*Homarus americanus*) (Stewart *et al*. 1967) and in the blue crab (*Callinectes sapidus*) (Johnson 1976) when harmful bacteria were present in the rearing media. THC were also lower in the moribund *P. cygnus* than in the healthy individuals (Jussila *et al.* 1997). The similar results were achieved in our study when *P. latisulcatus* were exposed to *V. harveyi*. The HCs are the most numerous of haemocytes (Sequeira *et al.* 1995) and are chiefly involved in phagocytosis (Johansson *et al.* 2000). In our study, the higher HC were produced in *P. latisulcatus* when they were exposed to *V. harveyi* infections. Rowley and Powell (2007) that the elevated levels of phagocytosis are partial explanation for enhancing immune response of prawns.

In addition, during the challenge period, the decreases in THC, SGC and GC, and the increases in clotting time, bacteraemia and intestinal bacterial load may be consequences of immune degradation due to exposure to *V. harveyi*. Thus, bacterial infectious symptoms viz. erosion of appendages and black gills appeared. Therefore,

the probiotics could not counteract the effects of the *V. harveyi* infection even at low a concentration of 10^3 CFU/mL.

V. harveyi at 10^7 CFU/mL was more harmful than lower concentrations. The resistibility of the probiotic-fed prawns to *V. harveyi* at 10^3 CFU/mL within 36 h of challenge has indicated that *P. synxantha* and *P. aeruginosa* can be used as appropriate probiotics for the cultivation of juvenile *P. latisulcatus*. Although the probiotic-fed prawns were healthier than the prawns not fed probiotics, the immune responses of the prawns subjected to *V. harveyi* challenge were not improved, while only the survival of the probiotic-fed prawns was longer than those not fed probiotics. The customised probiotics could not counteract the effects of *V. harveyi* in the improvement of immune responses of the prawns.

CHAPTER 7

ENCAPSULATION OF *ARTEMIA* WITH CUSTOMISED PROBITICS [5]

7.1 INTRODUCTION

Ozone is known as a powerful oxidising agent and an effective disinfectant against viral, bacterial and fungal pathogens in aquatic systems (Suantika *et al.* 2001; Summerfelt and Hochheimer 1997) and is effective in crustacean aquaculture (Danald *et al.* 1979; Theisen *et al.* 1998). It is used routinely for the disinfection of crustacean eggs (Coman *et al.* 2005; Sellars *et al.* 2005), but may be toxic to larvae (Tango and Gagnon 2003). Though marine crustaceans appear tolerant to low levels of ozonation (Blogoslawski *et al.* 1977; Jiang *et al.* 2001; Meunpol *et al.* 2003), excessive ozonation causes deformities and eventual death (Ritar *et al.* 2006). A non-toxic level of ozonation for southern rock lobster (*Jasus edwardsii*) larvae and the prevention of contamination of micro-organisms is from 400 to 500 mV of oxidation-reduction potential (Ritar *et al.* 2006). Therefore, ozone was used to sterilise seawater as a medium for use in the encapsulation in this study.

In addition, one of the common media for the culture bacteria is tryptone soya broth (TSB), which has a long history of use in the microbiology area. In aquaculture, TSB was used for studies on bacteriology in fish and prawns (Chand and Sahoo 2006; Chen *et al.* 2008; Cheng *et al.* 2003; Suhalim *et al.* 2008) and was used effectively to multiply the customised probiotics in our previous studies (Hai *et al.* 2009a; Hai and Fotedar 2009; Hai *et al.* 2007). TSB was employed as a medium for encapsulation capacity of *Artemia* in this study.

Artemia are indispensable food organisms in the industrial larviculture of fish and crustaceans (Lee and Ostrowski 2001; Liao *et al.* 2001; Marte 2003; Shields 2001) owing to their small size, slow swimming behaviour, rapid reproduction rate, ability to be cultured at high density (Lubzens 1987; Lubzens *et al.* 1989; 2001), and usefulness for transferring probiotic bacteria to host aquatic animals (Makridis *et al.*

[5] Paper is in press Aquaculture Research (*Appendix 8*)

2000; Ziaei-Nejad *et al.* 2006). Unfortunately, supplying live food in rearing systems can introduce opportunistic pathogenic bacteria, which cause massive mortalities of fish and crustacean larvae (Keskin *et al.* 1994; Lightner 1983; Nicolas *et al.* 1989). One solution for this problem is to replace opportunistic pathogenic bacteria with other less aggressive probiotic bacteria (Makridis *et al.* 2000). A positive effect of probiotics is their ability to outcompete other unwanted bacteria (Austin *et al.* 1995). Although enriching live food with probiotics has a benefit to the hosts by enhancing the indigenous microflora (Havenaar *et al.* 1992), overdoses of probiotics can induce immunosuppression (Sakai 1999). To avoid negative effects of an overdoses of probiotics such as immunosuppression (Sakai 1999), the quantity of probiotics in live food needs to be optimised.

Several bacterial species are proven probiotics for use in aquaculture, as they improve the growth and survival as well as the immunity of hosts (Rengpipat *et al.* 1998b; Rengpipat *et al.* 2000; Ziaei-Nejad *et al.* 2006). Two probiotics *Pseudomonas synxantha* and *P. aeruginosa* that showed high inhibition of growth of 15 *Vibrio* spp. isolated from western king prawns (*Penaeus latisulcatus*) and eight pathogenic *Vibrio* spp. from other aquatic animals (Hai *et al.* 2007), and improved the immune responses of western king prawns (Hai *et al.* 2009a; Hai and Fotedar 2009) were employed for this study. However, it is imperative to determine the optimal encapsulation capacity of *Artemia* nauplii of these probiotics as an effective way to convey them to the *P. latisulcatus* aquaculture. The aim of this study was to determine the encapsulation media and encapsulation capacity rate of *Artemia* nauplii with the customised probiotics for use in the cultivation of *P. latisulcatus*. The encapsulation capacity of *Artemia* nauplii was measured by recording their survival and the probiotic loads.

7.2 MATERIALS AND METHODS

7.2.1 General plan

Seawater at 30-35 ppt was ozonated by an ozone generator (capacity of 2 g/h, ZOX, model AQ-2KP, Enviroplus company, Western Australia). The sterilisation effectiveness of seawater was evaluated by counting the total bacterial load using a

serial dilution with normal saline lawn onto marine salt agar (MSA) (Hai *et al.* 2009a) at the oxidation-reduction potential (ORP) of 300, 400, 500, 600 and 700 mV (Ritar *et al.* 2006). When the total bacterial count equalled zero, the corresponding ORP was recorded and used as a benchmark for completion of the sterilisation process for further trials. The sterilisation was repeated three times.

There were total 7 trials in this study (Table 7.1). In the trials 1 and 2, *Artemia* nauplii were fed the probiotics *P. synxantha* and *P. aeruginosa* at 0 (control), 10^3, 10^5 and 10^7 CFU/mL, respectively in a medium of OW. In the trials 3 and 4, the encapsulation of *Artemia* nauplii with *P. synxantha* and *P. aeruginosa* at 0, 10^3, 10^5 and 10^7 CFU/mL was performed in a medium of TSB. The trials 1, 2, 3 and 4 were set up as Latin Square designs with four replicates for each probiotic concentration. In the trials 5 and 6, *Artemia* nauplii were fed *P. synxantha* and *P. aeruginosa* at 10^5 CFU/mL in the mixture media of OW and TSB at 25:75, 50:50 and 75:25 v/v, respectively.

Table 7.1 Description of trials

Trial	Description
1	Medium of OW: *P. synxantha* at 0, 10^3, 10^5, 10^7 CFU/mL
2	Medium of OW: *P. aeruginosa* at 0, 10^3, 10^5, 10^7 CFU/mL
3	Medium of TSB: *P. synxantha* at 0, 10^3, 10^5, 10^7 CFU/mL
4	Medium of TSB: *P. aeruginosa* at 0, 10^3, 10^5, 10^7 CFU/mL
5	*P. synxantha* at 10^5 CFU/mL in the mixture of OW:TSB at 25:75, 50:50 and 75:25 v/v, respectively.
6	*P. aeruginosa* at 10^5 CFU/mL in the mixture of OW:TSB at 25:75, 50:50 and 75:25 v/v, respectively.
7	Mixtures of *P. synxantha* and *P. aeruginosa* at 30:70, 50:50 and 70:30 v/v, respectively, in the mixture of OW:TSB at 75:25 v/v, respectively.

In trial 7, the mixtures of the same inoculum of 10^5 CFU/mL of the two probiotics *P. synxantha* and *P. aeruginosa* at 30:70, 50:50 and 70:30 v/v, respectively were used to feed *Artemia* in the mixture media of OW and TSB at 75:25 v/v. The trials 5, 6 and 7 were also set up as Latin Square designs but only in triplicate for each media mixture of OW and TSB and each mixture of *P. synxantha* and *P. aeruginosa*. The trials lasted for 72 h with the exception of trials 3 and 4, which lasted only for 48 h.

All the 7 trials were set up in a water bath, in which a heater (Sonpar®, Model: HA-200, Zhongshan, Guangdong, China) and a pump (Accent pump WWA1000, Waterwerks Australia Pty Ltd, Victoria, Australia) were installed on one side of the water bath, to maintain water circulation and at a constant temperature of 25 ^{o}C. During the trial periods, aeration was continuously supplied through holes drilled in the lids of the containers, light was provided by an electric light (Sylvania Gro-Lux F36W/Gro, Germany) and the culture media was neither exchanged nor replenished. Five hundred millilitre plastic beakers were filled with 200 mL of media of either ozonated water (OW) or TSB (OXOID, Australia) plus NaCl 2% (Buller 2004), or mixtures of OW and TSB in the ratios of 25:75, 50:50 and 75:25 v/v, respectively.

The customised probiotics *P. synxantha* and *P. aeruginosa* from available commercially available probiotic product in our previous study (Hai *et al.* 2007) were reconstituted on MSA from the cultures stored at -80 ^{o}C. A standard curve was prepared from optical density measurement of cells diluted in normal saline solution and the corresponding total bacterial counts on MSA. The standard curve constructed by plotting optical density as a function of total bacterial counts was used to estimate the bacterial cell density as in our previous studies (Hai *et al.* 2007). The two probiotics at equal concentration of 10^5 CFU/mL were mixed at 30:70, 50:50 and 70:30 v/v respectively for trial 7, an encapsulation with the mixture of the two probiotics in appropriate mixture of OW and TSB.

Artemia cysts "Salt Creek", Inc., USA were hydrated in freshwater for 1 h, then immersed in 50% chlorine solution for 1 min, rewashed in OW, then incubated in a hatching cone with OW at 25 ^{o}C, 30 ppt, pH of 8.0 (Lavens and Sorgeloos 1996) at a cyst density of 1.5 g/L (Aragão *et al.* 2004). After 24 h of incubation, hatched *Artemia* nauplii were separated and collected from the unhatched and empty shells, then quickly washed with OW and transferred to 500 mL plastic beakers at 250 nauplii/mL. This stocking density was recommended by Czesny *et. al.* (1999) and Aragão *et al.* (2004).

The bacterial load in the media and *Artemia* nauplii was determined at commencement of the trials and after a 12 h interval. *Artemia* nauplii samples were

rinsed in distilled water, washed with 0.1% benzalkonium chloride for 30 second, then rinsed again in distilled water to remove the external bacteria. Fifty *Artemia* nauplii were ground in Eppendorf tubes with micropestles. The homogenised samples were diluted serially with sterilised normal saline solution and total bacterial counts were performed on MSA plates. Plates were incubated for 24 h at 25 ^{o}C. The dilution resulting in 30-300 colonies per plate was used to calculate the colony forming units per mL.

7.2.2 Data collection and analyses

The assumption was made that the bacterial multiplication ability was equal in all trials. The probiotic loads in *Artemia* and media were calculated by the differences between the bacterial loads in all treatments where probiotics were applied and in controls where no probiotics were applied. The averaged bacterial loads in the controls of trials 1 and 3, 2 and 4 acted as the control for trials 5 and 6, respectively. The averaged bacterial loads in the controls of trials 5 and 6 acted as the control for trial 7.

Survival of *Artemia* nauplii was measured by counting samples from their population in 2 mL of the media in triplicate, and then calculated using the following equation:

$$\text{Survival (\%)} = (N_t - N_o) \times 100$$

Where: N_t and N_o are the average number of *Artemia* at time 't' and the commencement of the trials, respectively.

All collected data were presented as means ± SE (standard error). SPSS statistical package version 14.0 was used to conduct one way ANOVA (analysis of variance). LSD (Least significant difference) post hoc tests were used to determine any significant differences between the tested variables of the probiotic loads in the media and in *Artemia*, and *Artemia* survival. The Games-Howell post hoc test was used when variances were not homogenous. All significance tests were performed at P=0.05.

7.3 RESULTS

7.3.1 Sterilising seawater for encapsulation

The bacterial loads (CFU/mL) in the ozonated seawater were negatively correlated with ORP (mV) (Figure 7.1). The sterilising process was complete when the bacterial load dropped to zero and when the corresponding ORP was 700 mV. The sterilising process for 50 L of seawater took 7-8 min.

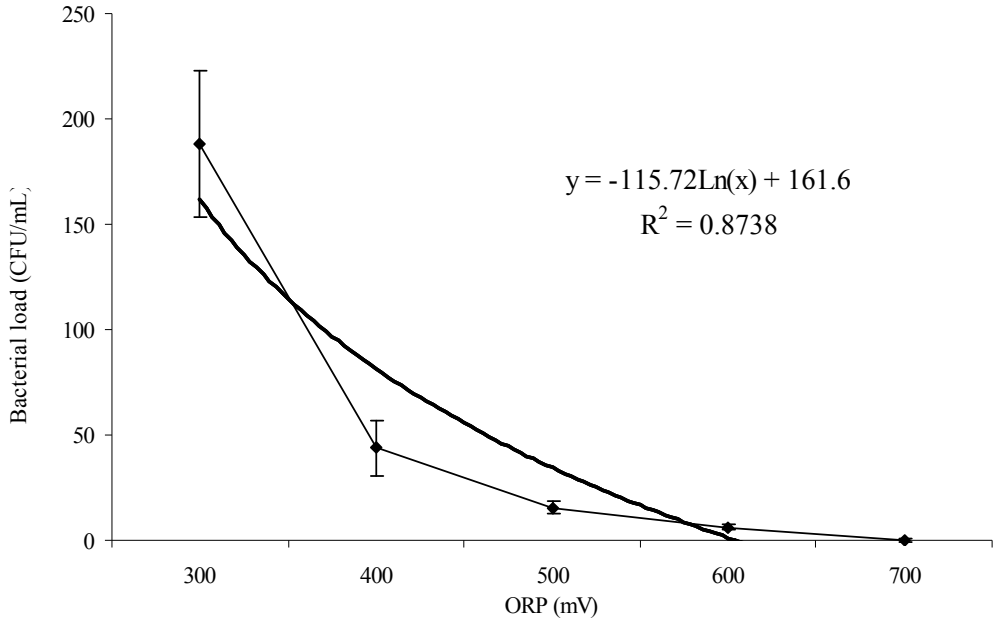

Figure 7.1 Relationship between the bacterial load (CFU/mL) and ORP (mV)

7.3.2 Encapsulation in OW

The *Artemia* survival decreased significantly at all densities of both probiotics. At 72 h of encapsulation, the highest (35 ± 1%) and lowest (29 ± 1%) *Artemia* survival were achieved when *Artemia* were fed *P. synxantha* and *P. aeruginosa* at 10^5 and 0 CFU/mL. The probiotic loads in *Artemia* reached a peak of 3.7 x 10^4 or 3.9 x 10^4 CFU/nauplius at 60 h of encapsulation, and did not change significantly beyond 60 h of encapsulation at all densities of *P. synxantha* and *P. aeruginosa*, respectively (Figure 7.2I and 7.2II). The probiotic loads in *Artemia* were significantly higher when the densities of the customised probiotics increased from 10^3 to 10^7. The probiotic loads in OW did not significantly change when the probiotics were applied

at 10^3 and 10^5 CFU/mL; whereas, at 10^7 CFU/mL, the probiotic loads increased significantly up to 36 h or 24 h, then decreased significantly for the rest of the encapsulation periods with *P. synxantha* and *P. aeruginosa*, respectively.

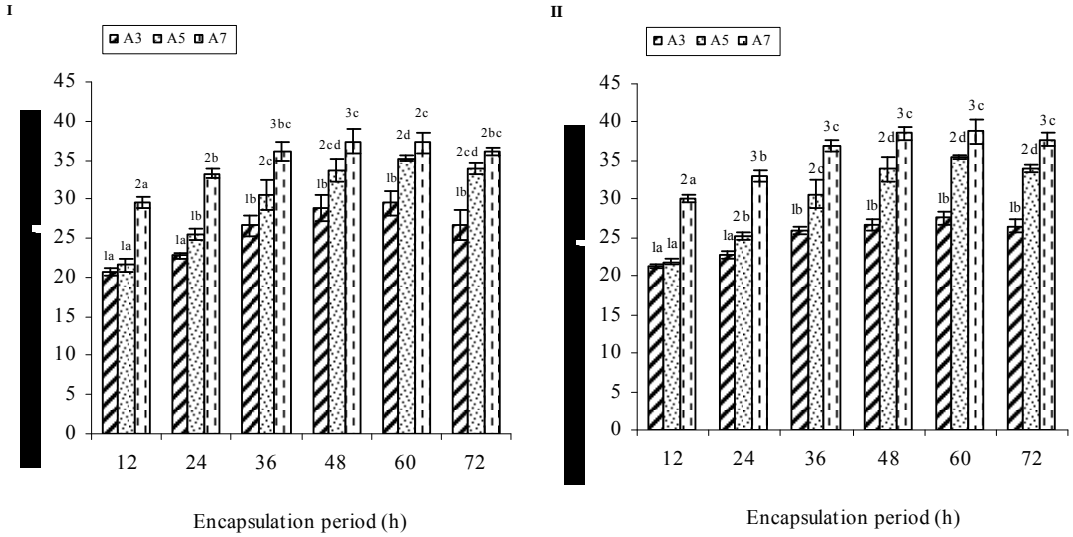

Figure 7.2 Probiotic loads (Mean ± SE) in *Artemia* nauplii (CFU/nauplius) when *Artemia* nauplii were fed *Pseudomonas synxantha* (I) and *P. aeruginosa* (II) at 10^3 (A3), 10^5 (A5) and 10^7 (A7) in OW. Bars with different letters or numbers are significantly different between encapsulation times or between the probiotic densities

7.3.3 Encapsulation in TSB

At the end of encapsulation, the *Artemia* survival was low (21-24%) and the *Artemia* survival decreased significantly at all probiotic densities, but was not significantly different between probiotic densities. Over the encapsulation periods, the probiotic load in *Artemia* increased significantly in all probiotic densities (Figure 7.3I and 7.3II). At 48 h of encapsulation, although the probiotic load in TSB showed no significant differences between the probiotic at 10^3 and 10^5, the probiotic loads in *Artemia* were significantly different. The probiotic load in *Artemia* fed the probiotic at 10^5 and 10^7 CFU/mL was higher than those fed the probiotic at CFU/mL.

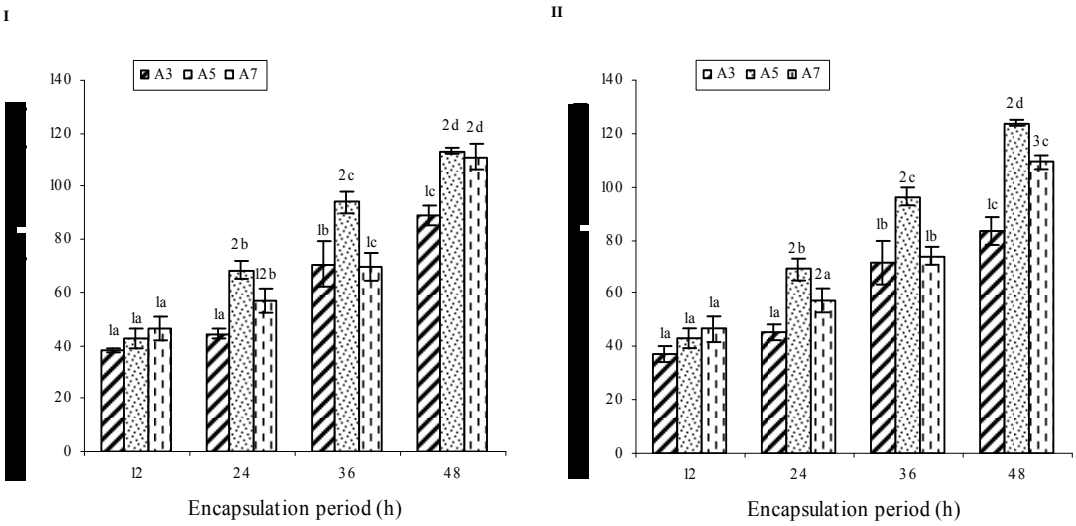

Figure 7.3 Probiotic loads (Mean ± SE) on *Artemia* nauplii (CFU/nauplius) when *Artemia* nauplii were fed *Pseudomonas synxantha* (I) and *P. aeruginosa* (II) at 10^3 (A3), 10^5 (A5) and 10^7 (A7) in TSB. Bars with different letters or numbers are significantly different between encapsulation times or between the probiotic densities

7.3.4 Encapsulation in the mixture of OW and TSB

The *Artemia* survival in the three mixtures of OW and TSB decreased significantly. The highest survival was 67 and 66% when fed *P. synxantha* and *P. aeruginosa* in the mixture of OW and TSB at 75:25 v/v. respectively From 36 h onward, the probiotic loads in *Artemia* increased significantly in all mixtures of OW and TSB (Figure 7.4I and 7.4II). From 60 h onward, the probiotic loads in *Artemia* in the mixtures of OW and TSB at 25:75 and 50:50 v/v respectively were significantly lower than those in the mixture of OW and TSB at 75:25 v/v. At 72 h, the encapsulation with *P. synxantha* and *P. aeruginosa* in the mixture of OW and TSB at 75:25 v/v resulted in the highest probiotic loads of 50 x 10^3 and 51 x 10^3 CFU/nauplius. Over the encapsulation periods, the various mixtures of OW and TSB did not affect significantly the probiotic loads in the media.

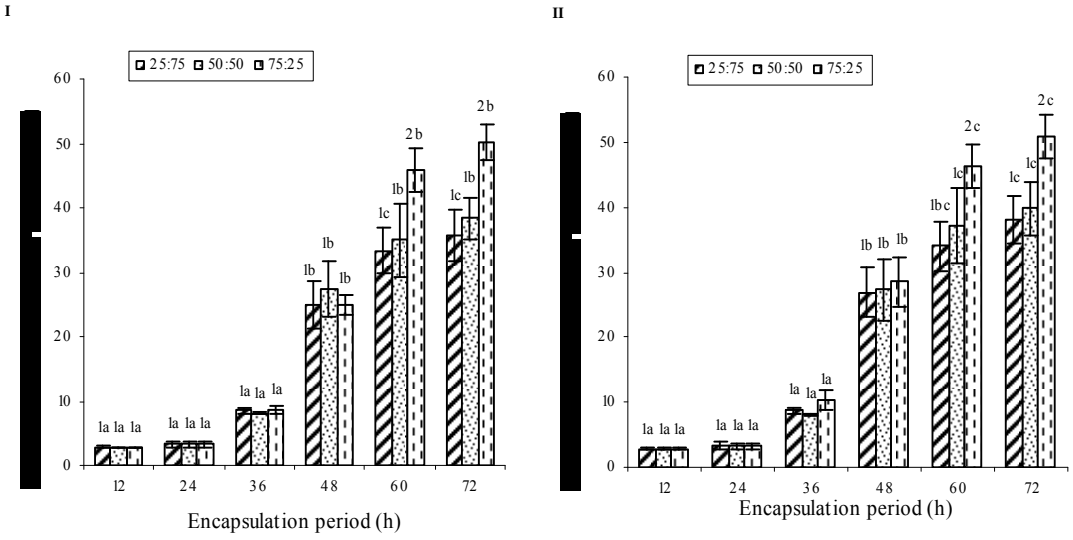

Figure 7.4 Probiotic loads (Mean ± SE) on *Artemia* nauplii (CFU/nauplius) when *Artemia* nauplii were fed *Pseudomonas synxantha* (I) and *P. aeruginosa* (II) in the mixture of OW and TSB at 25:75, 50:50 and 75:25 v/v respectively. Bars with different letters or numbers are significantly different between encapsulation times or between the probiotic densities

7.3.5 Encapsulation with the mixture of two probiotics in the appropriate mixture of OW and TSB

The *Artemia* survival and probiotic load in *Artemia* and in media did not change significantly between different probiotic mixtures. During the encapsulation period, the *Artemia* survival decreased significantly in all probiotic mixtures. After 24 h of encapsulation, the probiotic loads in *Artemia* in all probiotic mixtures increased significantly. The highest probiotic load in *Artemia* (5.0×10^4 CFU/nauplius) and the highest *Artemia* survival (74%) were achieved at 72 h of encapsulation with a mixture of *P. synxantha* and *P. aeruginosa* at 30:70 v/v, in a mixture of OW and TSB at 75:25 v/v, respectively (Table 7.2).

Table 7.2
Probiotic loads (mean± SE) in *Artemia* nauplii (CFU/nauplius), media (CFU/mL) and survival (%) of *Artemia* nauplii fed the mixtures of *Pseudomonas synxantha* and *P. aeruginosa* (10^5 CFU/mL) at 70:30, 50:50 and 30:70 v/v in the mixture of OW and TSB at 75:25 v/v

	Encapsulation period (h)	Mixture of *Pseudomonas synxantha* and *Pseudomonas aeruginosa* (v/v)		
		70:30	**50:50**	**30:70**
Probiotic loads in *Artemia*	12	$_{1}0.27 \pm 0.03 \times 10^{4a}$	$_{1}0.28 \pm 0.02 \times 10^{4a}$	$_{1}0.28 \pm 0.02 \times 10^{4a}$
	24	$_{1}0.31 \pm 0.05 \times 10^{4a}$	$_{1}0.32 \pm 0.04 \times 10^{4a}$	$_{1}0.32 \pm 0.04 \times 10^{4a}$
	36	$_{1}1.59 \pm 0.2 \times 10^{4b}$	$_{1}1.67 \pm 0.4 \times 10^{4b}$	$_{1}1.74 \pm 0.3 \times 10^{4b}$
	48	$_{1}2.59 \pm 0.4 \times 10^{4c}$	$_{1}3.36 \pm 0.4 \times 10^{4c}$	$_{1}2.95 \pm 0.5 \times 10^{4c}$
	60	$_{1}4.26 \pm 0.3 \times 10^{4d}$	$_{1}4.36 \pm 0.5 \times 10^{4d}$	$_{1}4.73 \pm 0.3 \times 10^{4d}$
	72	$_{1}4.21 \pm 0.3 \times 10^{4d}$	$_{1}4.38 \pm 0.5 \times 10^{4d}$	$_{1}4.98 \pm 0.4 \times 10^{4d}$
Probiotic loads in Media	12	$_{1}2.37 \pm 0.2 \times 10^{6a}$	$_{1}2.30 \pm 0.2 \times 10^{6a}$	$_{1}2.60 \pm 0.2 \times 10^{6a}$
	24	$_{1}2.43 \pm 0.3 \times 10^{6a}$	$_{1}2.57 \pm 0.5 \times 10^{6a}$	$_{1}2.83 \pm 0.1 \times 10^{6a}$
	36	$_{1}2.73 \pm 0.6 \times 10^{6a}$	$_{1}3.00 \pm 0.3 \times 10^{6a}$	$_{1}3.53 \pm 0.6 \times 10^{6a}$
	48	$_{1}2.60 \pm 0.7 \times 10^{6a}$	$_{1}3.23 \pm 0.6 \times 10^{6a}$	$_{1}3.50 \pm 0.9 \times 10^{6a}$
	60	$_{1}2.57 \pm 0.8 \times 10^{6a}$	$_{1}3.70 \pm 0.9 \times 10^{6a}$	$_{1}3.27 \pm 0.7 \times 10^{6a}$
	72	$_{1}2.90 \pm 0.7 \times 10^{6a}$	$_{1}3.63 \pm 0.6 \times 10^{6a}$	$_{1}3.47 \pm 0.6 \times 10^{6a}$
Survivals of *Artemia* nauplii	0	$_{1}100.00 \pm 0.00^{a}$	$_{1}100.00 \pm 0.00^{a}$	$_{1}100.00 \pm 0.00^{a}$
	12	$_{1}92.96 \pm 1.80^{b}$	$_{1}94.01 \pm 1.20^{b}$	$_{1}92.24 \pm 0.49^{b}$
	24	$_{1}84.56 \pm 0.67^{c}$	$_{1}86.19 \pm 0.58^{c}$	$_{1}85.23 \pm 0.00^{c}$
	36	$_{1}78.88 \pm 1.33^{cd}$	$_{1}79.79 \pm 0.42^{d}$	$_{1}80.68 \pm 0.69^{d}$
	48	$_{1}75.32 \pm 2.94^{de}$	$_{1}73.66 \pm 1.25^{e}$	$_{1}78.26 \pm 0.00^{e}$
	60	$_{1}70.95 \pm 2.76^{ef}$	$_{1}68.48 \pm 2.24^{f}$	$_{1}75.84 \pm 0.53^{f}$
	72	$_{1}68.35 \pm 2.46^{f}$	$_{1}65.33 \pm 0.94^{f}$	$_{1}74.23 \pm 0.56^{g}$

Values for probiotic loads and survivals in any one column not followed by the same superscript letters are significantly different at $P<0.05$. Values in any one row in the same probiotic loads or survivals not preceded by the same subscript numbers are significantly different at $P<0.05$.

7.4 DISCUSSION

Ozonation of a culture medium is an effective means of reducing bacterial diseases (Ritar *et al.* 2006). Moreover, a major encumbrance in the use of ozonation for seawater is the difficulty in measuring accurately the formation of ozone by-products (OBP) (Ritar *et al.* 2006). The content of OBP is estimated indirectly via ORP readings, which measures the potential of the seawater to oxidise or reduce, and is an indication of its ability to disinfect against micro-organisms or to kill aquaculture animals (Tango and Gagnon 2003). In our study, the ozone level required to sterilise seawater was found to be at ORP of 700 mV. Although excessive ozonation causes deformities and eventual death of animals (Ritar *et al.* 2006), ozonation at ORP level of 700 mV met the purpose for sterilisation of seawater. In this study, the use of OW to incubate *Artemia* cysts reduced unwanted bacterial loads in *Artemia* nauplii. The

unwanted bacteria remained below 2.8 x 10^3 CFU/nauplius (control). By contrast to another study (Ritar *et al.* 2004), the bacterial loads were 15 x 10^3 CFU/nauplius immediately after hatching.

One way to convey probiotics to a host is via enrichment/encapsulation of live foods which is ultimately fed to the host. *Artemia* nauplii are used as a vector for the transfer of specific components into cultured larvae (Merchie 1996). In our study, *Artemia* nauplii were used to encapsulate and convey *P. synxantha* and *P. aeruginosa* to western king prawns. Other probiotic enrichments include *Pediococcus acidilactici* and *Saccharomyces cerevisiae* (Gatesoupe 2002), *Lactobacillus sporogenes* (Venkat *et al.* 2004), and *Bacillus* spp. (Ziaei-Nejad *et al.* 2006).

It is possible to replace the opportunistic pathogenic bacteria present in live food organisms with less-aggressive bacteria, which can dominate the other bacterial flora of live food up to 24 h (Makridis *et al.* 2000). Two customised probiotics *P. synxantha* and *P. aeruginosa* with high growth inhibition of 15 *Vibrio* spp. isolated from healthy western king prawns and eight pathogenic *Vibrio* spp. isolated from other aquatic animals (Hai *et al.* 2007) were used in this study. In recent studies (Marques *et al.* 2006b; Soltanian *et al.* 2007b), probiotic bacteria helped to protect *Artemia* against pathogenic bacteria of *Vibrio campbellii* and *V. proteolyticus*. Generally, enrichments become functional and increase the quality of *Artemia* when enrichment bacteria reach sufficient levels in *Artemia*. The retention of probiotics in *Artemia* may function in improvement of the immune system of *Artemia*, and in conveyance of the probiotics to target organisms.

The use of either OW or TSB alone for *Artemia* nauplii encapsulating the customised probiotics failed to achieve high probiotic load in *Artemia* and high *Artemia* survival. The poor survival of *Artemia* in OW may be due to inadequate and lack of nutrition provided in OW medium, although newly-hatched *Artemia* nauplii do not feed much external food. Therefore the quality of *Artemia* nauplii depend on their enrichment diet adhered to the surface of *Artemia* nauplii (Gatesoupe 1991). The survival of *Artemia* in TSB was lower than in OW, but the probiotic loads in *Artemia* in TSB were nearly four times that in OW. It is because that TSB is a good medium for the

customised probiotics growth (Hai *et al.* 2009a; Hai and Fotedar 2009). Therefore, the probiotic bacteria proliferated and accumulated in *Artemia* nauplii during the encapsulation period. Although the probiotic load in *Artemia* in all treatments reached nearly three times their initial densities, the *Artemia* survival was low at 48 h of encapsulation. This could be attributed to problem of *Artemia* not swimming freely in the viscous TSB. Therefore, OW and TSB used on their own were not appropriate media for *Artemia* nauplii encapsulation.

On the other hand, the combination of OW and TSB improved both the survival of *Artemia* and the probiotic loads in *Artemia* fed individually *P. synxantha* and *P. aeruginosa*. The combination of media may allow the *Artemia* to swim freely in the less viscous medium than OW or TSB alone, and allow the probiotics to proliferate while maintaining *Artemia* growth in the combined media. The mixture of OW and TSB at 75:25 v/v was optimal for producing the highest probiotic loads in *Artemia* nauplii and the highest *Artemia* survival. The combination of *P. synxantha* and *P. aeruginosa* was optimal at 30:70 v/v with the higher survival of *Artemia* and higher probiotic loads in *Artemia*.

It was shown previously that developing techniques to reduce bacterial contamination in *Artemia* is an important step in the process of administering probiotic. The accumulation of bacteria in *A. franciscana* depends on the concentrations of the initial bacterial suspension (Makridis *et al.* 2000). In our study, both *P. synxantha* and *P. aeruginosa* at 10^5 CFU/mL were effective for *Artemia* encapsulating in OW and TSB. Further, the probiotic loads in *Artemia* and *Artemia* survival when fed a combination of equal concentration of *P. synxantha* and *P. aeruginosa* at 10^5 CFU/mL were at a higher level than those fed *P. synxantha* and *P. aeruginosa* individually. By comparison, 5 x 10^7 CFU/mL was reportrd by Makridis *et al.* (2000) where two *Vibrio* spp. isolated from halibut (*Hippoglossus hippoglossus*) larvae that inhibited pathogenic *vibrio* strains were effectively grazed by *A. franciscana* for 60 min. The probiotic density of 10^5 CFU/mL was also reported by Moriarty (1998), Vijayan et al. (2006), Ziaei-Nejad *et al.* (2006), Hai *et al.* (2009a) and Hai and Fotedar (2009). At this density, a combination of OW and TSB at 75:25 v/v provided an appropriate medium for *Artemia* nauplii encapsulating with higher probiotic load.

In our study, the probiotic load in *Artemia* in OW or TSB alone, or in the combination of these media was the same as conducted by Gatesoupe (2002) on enrichment with *Pediococcus acidilactici* and *Saccharomyces cerevisae*. Previous studies found that the capacity of *Artemia* to bioencapsulate bacteria was 2.4 x 10^3 to 1.2 x 10^5 CFU/*Artemia* (Campbell *et al.* 1993; Gomez-Gil *et al.* 1998; Makridis *et al.* 2000).

Generally, the most appropriate enrichment technique for newly hatched *Artemia* is commonly applied for a 24 h period after hatching (Ando *et al.* 2004; Han *et al.* 2000; Makridis *et al.* 2000; Sorgeloos *et al.* 2001; Stewart *et al.* 2001; Woods 2003). In our study, the encapsulation time of 48 h was longer than that in other studies conducted by Moren *et. al* (2005), Evjemo et al. (2001), Han et al. (2000) and Ritar et al. (2004). *Artemia* enrichment periods vary with different enrichment probiotics (Gatesoupe 1991). The nutrition value of *Artemia* nauplii decreases if applied to later stages of 8-h post hatched. In our study, *Artemia* nauplii grew bigger and encapsulated more probiotics by time. A 48-h encapsulation was the effective time to harvest the *Artemia* in a combination of *P. synxantha* and *P. aeruginosa* at 30:70 v/v in the mixtures of OW and TSB as the nutrition value of *Artemia* is believed to increase with a larger number of probiotics encapsulated, and the *Artemia* survival was high.

In this study, the best encapsulation period of *Artemia* was 48 h using a medium of OW and TSB at 75:25 v/v with the probiotics *P. synxantha* and *P. aeruginosa* at ratio of 30:70 v/v respectively and at the same inoculum of 10^5 CFU/mL.

CHAPTER 8

GENERAL DISCUSSION[6]

Western king prawns are widely distributed throughout the Indo-west Pacific region (Dore and Frimodt 1987), and throughout warm and temperate waters of the Australian coast (Grey *et al.* 1983). They are considered a prospective species for aquaculture since 1987 (Kathirvel and Selvaraj 1987) because of their established markets in Asia and Australia (Andrews and Bowen 1992) and their ability to tolerate a wide range of environmental conditions such as salinity and temperature (Penn 1980; Ramasamy and Pandian 1984). Biological characteristics of this species such as spawning and fecundity (Penn 1980) or behaviour in effect of food dispersion and crowding (Rasheed and Bull 1992) have been studied. Recent studies on the culture of this species have been undertaken in Australia by Sang and Fotedar (2004a; 2004b), Prangnell and Fotedar (2005; 2006b), Prangnell (2007) and *Hai et al.* (2007), *Hai et al.* (2009a), Hai and Fotedar (2009). The prawn cultivation has intensified, which has led to serious losses because of the spread of diseases and the deterioration of environmental conditions (Bondad-Reantaso *et al.* 2005; Rodríguez *et al.* 2007). As a substantial increase in the regular use of chemical additives and veterinary medicines as preventative and curative measures for disease, leads to antimicrobial resistance among pathogenic bacteria (Bachère 2000, 2003; Nomoto 2005), past research has shown that one of the benefits of probiotics could be to replace these chemicals and antibiotics (Li *et al.* 2006; Rengpipat *et al.* 1998a) and therefore, assist in the protection of disease-free aquacultured species (Gullian *et al.* 2004; Moriarty 2003; Rengpipat *et al.* 2000). Non-pathogenic strains of *Vibrio*, *Aeromonas, Pseudomonas* and *Alteromonas* that are known to have antagonistic properties to pathogenic strains, can be used as probiotic strains (Irianto and Austin 2002a; Moriarty 2003).

Although probiotics offer a promising alternative to chemicals and antibiotics in marine prawn culture (Li *et al.* 2006; Rengpipat *et al.* 1998a; Vaseeharan and Ramasamy 2003), the selection of probiotics for specific marine aquatic species has to be considered carefully in order to make them species specific. Research needs to

[6] Paper published in Fish & Shellfish Immunology 27 (2009) 100-104 (*Appendix 6*)

be undertaken on the particular culture conditions of the selected species in order to understand the rationale, preparation and hazards of probiotics (Wang *et al.* 2008a) from inappropriate species/strains of bacteria selection and/or usage of inadequate population densities (Balcázar *et al.* 2006a; Moriarty *et al.* 2005). This study reviews our past research on the usage of *P. synxantha* and *P. aeruginosa* on the cultivation of western king prawns in the light of published literature.

8.1 CUSTOMISING PROBIOTICS (Hai *et al.* 2007)

The inhibition test between the probiotic bacteria isolated from commercially available probiotic products and the *Vibrio* spp. isolated from healthy western king prawns and other aquatic animals was used to evaluate potential probiotics for use in the culture of the prawns and to examine the effects of these probiotics on normal and pathogenic microflora. Eight virulent *Vibrio* spp. isolated from aquatic animals and 15 *Vibrio* spp., the dominant normal microflora (93.75%), isolated from the prawns, were used for the test of inhibition impact of the selected probiotics on both the virulent and normal microflora. *Vibrio* spp. in their active state have an ability to release organic acids (Moriarty *et al.* 2005) and become resistant to antibiotics such as chloramphenicol, furazolidone, oxytetracycline and streptomycin, and become more virulent than the past (Moriarty 1999).

Some bacterial species produce a wide range of antagonistic compounds that can be valuable as probiotics (Moriarty 2003). Some probiotics are advertised to contain *Clostridium* spp., *Pseudomonas putida, P. aeruginosa*, but they are usually ineffective in reducing pathogenicity in aquatic species (Moriarty 2003). In our study, only two genera *Bacillus* and *Pseudomonas* were isolated and identified from the two commercial probiotics tested. *Bacillus* spp. are used commercially as probiotics in aquaculture, mainly for prawns (Moriarty 2003), while *Pseudomonas* acts as a potential probiotic for marine prawns and has caused growth inhibition of a number of pathogens such as *Salmonella, Staphylococcus aureus* and *Vibrio parahaemolyticus, V. harveyi, V. fluvialis, Photobacterium damselae* (previously *V. damsela*), *V. vulnificus* and *Aeromonas* spp. (Chythanya *et al.* 2002; Oblinger and Kreft 1990; Vijayan *et al.* 2006). Our research indicated that *Pseudomonas*

synxantha and P. aeruginosa were more effective in inhibiting to bacteria isolated from *P. latisulcatus* (Hai *et al.* 2007).

Of the five methods tested: BLIS, modified BLIS, disc-diffusion, well-diffusion and co-culture, employed for selecting probiotics, the modified BLIS method proved to be the most suitable for determining not only sensitivity/resistance but also the degree of sensitivity between the different *Vibrio* strains and probiotics. The modified BLIS method is also simple and practical compared to the other methods. At the same bacterial cell-density of 10^3 CFU/mL, this method allows a comparison of inhibitory effectiveness for different probiotics tested on the same range of pathogenic bacteria. Probiotics at 10^3 CFU/mL were found to be effective in inhibiting bacteria when tested by the BLIS, modified BLIS and co-culture methods. Probiotic concentrations were required at higher levels to inhibit bacteria using the well-diffusion and disc-diffusion methods. If *Bacillus* strains (probiotics) produced antimicrobial compounds that can inhibit bacteria, then the mortality rates of bacteria could increase (Moriarty 1999). Moreover, probiotics must be applied in advance of the pathogen, because the longer incubation period for the probiotics before the addition of the test bacterium produced greater inhibition zones between the probiotics and *Vibrio* spp. in the modified BLIS, well-diffusion and disc-diffusion methods. If the growth of probiotics is rapid in a chosen medium, an inhibition test might only require one day of incubation before the addition of the test bacteria. Based on our studies of the inhibition tests, the recommended protocol for the selection of probiotics for aquatic animals is the modified BLIS method (Hai *et al.* 2007).

8.2 THE CUSTOMISED PROBIOTICS ON THE CULTIVATION OF THE WESTERN KING PRAWNS

8.2.1 Application methods

Administration methods for probiotics need to be considered to get the desired results (Sakai 1999). Probiotics can be either administered directly into the rearing water or supplemented with the formulated feed. Similar to the results of Skjermo and Vadstein (1999) and Azad *et al.* (2005), in our study (Hai *et al.* 2009a), both

probiotic application methods of supplementation with the formulated feed and direct application into the rearing media, were effective in the transfer of the probiotics into the prawns, the former being more practical than the latter in reality. However, the survival and SGRs were higher when the probiotics were supplemented with the formulated feed compared to the probiotics applied into the rearing medium. The prawns that received the probiotics supplemented with the formulated feed were healthier than those receiving the probiotics via the rearing medium as indicated by higher THCs observed in the former.

Probiotics can be applied in mono or mixed culture (Havenaar *et al.* 1992). A two-probiotic combination improved the growth of prawns (Gatesoupe 2002) and increased the growth of catla (*Catla catla*) larvae and mrigal (*Cirrhinus mrigala*) fry (Mohanty *et al.* 1996). On the contrary, in our study, the two-probiotic combination did not significantly increase the SGRs of the prawns compared to the individual probiotics, but the prawns exposed to the combined probiotics either through the formulated feed or rearing media had lower HC proportion and higher GC and SGC proportions, and lower bacterial load in the haemolymph, hence were healthier than those exposed to the individual probiotics. *P. aeruginosa* was more effective in improving the prawn health than *P. synxantha*.

V. harveyi is regarded as an opportunistic pathogen (Saeed 1995). Application of probiotics in advance can prevent the amplification and detrimental effects of *V. harveyi* on the host as the opportunistic pathogen *V. harveyi* has less power under these conditions. Probiotics should be administered at an early stage in the life of the host (Skjermo and Vadstein 1999). *V. harveyi* from broodstock, water, *Artemia* or even bacterial biofilms on the surface of plastic or cement tanks (Abraham and Palaniappan 2004; Karunasagar *et al.* 1996) may lead to a disease outbreak under stressful conditions (Thaithongnum *et al.* 2006). Probiotics application is an important factor in manipulating the indigenous microbiota communities (Balcázar *et al.* 2006a), in which a small impact on the growth and mortality of one species may lead to change of species dominance in the communities (Moriarty 1998). The customised probiotics showed a high inhibition test to *V. harveyi* (Hai *et al.* 2007). The probiotic-fed prawns were healthier than those fed without the probiotics (Hai *et al.* 2009a). In our study (Hai *et al.* unpublished), the probiotic-fed prawns survived

longer than those not fed probiotics when challenged with *Vibrio harveyi*. Moreover, the probiotics have reduced the effects of *V. harveyi*, allowing the prawns to survive up to 60, 108 and 156 h of challenge with *V. harveyi* at 10^7, 10^5 and 10^3, respectively (Hai *et al.* unpublished). The probiotic-fed prawns died if the challenge with *V. harveyi* at 10^3 CFU/mL lasted longer than 36 h (Hai *et al.* unpublished). Therefore, the *V. harveyi* influenced the prawn survival as an opportunistic bacterium in other penaeid prawns.

Overdosage or prolonged administration of probiotics can induce immunosuppression in fish (Sakai 1999), but the effects of overdosage in prawns are yet be confirmed. Supplementing a combination of two probiotics (10^5 CFU/mL) at 20 mL/kg, Bio-Mos® at 0.5% and ß-1,3-D-glucan at 0.2% with the formulated feed improved the SGR, survival, FCR and immune response in the prawns (Hai and Fotedar 2009). The probiotic helped the prawns to digest the feed. The prawns consumed less feed leading to significantly lower FCRs when the prawns were fed the probiotics. The SGRs of the prawns in our studies were higher than those reported by Prangnell and Fotedar (2005). Similar to the studies conducted by several authors (Ai *et al.* 2007; Chang *et al.* 2003; Fritts and Waldroup 2003; Kocher *et al.* 2005; López *et al.* 2003; Waldroup *et al.* 2003), in our study (Hai and Fotedar 2009), the probiotics increased the SGR, survival and surface structure of the prawn intestines and decreased the FCR of the prawns compared to those without the probiotics. Changing intestinal morphology of smaller crypts with a larger number leads to an increase in the surface structure of the prawn intestines. The nutrient absorption was better when the prawns were fed probiotics leading to a lower FCR than those fed without the probiotics (Hai and Fotedar 2009).

8.2.2 Improvement in growth, survival and health of the prawns

Immunostimulants are additives and adjuvants, and include probiotics and prebiotics. Probiotics play a role as common immunostimulants in improving the growth, survival, food conversion ratio (FCR) and immune responses of the prawns (Hai *et al.* 2009a). Probiotics and prebiotics (Bio-Mos® and ß-1,3-D-glucan) supplemented with the formulated feed provided similar effects on these parameters (Hai and Fotedar 2009). Prebiotics are non-digestible substances, whereas probiotics are live

organisms and digestible substances (Douglas and Sanders 2008; Reid 2006). Both Bio-Mos® and ß-1,3-glucan are derived from yeast (*Saccharomyces cerevisiae*) cell wall (Couso *et al.* 2003; Waldroup *et al.* 2003). Several strains of baker's yeast are excellent sources of ß-glucan (Chang *et al.* 2003), and good immune enhancers in crustaceans (Ai *et al.* 2007). Recently, Bio-Mos® has been tested and promoted as a potential alternative to antibiotics and chemicals used in the pig, broiler and poultry industries (Iji *et al.* 2001; Kocher *et al.* 2005; Staykov *et al.* 2007; Valancony *et al.* 2001; Waldroup *et al.* 2003). ß-1,3-glucan is used in small dosages in humans, while probiotics are used not only in humans (Douglas and Sanders 2008; Mombelli and Gismondo 2000; Sullivan and Nord 2002) but also in aquatic animals (Irianto and Austin 2002a). Immunostimulants, either probiotics or prebiotics, enhance the disease resistance, growth of the hosts (Skjermo *et al.* 2006; Smith *et al.* 2003), act as effective alternatives to antibiotics and chemicals, and play the role of alarm molecules to activate the immune system (López *et al.* 2003). Other benefits derived from the use of immunostimulants for the host have been mentioned by several authors (Douglas and Sanders 2008; Reid 2008; Verschuere *et al.* 2000; Wang *et al.* 2008a).

Immunostimulants can promote immune boosting effects (Reid 2008) and improve the welfare, health and production of aquatic animal larvae (Rautava *et al.* 2002; Viljanen *et al.* 2005). Probiotics enhance the immune response of aquatic animals (Verschuere *et al.* 2000) and confer immunomodulatory effects and reduce allergic tendency (Waldroup *et al.* 2003). Total haemocyte counts (THCs) and different haemocyte counts (DHCs) play important roles in the crustacean immune response and are used as indicators for the health of aquatic animals (Jussila *et al.* 1997). THCs decreased when western rock lobsters (*Panulirus cygnus*) were exposed to air (Fotedar *et al.* 2001), when prawns (*Macrobrachium malcolmsonii*) were exposed to NO_2-N at 0.063 and 0.314 mg/L for 120 h (Chand and Sahoo 2006), and when white prawns (*Litopenaeus vannamei*) were exposed to 488 μg/L sulfide (Hsu and Chen 2007). THCs in American lobster (*Homarus americanus*) (Stewart *et al.* 1967) and in the blue crab (*Callinectes sapidus*) (Johnson 1976) declined in the presence of harmful bacteria and THCs were lower in moribund western rock lobsters than in the healthy ones (Jussila *et al.* 1997), but in our studies (Hai *et al.* 2009a; Hai and Fotedar 2009), the THCs of the prawns fed the probiotics were higher than those in

the controls. Therefore, the probiotics stimulated the prawns to produce more THCs, thus improving the health of prawns. Similarly, when exposed to mitogenic stimulation, the haemocytes of kuruma prawns (*Penaeus japonicus*) proliferated up to sixfold compared to haemocytes in non-stimulated individuals (Sequeira *et al.* 1996).

The response of DHC to different stressors is not fully understood, as previous reports have produced inconsistent results while using DHC as stress indicators in crustaceans (Johansson *et al.* 2000; Jussila *et al.* 1997). DHC has been reported to be dependent on sex, moult cycle, investigative methods and exposure time to stressors (Bauchau 1981; Sequeira *et al.* 1995). The probiotics decreased the hyalinocyte (HC) proportion and increased the semi-granulocyte (SCG) and granulocyte (GC) proportions of the prawns (Hai *et al.* 2009a; Hai and Fotedar 2009). These changes revealed that the prawns were healthier when they were fed the probiotic. The decrease in the SGC proportion contradicted an earlier study by Jussila *et al.* (1997) as the HC, SCG and GC proportions are considered to be volatile and their proportion in haemocyte could be time-dependant (Fotedar *et al.* 2006). SGCs and GCs have similar effects on cytotoxicity and storage and release of the prophenoloxidase system (Hose *et al.* 1990; Johansson *et al.* 2000; Martin *et al.* 1996). HCs are chiefly involved in phagocytosis (Johansson *et al.* 2000) and decreased with the application of the probiotics in our studies (Hai *et al.* 2009a; Hai and Fotedar 2009). The prawns not exposed to the probiotics were more stressed and produced relatively more HCs compared to those exposed to the probiotics. HCs were the most numerous of the haemocytes in kuruma prawns (Sequeira *et al.* 1995), western rock lobster (Jussila *et al.* 1997) and this was reflected where the HC proportion was 44.17 ± 3.66 to 70.83 ± 3.06% of the total haemocytes (Hai *et al.* 2009a). Similarly, the HC proportion of healthy red western rock lobsters was lower than those of the moribund ones (Jussila *et al.* 1997). The HC proportion of white prawns declined by 21 and 31% for 24 and 48 h exposure to 488 μg/L sulfide, respectively (Hsu and Chen 2007).

Aquatic animals come into contact with numerous bacteria in their habitat. Their haemolymph is also not sterile (Welsh and Sizemore 1985). A high bacterial load in the haemolymph indicates a decline in the immune capacity and an increase in

susceptibility to infection (Fotedar *et al.* 2001) as this is the case in the exposure of western rock lobster to air (Fotedar *et al.* 2001), and subjecting blue crabs (*Callinectes sapidus*) to the stresses of commercial capture, handling and transport (Welsh and Sizemore 1985). Foreign particles invading the haemolymph impairs the clotting time (Durliat and Vranckx 1983). High clotting times indicate a decline in resistance to pathogens, pointing to a lower immune status (Fotedar *et al.* 2001). Higher clotting times were found in air-exposed western rock lobster groups (Fotedar *et al.* 2001) compared to the lobsters not exposed to the air. In our studies (Hai *et al.* 2009a; Hai and Fotedar 2009), the probiotics reduced the bacterial load in the prawn haemolymph, the clotting times of the prawns were higher in the controls than those in other treatments. Similarly, the probiotics act as immunostimulants to enhance the defence potential of prawns against bacterial infection (Chang *et al.* 2000; Song and Hsieh 1994; Song *et al.* 1997).

8.2.3 Use as alternatives to antibiotics

The effectiveness of the use of probiotics needs to be investigated (Wang *et al.* 2008a). *V. harveyi* as the common harmful bacteria in marine animals was used for this investigation. Only 51.50% of *P. monodon* larvae survived at 48 h of challenge with *V. harveyi* at 10^3 CFU/mL (Prayitno and Latchford 1995). *V. harveyi* is dominant flora and accounts for up to 94.05% of luminous bacteria in all hatcheries (Abraham and Palaniappan 2004). Reducing the risk of *V. harveyi* contamination in postlarvae prevents losses caused by *V. harveyi* (Thaithongnum *et al.* 2006). Disease outbreaks caused by *V. harveyi* at 10^2-10^3 CFU/mL were observed in prawn hatcheries (Lavilla-Pitogo *et al.* 1990). The immersed challenge of *V. harveyi* at 10^2-10^4 cells/mL caused significant mortalities of *P. monodon* larvae and postlarvae (Karunasagar *et al.* 1994; Lavilla-Pitogo *et al.* 1990; Prayitno and Latchford 1995). In our study (Hai *et al.* unpublished), most of the probiotic-fed prawns died within 60 h of challenge with *V. harveyi* at 10^7 CFU/mL. The resistibility of the probiotic-fed prawns to *V. harveyi* at 10^3 CFU/mL lasted for 36 h of challenge. Although the probiotics appeared in the prawns before *V. harveyi*, the probiotic-fed prawns could not fully resist *V. harveyi*.

Probiotics can be used as a suitable alternative to antibiotics (Rekiel *et al.* 2007). Probiotic *Bacillus* spp. can be a suitable alternative to the prophylactic use of antibiotics and chemicals (Decamp *et al.* 2008) as they produce antibiotic compounds to compete for nutrients and sites and inhibit other bacteria if probiotics are present in high numbers (Moriarty 1998). Similar results can be seen in studies on Bio-Mos®, ß-1,3-glucan and probiotics conducted by several authors (Couso *et al.* 2003; Itami *et al.* 1998; Le Moullac *et al.* 1997; Loddi *et al.* 2004; Waldroup *et al.* 2003). In our studies (Hai *et al.* 2009a; Hai and Fotedar 2009), the use of the probiotics maintained a higher bacterial load in the prawn intestines and a lower bacterial load in the prawn haemolymph compared to those fed the prebiotics or without any immunostimulants. The direct use of probiotic bacteria contributed to the increase in the total bacterial counts in the prawn intestines, but the bacterial contamination in the haemolymph of the prawns was reduced. The customised probiotics can be used as an alternative to antibiotics in the culture of the prawns.

8.2.4 A strategy for use of customised probiotics

Artemia nauplii can convey customised probiotics *Pseudomonas synxantha* and *P. aeruginosa* to *P. latisulcatus* through encapsulation. The appropriate medium for encapsulation of probiotics by *Artemia* nauplii was the mixture of OW and TSB at 75:25 v/v respectively; whereas, the use of OW or TSB alone was not effective. *Artemia* nauplii most effectively encapsulated the customised probiotics at 10^5 CFU/mL. The results indicate that the encapsulation of *Artemia* nauplii is optimised by using a combination of *P. synxantha* and *P. aeruginosa* at 50:50 v/v in a media mixture of OW and TSB at 75: 25 v/v. *Artemia* should be harvested at 48 h when survival is still high (78%) and the probiotic load in *Artemia* is high (3 x 10^4 CFU/nauplius).

8.3 CONCLUSIONS

Overall, supplementing formulated feed with probiotics was advantageous as well as practical. *P. aeruginosa* improved the health of the prawns better than that of *P. synxantha*. The combination of both probiotics offered more advantages for improving health than the individual probiotics. *P. synxantha* and *P. aeruginosa* have

been proved as appropriate probiotics for the cultivation of the prawns through evidence of improvements in the immune response, survival and growth of the prawns subjected to *V. harveyi* challenge, although the application of the probiotics could not completely resist the *V. harveyi* injection. Our achievement from a series of the research project leads to the recommendation that *P. synxantha* and *P. aeruginosa* can be used as appropriate probiotics and as an alternative to antibiotics, in the cultivation of *P. latisulcatus*.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

9.1 CONCLUSIONS

Based on the results of the experiments conducted for this research project, the hypothesis (H_o), that there is no significant difference in the physiological and immune responses, in terms of growth, survival, food conversion ratio total haemocyte count, different haemocyte count, clotting time, bacteraemia and bacterial load in intestines, as well as tolerant capacity to harmful bacteria *V. harveyi,* on *P. latisulcatus* reared with and without customised probiotics, is rejected. The following summary in the form of conclusions demonstrates that the objectives of the research have been achieved (The most relevant objectives follow each conclusion in brackets):

1. Modified BLIS method is the most appropriate method for testing the inhibition capacity of probiotics to harmful bacteria (Objective 1).
2. *Pseudomonas* spp. may be either harmful or beneficial probiotic bacteria for aquatic species. However, *P. synxantha* and *P. aeruginosa* are the appropriate and suitable probiotics for the cultivation of juvenile *P. latisulcatus* (Objective 1).
3. A protocol for selecting appropriate probiotics using modified BLIS method for use in aquaculture is established (Objective 2).
4. The customised probiotics, *P. synxantha* and *P. aeruginosa* show higher inhibition to vibrios compared to other isolated probiotic strains (Objective 3).
5. Supplementing formulated feed with the probiotics was an effective method to introduce probiotics to the target species than the direct application into the rearing medium (Objective 3).
6. The customised probiotics at 10^5 CFU/mL was the most effective tested concentration for the cultivation of juvenile *P. latisulcatus* (Objective 4 and 5).

7. It is safe for juvenile *P. latisulcatus* to be exposed to the customised probiotics at high concentrations of 10^5 and 10^7 CFU/mL (Objective 4).
8. Application of a combination of the two customised probiotics *P. synxantha* and *P. aeruginosa* at equal concentration of 10^5 CFU/mL was more effective than the separate individual probiotic applications (Objective 5).
9. Probiotic *P. aeruginosa* is more effective in improving the survival and health of *P. latisulcatus* than *P. synxantha* (Objective 5).
10. The customised probiotics show their effectiveness by reducing the mortalities of juvenile *P. latisulcatus* after 28 days of exposure (Objective 5).
11. The customised probiotics improve the physiological and immune responses of juvenile *P. latisulcatus*, compared to the prawns which receive no supplementation of the customised probiotics (Objective 5).
12. The customised probiotics alter the physiology and enhance the immune response of juvenile *P. latisulcatus.* (Objective 5).
13. The customised probiotics have the similar effects on the physiological and immune responses of *P. latisulcatus* as prebiotics (ß-1,3-D-glucan and Bio-Mos®) (Objective 6).
14. The probiotic-fed *P. latisulcatus* show longer 100% survival-hours than the unfed *P. latisulcatus* when these prawns are challenged with *V. harveyi* at 10^3, 10^5 and 10^7 CFU/mL (Objective 7).
15. For a challenge longer than 36 h, the probiotic-fed *P. latisulcatus* can not completely resist *V. harveyi* even at a low concentration of 10^3 CFU/mL. (Objective 7).
16. The highest *Artemia* survival and probiotic load in *Artemia* were achieved at 48 h of the enrichment (Objective 8).
17. The suitable combination of the customised probiotics, *P. synxantha* and *P. aeruginosa* at equal concentration of 10^5 CFU/mL was 30:30 v/v, respectively (Objective 8).
18. The ideal combination of OW and TSB suitable for effective enrichment of *Artemia* was 75:25 v/v, respectively (Objective 8).
19. The concept of "one size fits all" is not applicable for probiotics as they are species specific and dependant on various other variables (concentrations, environmental factors and interation with other bacteria) (Objectives 1-8).

20. The development of probiotics applicable to commercial use in aquaculture is a multi-step and multi-disciplinary process requiring both empirical and fundamental research. (Objectives 1-8).
21. Customising probiotics involves a cumbersome process of isolation, identification, purification and then challenging with existing harmful bacteria, compared to commercially available probiotic products (Objectives 1-8).

9.2 RECOMMENDATIONS

Based on the research outcomes, some recommendations for further research are summarised below (Objective 9):

1. Study on the effects of the customised probiotics on other immune parameters such as ProPO, synthesise cells, agglutinating, phenoloxidase, haemocyte phagocyte and antibacterial activities, haemolymph protein profile, cell adhesion, superoxide anion production, levels of glucose and lactate in the plasma is important in order to get a complete picture of the immune responses of the probiotic-fed prawns. Hence, future study should incorporate all these parameters.
2. The study which investigates the effects of the customised probiotics on improving digestive enzymes of the juvenile *P. latisulcatus* is recommended.
3. The study on "intestinal epithelial changes" of juvenile *P. latisulcatus* when they are exposed to the customised probiotics using SEM and histological methods is warranted.
4. The study involving a study on interaction between the use of pro and prebiotics is also recommended.
5. Investigation on the resistant capacity of the probiotic-fed prawns to other known vibrios is also warranted.
6. Studies on the effectiveness of the customised probiotics at commercial or large scale are needed for complementing a complete picture of effectiveness of the customised probiotics on the cultivation of *P. latisulcatus*. Therefore, results from this research should be validated under the field conditions.

7. The target species should be extended to other commercially important species. For example studies on the effects of the customised probiotics on other penaeid prawns such as *P. monodon*, *L. vannamei* and *P. japonicus* or even in marine fish species should be conducted and compared with each other.
8. As the production of the species specific probiotics could be costly, it is essential to develop an economic model for the application of probiotics for the commercial cultivation of *P. latisulcatus.*
9. Transferral process of genes between the customised probiotics and other bacterial species in the cultured prawn species need to be investigated.

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