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Physiological responses of customized probiotic fed marron, *Cherax cainii* (Austin, 2002)

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DECLARATION

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature : 

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LIST OF ABBREVIATIONS

A10 Code for *Bacillus mycoides* from marron intestine
A12 Code for *Shewanella* sp. from marron intestine
ANOVA Analysis of Variance
AQ2 Code for *Bacillus* sp. from commercial probiotic from Aquasonic Pty. Ltd
BA Blood agar
BI Biomass increment
Cal/g Calorie per gram
CARL Curtin Aquatic Research Laboratory
CFU/mL Colony forming unit per millilitre
cm centimetre
DHC Differential haemocyte count
DO Dissolve Oxygen
FCR Feed conversion ratio
g Gram
GC Granulocyte(s), granular cell(s)
GPx Glutathione peroxide
HBR Haemolymph bacteraemia rank
HC Hyalinocytes/ hyaline cell
HM% Moisture content of hepatopancreas
HSIdry Dry hepatosomatic index
HSIwet/Hiw Wet hepatosomatic index
IBR Intestine bacteria rank/intestinal bacteria population/density
kg Kilogram
L Litre
LSD Least Significant Difference
mg/L Milligram per litre
Min Minute
mL Millilitre
MOS Mannan oligosaccharide
NA Nutrient Agar
NRR Neutral red retention
OC Osmoregularoty capacity
PM3 Code for *Bacillus subtilis* from commercial probiotic from Enviroplus
PM4 Code for *Bacillus sp* from commercial probiotic from Enviroplus
PO Phenoloxidase
ppt Parts per thousand
ProPO Prophenoloxidase
SE Standard error
SGC Semi-granulocytes/ semi-granular cell(s)
SGR Specific growth rate
SPSS Statistical package for the social science
TCBS Thiosulfate-Citrate-Bile Salt Sucrose Agar
THC Total haemocyte count
Tiw Wet tail muscle index
TM% Moisture content of tail muscle
TMIdry Dry tail muscle index
WA Western Australia

LIST OF ANIMAL SPECIES

Abalone, *Haliotis discus hannai*
Abalone, *Haliotis gigantea*
Abalone, *Haliotis tuberculate*
American lobster, *Homarus americanus*
Arctic charr, *Salvelinus alpinus*.
Atlantic cod, *Gadus morhua*
Atlantic salmon, *Salmo salar*
Beluga, *Huso huso*
Black swordtail, *Xiphophorus helleri*
Channel catfish, *Ictalurus punctatus*
Cheek crayfish, *Orconectes limosus*
Chinese shrimp, *Fenneropenaeus chinensis*
Cobia, *Rachycentron canadum*
Common wolfish, *Anarhichas lupus*
Crab, *Cancer pagurus*
Crab, *Portunus trituberculatus*
Crayfish, *Cherax albidus*
Crayfish, *Cherax quadricarinatus*
Crayfish, *Pacifastacus leniusculus*,
European lobster, *Homarus gammarus L.*
Freshwater prawn, *Macrobrachium rosenbergii*
Gilthead seabream, *Sparus aurata*
Grouper, *Epinephelus coioides*
Indian white shrimp, *Fenneropenaeus indicus*
Japanese flounder, *Paralichthys olivaceus*
Koi, *Cyprinus carpio koi*,
Kuruma shrimp, *Penaues japonicas*
Marron *Cherax cainii / Cherax tenuimanus*
Nile tilapia, *Oreochromis niloticus*
Pacific white shrimp, *Litopenaeus vannamei*
Rainbow trout, *Oncorhynchus mykiss*
Red drum, *Sciaenops ocellatus*
Red swamp crayfish, *Procambarus clarkia*
Sea cucumber, *Apostichopus japonicus*
Seabream, *Sparus aurata* L.
Senegalese sole, *Solea senegalensis*
Shrimp, *Litopenaeus stylirostris*
Shrimp, *Penaeus japonicas*
Tiger shrimp, *Penaeus monodon*
Shrimp, *Litopenaeus vannamei*
Tilapia, *Oreochromis niloticus*
Trout, *Oncorhynchus mykiss*
Western king prawn, *Penaeus latisulcatus*
Western rock lobster, *Panulirus cygnus*
Yellow croaker, *Larimichthys crocea*
Zebrafish, *Danio rerio*

PREAMBLE

One of the purposes of conducting research in the aquaculture area is to increase its productivity by improving the growth, disease resistance and survival of the target aquatic organisms, in addition to reducing production costs through feed efficiency. Feed constitutes the largest variable cost in intensive fish production ranging from 40 to 60%, whereas disease is the major constraint of intensification of aquaculture. One of the promising emerging paradigms recently from research on nutrition is that prepared diets which may influence a cultured organism's health and resistance to stress and disease-causing agents (Burr et al., 2005) but should also improve growth and feed digestibility and efficiency. Most of these nutritional benefits have been demonstrated in aquatic species by exploring the role of probiotics and prebiotics in aquatic animal nutrition studies (Denev et al., 2009 ; Merrifield et al., 2010 ; Merrifield & Zhou, 2011 ; Ganguly et al., 2010).

Marron, *Cherax cainii* (Austin, 2002) is an economically viable aquaculture species in Western Australia, however the farm production is relatively very low and thus requires improvement in culture technique and productivity. The purpose of this research is to screen a potential probiotic candidate isolated from a number of adult healthy marron and from commercial probiotic products that could be used in marron culture. The selected strain should be able to improve the productivity of farmed marron by enhancing their growth, survival, health and immune status. The research relevant to this area is described in eleven chapters of this thesis.

Chapter 1 briefly introduces the role of aquaculture in meeting the demand of aquatic protein in the fast growing world population. It also discusses the updated research on marron culture in Western Australia, constraints in improving aquaculture productivity and potential application of the probiotic and prebiotics. This chapter also includes the aim, objectives and significance of the research.

Chapter 2 briefly presents a taxonomic classification and biology of marron. This chapter also reviews crustacean immunity, major constraints in aquaculture which has led to the use of probiotics in aquaculture. This section also describes the approach used to evaluate health status of marron.

Chapter 3 describes the probiotic selection process by comparing the two different sources of the probiotics i.e. host (healthy marron) and commercial probiotic products. The criteria used for this selection including pathogenicity to marron (feeding test), production of protease and amylase, API-Zym test and its antagonism ability towards common pathogens in crayfish and marron, namely *Vibrio mimicus* and *V. cholerae non-01*, are discussed in this chapter.

Chapter 4 evaluates the two sources of probiotic candidates on marron growth, survival, and health status and investigates the marron's immunity against virulent bacteria strains of *V. mimicus* through pathogen injection challenge tests during the post probiotic feeding period.

Chapter 5 explores the performance of the selected customised probiotic *Bacillus mycoides* on the gastro intestinal health status (GIT) of marron by measuring indicators such as intestinal bacteria population, hepatosomatic indices, microvilli condition and histological appearance of intestinal epidermis cells.

Chapter 6 further evaluates the stress resistance of marron fed a probiotic *B. mycoides* supplemented diet under a simulated transport test by measuring the health and immune status including THC, intestinal bacteria population, morbidity and mortality post 24 and 48h of simulated transport.

Chapter 7 examines the effects of feeding duration with probiotic *B. mycoides* supplementation, on the health status and immunity of the marron by evaluation of health indicators including intestinal bacteria population, THC, bacteraemia and hepatosomatic index.

Chapter 8 researches the possible synergistic effects of customised probiotic *B. mycoides* and prebiotic organic selenium (OS) on marron by comparing the effects of their isolated uses and the combined effect on the health and immune status of marron. This chapter also examines a direct effect of OS dispersant on probiotic *B. mycoides* population growth.

Chapter 9 evaluates the performance of the customised probiotic *B. mycoides* in vivo in a commercial earthen marron farm. This chapter compares the probiotic and commercial basal diet fed marron during a one year feeding trial.

Chapter 10 summarises and discusses the criteria for screen techniques of probiotics in the light of the previous research. This chapter also discusses all the laboratory scale (in vitro) and in vivo findings of the current research. An assessment of a customised probiotic isolated from the host is also discussed which leads to a series of conclusions, followed by the recommendations for future research.

ABSTRACT

Aquaculture has become an important economic activity in many countries, including marron culture in Western Australia. To optimize profitability and production, aquaculture is expanding into intensifying and diversifying, however under intensive culture conditions the animal may be exposed to various stressful conditions, and a link between stress and disease susceptibility may occur which may result in serious economic losses.

Prevention and control of diseases in aquaculture has been achieved by various methods. The use chemotherapeutants is the most widely criticized for human health issues. However, the use of probiotics as environmentally friendly immunostimulants in aquaculture has been more widely accepted.

Six strains of probiotic candidates from two sources (from the host or marron gastrointestinal tract origin (GIT) and two commercial probiotic products used in aquaculture) were screened using various tests and criteria proposed by most probiotic screening study's authors. Based on the results and criteria, *Bacillus mycoides* was selected as the most favourable probiotic candidate for marron. The strain was a predominant isolate from the GIT of a number of healthy adult farmed marron (recommended by most authors) which exhibits many favourable probiotic properties especially antagonism ability towards pathogenic bacteria of crayfish *Vibrio mimicus* and *V. cholerae* non-01, non-resistant to the majority of antibiotics, non-pathogenic to the host and exhibit a wide range of enzyme profiles.

A series of laboratory scale experiments and a commercial scale trial were performed to investigate beneficial effects of the selected probiotic candidates. Supplementation with the host derived customised probiotic *B. mycoides* at a rate 10^8/g of feed (as the common density used in *Bacillus* spp studies) significantly improved specific growth rate, survival, health and immune status of marron compared to probiotic-free fed marron. Application of the probiotic in marron diet also improved gastrointestinal health (GIT) of marron including number and height of microvilli, intestinal bacteria population, intestinal layer morphology and hepatosomatic indices.

Supplementation with the customised probiotic *B.mycoides* in marron diet also improved marron immune status and stress tolerance including higher THC, lower haemolymph

bacteria and no mortality occurred after challenging with the pathogen *Vibrio mimicus* injection test nor after 48h simulated transport test.

In conclusion, application of *Bacillus mycoides*, a predominant marron origin strain with favourable probiotic properties is recommended as an alternative feed additives and substitute to antibiotics in marron diets which may contribute numerous health benefits on marron culture.

CHAPTER 1. Introduction

1.1. Background

Aquacultured species are a vital source of protein and essential nutrients, especially for the communities in developing countries and will continue to be one of the most-traded food commodities worldwide (FAO, 2014). Today, more than 800 million humans suffer from chronic malnourishment, and the global population is expected to grow by another 2 billion to reach 9.6 billion by 2050. It is a significant challenge to feed such a growing population while conservating the natural resources for future generations.

Global fish production continues to outpace world population growth, and aquaculture remains one of the fastest-growing food producing sectors (FAO, 2014). In 2012, aquaculture set another production milestone and now provides almost half of all fish for human food. Aquaculture is also set to bridge the gap between seafood supply and demand in order to meet the world demand (FAO, 2009). This gap is projected to rise to 62 percent by 2030 as catches from wild capture fisheries level off and demand from an emerging global middle class substantially increases. To achieve the target, a responsible and environmentally friendly aquaculture practise (Sihag & Sharma, 2012) could generate lasting benefits for global food security and economic growth (FAO, 2014).

As seafood production relies more on aquaculture, a goal of global aquaculture is to maximize the efficiency of production to optimize profitability through intensifying and diversifying (Denev et al., 2009). However, intensification is always associated with disease problems as aquacultured animals under the conditions of intensification are subjected to high-stress conditions (Rollo et al., 2006; Cruz et al., 2012; Ige, 2013; Tapia-Paniagua et al., 2014). In addition with over-crowding conditions, susceptibility to disease is often intensified by the highly stressful environment such as high temperature, low oxygen or high ammonia concentrations and poor nutrition in production systems (Moriarty, 1997). Disease outbreak can limit food supply from the global fishery and aquaculture sectors (Stentiford et al., 2012) and can be a major constraint to the aquaculture industry (Kesarcodi-Watson et al., 2008; Sahu et al., 2008; 2012) including marron, *Cherax cainii* (Austin, 2002) industry in Western Australia.

Disease control in the aquaculture industry has been achieved by different methods using traditional ways, application of synthetic chemicals and antibiotics (Sahu et al., 2008) have been widely criticized for their negative impacts (Moriarty, 1997; Tinh et al., 2007; Sahu *et al.*, 2008). Thus, there is a growing need to control, prevent or minimize the devastating effect of disease in aquaculture without the use of toxic chemicals or antibiotics (Irianto & Austin, 2002) which is both cost-effective and environmentally safe (Moriarty, 1997; Gatesoupe, 1999; Sihag & Sharma, 2012).

The use of probiotics and prebiotics as immunostimulants, has been proposed for the control of disease in aquaculture, as they enhance resistance against pathogens by improving the immune system (Burr & Gatlin, 2005; Denev et al., 2009; Yousefian & Amiri, 2009; Ringø et al., 2010; Daniel & Zhou, 2011). The prebiotics commonly used and evaluated in aquatic animals to date includes inulin, fructooligosaccharides (FOS), mannan-oligosaccharides (MOS), galacto-oligosaccharides (GOS), xylooligo-saccharides (XOS), arabinoxylo oligosaccharides (AXOS), isomaltooligosaccharides (IMO) and GroBiotic-A (Burr & Gatlin, 2005; Denev et al., 2009; Yousefian & Amiri, 2009; Ganguly et al., 2010; Ringø et al., 2010; Daniel & Zhou, 2011), chitosan oligosaccharides (COS) and organic selenium (OS). In marron (*Cherax cainii*, Austin 2002), the prebiotics that have been evaluated include MOS (Sang et al., 2009; Sang & Fotedar, 2010b) and organic selenium (OS) (Nugroho & Fotedar, 2013b).

Probiotics have shown enough evidence to play an important role in aquaculture (Newaj-Fyzul et al., 2014) by becoming an integral part of the aquaculture practices to obtain high productivity (Nayak, 2010). Probiotics have been used as a strategy to overcome microbial problems of farmed aquatic animals (Skjermo et al., 2015) for successful and sustainable aquaculture (Gatesoupe, 1999; Kesarcodi-Watson et al., 2012; Sihag & Sharma, 2012). Numerous health benefits attributed to probiotics, have been demonstrated in various groups of aquatic animals such as fish, crustaceans and molluscs in many countries and a region including China, India, Ecuador, Egypt, Nigeria and South America which have been reviewed by numerous authors (Ringø & Gatesoupe, 1998; Gatesoupe, 1999; Ringø & Birkbeck, 1999; Gomez-Gil et al., 2000; Verschuere et al., 2000; Irianto & Austin, 2002; Burr & Gatlin, 2005; Austin, 2006; Balcázar et al., 2006; Farzanfar, 2006; Gómez et al., 2007; Das et al., 2008; Kesarcodi-Watson et al., 2008; Sahu et al., 2008; Tinh et al., 2008;

Wang et al., 2008; Denev et al., 2009; Ninawe & Selvin, 2009; Qi et al., 2009; Yousefian & Amiri, 2009; Hai et al., 2009b; Ganguly et al., 2010; Merrifield et al., 2010; Nayak, 2010; Prado et al., 2010; Dimitroglou et al., 2011; Kolndadacha et al., 2011; Lara-Flores, 2011; Aguirre-Guzmán et al., 2012; Cruz et al., 2012; Ibrahem, 2013; Lakshmi et al., 2013; Mohapatra et al., 2013; Pandiyan et al., 2013; Lazado et al., 2014; Michael et al., 2014; Newaj-Fyzul et al., 2014; Ghanbari et al., 2015; Hai, 2015). In Mediterranean teleosts, the probiotic benefits included stimulated immune responses, enhanced growth performance, feed utilisation, digestive enzyme activity, antioxidant enzyme activity, gene expression, disease resistance, larval survival, healthy gut morphology, modulated GI microbiota and mediated stress responses (Dimitroglou et al., 2011). Among those numerous health benefits, modulation of the immune system is the most reported benefit (Nayak, 2010), thus probiotic application currently targets disease prevention (Kesarcodi-Watson et al., 2012) notably against bacterial diseases (Newaj-Fyzul et al., 2014).

Although there is no report of massive losses in marron aquaculture caused by bacterial infection in Australia, the threat of virulent bacteria such as *Vibrio mimicus* which may cause fatal mortalities in crayfish *Cherax albidus* and *C. quadricarinatus* (Wong et al., 1995) should be seriously considered. With the growth of crayfish aquaculture worldwide and intensification of marron culture, the occurrence of disease outbreaks is likely to increase (Evans & Edgerton, 2002).

Marron is a farmed crayfish and the world's third largest freshwater crayfish, native to the south-west of Western Australia, capable of reaching 2 kg (Morrissy et al., 1990) and shows potential for intensive culture (Jussila, 1997; Fotedar, 1998). Marron have been introduced into South Africa, Zimbabwe, Japan, USA, China and the Caribbean as a commercial aquaculture species (Morrissy et al., 1990; David B Rouse & Izuddin Kartamulia, 1992; Sang et al., 2009). As a result of the domestic and global interest in marron farming, its production has been extended and research is currently in progress to enhance productivity (Morrissy, 1979; Lawrence & Morrissy, 2000; Sang et al., 2009; Alonso, 2010; Nugroho & Fotedar, 2013a).

In the United States, *Vibrio mimicus* and *V. cholerae* have caused rapid and fatal systemic infections of cultured crayfish *Procambarus clarkii*, whereas in Australia *V. mimicus* caused sporadic mass mortalities of commercially farmed yabbies *Cherax albidus* during postharvest purging (Wong et al., 1995). Stock mortalities of *C. quadricarinatus* (Eaves & Ketterer, 1994). *V.mimicus* and *V. cholerae* non-01 are opportunistic bacteria that cause septicaemia in crayfish and tail blister in marron (Eaves & Ketterer, 1994; Wong et al., 1995; Evans & Edgerton, 2002). Therefore, antagonistic ability towards these strains is important when screening a probiotic candidate for marron.

To date, studies on marron have included pond production (Morrissy, 1979), physiological aspects under intensive culture conditions (Jussila & Evans, 1996; Jussila, 1997), nutrition under different culture conditions (Fotedar, 1998, 2004), pigmentation (Sommer et al., 1991), genetic (Lawrence & Morrissy, 2000), water quality assessments (Rouse & Kartamulia, 1992), outdoor tank culture (Kartamulia & Rouse, 1992), grading (Qin, 2001), production constraints (Alonso, 2010), use of prebiotics such as mannan-oligosaccharide (MOS) (Sang et al., 2009; Sang & Fotedar, 2010b; Sang et al., 2011), β - 1,3 – glucan (Sang & Fotedar, 2010a) and more recently the organic selenium (OS) (Nugroho & Fotedar, 2013c, 2013d). However, there has been no study on the effects of the supplementation with probiotics in the marron diet. The present study was designed to examine the effects of different sources of customized probiotic-supplemented feeds on the physiological and immunological aspects of marron.

Results of the experiments hopefully contribute to the better understanding of disease management in marron farming and will improve marron farming productivity by increasing growth and survival.

1.2. Aim

The aim of the present study is to screen and customize probiotic candidates and investigate their effectiveness on the growth, survival, physiological and immunity of marron, *Cherax cainii.*

1.3. Objectives

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

1. To isolate, screen and customise probiotic candidates from healthy farmed marron and commercial probiotic products with favourable probiotic properties for marron *Cherax cainii* (Austin, 2002) culture.
2. To evaluate the effectiveness of the selected probiotic candidates by studying the physiological response of marron fed the probiotics under laboratory conditions.
3. To investigate the effect of the dietary supplementation with the customized probiotic on the gastrointestinal track (GIT) health status of marron.
4. To investigate dietary supplementation with the customized probiotic candidate on immunological competence of marron by challenging the marron with (i) potential pathogens and (ii) exposing to simulated transport stressor.
5. To explore the potential synbiotic effects of the customized probiotic with prebiotic organic selenium (OS) on marron.
6. To evaluate the probiotic supplementation period on marron health
7. To validate the laboratory scale research findings on commercial scale marron farming.

CHAPTER 2. Literature Review

2. 1. Background

Marron, *Cherax cainii* (Austin, 2002) is one of the important freshwater crayfish species native to Western Australia that has attracted global interest as a potential aquaculture species due to its positive attributes, including large harvest-size (up to 2 kg), high price, non-burrowing behaviour, simple life cycle and ease of live transport (Rouse and Kartamulia, 1992, Morrissy, 1979, Lawrence and Jones, 2002, Morrissy et al., 1990). International interest in marron farming has led to the species being introduced to several countries and a region including South Africa, Zimbabwe, Japan, USA, China, Caribbean and several Australian states (Morrissy et al., 1990, Lawrence and Jones, 2002).

In Western Australia, marron farming has been an emerging industry for decades (Alonso, 2010). In 2011/2012, marron production from semi-intensive culture in purpose-built pond systems provided around 60 tonnes and has the potential to expand significantly (Fletcher and Santoro, 2012) with around 201 members of the Western Australia Marron Growers Association (Alonso, 2010). With increasing marron market demand, production has widely been extended and the research is currently in progress to enhance its productivity (Morrissy, 1979, Alonso, 2010)

2.2. Taxonomy and biology

The systematic classification of marron, *Cherax cainii* (Austin, 2002) is as follows;

Kingdom : Animalia
Phylum : Arthropoda
Subphylum : Crustacea
Class : Malacostraca
Order : Decapoda
Suborder: Pleocyemata
Infraorder: Astacidea
Family : Parastacidae
Genus : *Cherax*
Species: *Cherax cainii* (Austin, 2002)

Two types of marron are found in Western Australia namely hairy marron (*Cherax tenuimanus*) and smooth marron (*Cherax cainii*) (Bryant and Papas, 2007). Smooth marron are found in most southwest rivers and dams, whereas hairy marron are listed as critically

endangered and only found in the upper reaches of The Margaret River (Department of Fisheries Western Australia, 2015).

In aquaculture, understanding the biology of the animal is crucial in order to provide optimum environmental requirements cultivation. Survival of marron in grow out pond depends largely on providing optimal environmental requirements (Lawrence and Jones, 2002). Among the requirements, water temperature is the most important environmental variable as it directly affects metabolism, oxygen consumption, growth, moulting and survival (Le Moullac and Haffner, 2000).

Knowledge of the natural diet of marron is also crucial, to obtain maximum growth rate. Marron is a species of Parastacidae, which are defined as omnivorous detritivores, opportunistically ingesting a range of food items including small invertebrates, fish eggs, fish larvae and algae (O'Brien, 1994). Ideally, a diet should provide maximum growth and minimal (O'Brien and Davies, 2000). It is also noted that a clay bottom of culture ponds provides a suitable condition for marron culture (Morrissy et al., 1990).

2.3. Crustacean Immunity

Crustaceans live in an environment often rich in different parasites and pathogens, thus they must have an efficient defence mechanism against invading pathogenic organisms (Soderhall and Cerenius, 1992). Understanding the immune criteria as enhancement of non-specific defence responses against bacterial and viral diseases is the most effective strategy for sustainable aquaculture production (Bachere, 2003).

A number of defence mechanisms as part of the crustacean immune system have been well documented (For review see; Bachere, 2000, Soderhall and Cerenius, 1992, Liu et al., 2009, Hauton, 2012, Vazquez et al., 2009, Smith and Chisholm, 1992). In general, host defence reactions in crustaceans are divided into three categories which are physico-chemical, humoral and cellular defence mechanism. Component of crayfish host defence systems have been described (Table 2.1), but most of the reactions are haemocytes origins and thus cellular defence usually occur (Soderhall and Soderhall, 2002).

Table 2.1 Component of crayfish host defence systems (Soderhall and Soderhall, 2002).

Physicochemical barrier	External skeleton Melanisation Proteinase inhibitors Chitinase inhibitors
Cellular	Phagocytosis Encapsulation Cytotoxicity
Humoral	ProPO system Antibacterial peptide Agglutinins Proteinase inhibitors

The defence mechanism is activated when pathogen-associated molecular patterns are recognized by soluble or by cell surface host proteins, including lectins, antimicrobial, clotting, and pattern recognition proteins, which in turn activate cellular or humoral effector mechanisms to destroy invading pathogens (Vazquez et al., 2009). The target and the haemocytes which involved in various defence mechanism in crustacean has been documented by Vazquez *et al.*(2009).

Table 2.2. Effector defence mechanisms in crustaceans (Vazquez et al., 2009)

Defence mechanisms	Cellular population involved	Target
ProPO	Semigranulocytes, hemocytes with big refractile granules	Bacteria and fungi
Antimicrobial proteins	Hemocytes with granules	Bacteria and fungi
Phagocytosis	Hyalinocytes, semigranulocytes	Bacteria and micro-organisms <10 µm
Encapsulation	Semigranulocytes hemocytes with big refractile granules	Fungal spore and yeast, organisms >10 µm
Lectins	Hyalinocytes, semigranulocytes and hemocytes with refractile granules	Distinguish between the self and non-self particles, inducing agglutination and phagocytosis
Clottable protein	Clottable proteins from hemocytes	Bacteria and fungi

In crustacean, the haemocytes play a significant role in immunity (Soderhall and Cerenius, 1992, Bachere, 2000, Vazquez et al., 2009) including in crayfish and crabs (Johansson et al., 2000). In immunity, the haemocyte defence mechanisms including recognition, phagocytosis, encapsulation, storage and release of the proPO system and cytotoxicity (Johansson et al.,

2000, Soderhall and Cerenius, 1992, Sritunyalucksana and Soderhall, 2000), antimicrobial proteins, lectins and clottable protein (Vazquez et al., 2009). In general, there are three types of haemocytes in crustacean namely hyalinocytes, semi-granulocytes and granulocytes (Bauchau, 1981, Johansson et al., 2000).

Table 2.3. Three different haemocyte types in crustaceans (Bauchau, 1981, Johansson et al., 2000)

Criteria	Hyalinocytes	Semi-granulocytes	Granulocytes
Shape	round or oval	oval or spindle	oval
Nucleus	central, round, large	central or eccentric, oval, lobed	eccentric, kidney shaped
Endoplasmic reticulum	smooth, rough, scarce	smooth, rough, abundant	smooth, rough, moderate
Free ribosomes	present	abundant	Moderate
Golgi	0 or 1	1 or more	0 or 1
Granules	0 or few	moderate	abundant
Lysosomes	-	present	present
Mitochondria	moderate	abundant	abundant

The haemocytes contain many immune-related genes (Shi et al., 2010) and antibacterial activity which very effective against both gram-positive and gram-negative organisms (Chisholm and Smith, 1995, Le Moullac and Haffner, 2000). The haemocytes also contain glutathione-S-transferase (GST), glutathione peroxidase (GPx) enzymes which protect the body from damaged by free radicals as observed in marron (Nugroho and Fotedar, 2013a). Phagocytosis activity of crayfish and crab haemocytes have been described by Johansson *et al*. (2000)(Table 2.4).

Tabel 2.4. Crayfish and crab haemocytes (Johansson et al., 2000)

Hyaline cell	Phagocytosis
Semi granular cell	Encapsulation Phagocytosis (Limited) Storage and release of the ProPO system Cytotoxicity
Granular cell	Storage and release of the ProPO system Cytotoxicity

The crayfish haemocytes eliminate bacteria, spores, foreign particle and yeast (Soderhall et al., 1984, Vazquez et al., 2009) including parasitic fungus, *Aphanomyces astacii* in Crayfish,

Pasifastacus leniusculus (Persson et al., 1987). Thus, a low circulating haemocyte number in crustaceans is strongly correlated with a greater sensitivity to pathogen (Soderhall et al., 2003, Le Moullac and Haffner, 2000) including marron (Jussila et al., 1999, Sang et al., 2009).

2.4. Major constrains in aquaculture

Disease outbreak is the major constrain in aquaculture industry worldwide particularly infectious disease (Newaj-Fyzul et al., 2014, Batista et al., 2015) as a result of low immunity (Nayak, 2010) and low stress tolerance (Rollo et al., 2006, Lund et al., 2012). Stress routinely occur in aquaculture which lead to mortalities and cause significant economic losses (Dagar et al., 2010). The link between stress and higher susceptibility to diseases has been established (Tapia-Paniagua et al., 2014b) , therefore improve immunity of aquatic animal is extremely important to ensure a successful aquaculture (Bachere, 2000)

In Australia, bacteraemia has been associated with mortalities in crayfish (Eaves and Ketterer, 1994, Edgerton and Owens, 1999), as a result of exposure to environmental stressors (Jussila et al., 1997, Evans and Edgerton, 2002, Sang et al., 2009). In crayfish, three main categories of bacterial disease have been found that is bacteraemia which involve haemolymph and internal organs; infections of the exoskeleton by chitinoclastic bacteria; and gill infections by filamentous bacteria (Jussila, 1997b).

In marron aquaculture, fungus and parasites are dominant disease causing agents however prevalence of infection and incidence of mortality are relatively low (Langdon, 1991a, Herbert, 1987). *Epistylis* and *Temnocephala* are two epibionts which are commonly found in marron, caused by poor water quality, particularly in unaerated ponds containing excessive organic matter (Lawrence and Jones, 2002). These epibionts can decrease growth rates and reduce consumer appeal (Morrissy et al., 1990). *Vibrio mimicus* and *V. cholerae* non-01 are opportunistic bacteria that cause septicaemia in crayfish and tail blister in marron (Eaves and Ketterer, 1994, Wong et al., 1995, Evans and Edgerton, 2002) while *Vibrio mimicus* has emerged as a dominant bacterial pathogen of freshwater crayfish in aquaculture (Wong et al., 1995, Evans and Edgerton, 2002).

In Asia, antimicrobials, parasiticides, feed additives and probiotics are heavily used to improve the health status of the cultured organisms and to prevent or treat disease outbreaks where sixty different veterinary medicinal ingredients were recorded (26 antibiotics, 19 disinfectants and 15 parasiticides)(Rico et al., 2013). Newaj-Fyzul et al. (2014) summarized various method used for disease control in many developing countries (Table 2.5).

Table 2.5. Methods of controlling bacterial fish diseases in aquaculture (Newaj-Fyzul et al., 2014)

Method	Comment
Husbandry/management	Includes improved hygiene including sanitary disposal of dead animals; do not overstock and over feed
Movement restrictions	Effective at preventing the spread of diseases; essential to have governmental support
Genetically resistant stock	Emotive if it involves genetic modification; useful if selecting naturally disease resistant strains
Dietary supplements	Effective with compounds such as vitamin C
Nonspecific immunostimulants	Success with some products, such as β-1,3-glucans
Vaccines	Available commercially for a minority of diseases
Probiotics	A wide range of probiotics has been considered for use in aquaculture
Prebiotics	Compounds that support the growth of probiotics; of increasing interest to aquaculture
Medicinal plant products	A wide range of plants considered particularly in China and India; may be immunostimulatory
Water disinfection	Involves chemicals which may be effective at reducing or eliminating populations of pathogens
Biological control	The application of inhibitory micro-organisms often to water; may be effective but some concerns over the fate of the inhibitors
Antimicrobial compounds	There are emotive issues in many countries about the non-medical use of medicinal compound

In addition to disease outbreak as a major constraint in aquaculture (Stentiford et al., 2012), the feed efficiency is also a crucial aspect as the feed constitute 40–60% of the total production costs in aquaculture (Fotedar, 2004, Burr and Gatlin, 2005). As disease outbreak and feed efficiency are the two major issues in commercial aquaculture development, thus the need for increased immunity, stress tolerances, disease resistance while improve feed digestion efficiency has led the use of probiotics in aquaculture practices (Cruz et al., 2012).

2.5. Probiotics in aquaculture

Probiotics, which are regarded as micro-organisms administered orally leading to health benefits of the host (Fuller, 1989), are now used extensively and play a crucial role in various groups of aquaculture species (Nayak, 2010, Denev et al., 2009, Merrifield et al., 2010b).

The research of probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture (Gatesoupe, 1999). Today, numerous reviews on probiotic

use in aquaculture have been documented (see section 1.1), but its role in different aquaculture species is continuously being explored (Zheng et al., 2016). Various strains of probiotics proposed as biological control agents in aquaculture belong to the lactic acid bacteria (*Lactobacillus* and *Carnobacterium*), *Bacillus, Vibrios, Pseudomonas* although other genera including *Aeromonas* and *Flavobacterium* (Balcázar et al., 2006), *actinobacters* (Das et al., 2008), yeast and fungus (Gomez-Gil et al., 2000) have also been mentioned (For review see ; Verschuere et al., 2000, Balcázar et al., 2006, Newaj-Fyzul et al., 2014).

In Atlantic cod, the areas regarded as probiotic confer beneficial health effects to the fish are modulation of intestinal microbial community, growth enhancements, nutritional contribution and stress tolerance improvement (Lazado et al., 2014), whereas in Mediterranean teleosts, the probiotic health benefits includes, enhance growth performance, feed utilisation, digestive enzyme activities, modulate GI microbiota and mediate stress responses, survival rate, gut morphology, antioxidant enzyme activities, gene expression, disease resistance, stimulate immune responses (Dimitroglou et al., 2011). In salmonids, probiotics providing benefits to the host primarily via the direct or indirect modulation of the gut microbiota by increased favourable bacteria (e.g. lactic acid bacteria and certain *Bacillus* spp.) in the gastrointestinal tract (GIT) (Merrifield et al., 2010b).

In addition, the probiotics have also been successfully improved immunity and stress tolerance of various aquaculture groups including molluscs (Kesarcodi-Watson, 2009, Kesarcodi-Watson et al., 2012, Jiang et al., 2013) shrimps (Bachere, 2000, Farzanfar, 2006, Ninawe and Selvin, 2009, Lakshmi et al., 2013, Tseng et al., 2009, Rengpipat et al., 2000) and fishes (Rollo et al., 2006, Tapia-Paniagua et al., 2014a, Ridha and Azad, 2015, Sun et al., 2010) (For review see;Nayak, 2010). Probiotic treatments are particularly desirable during the larval stages, as this stage they start feeding even though the digestive tract is not yet fully developed and the immune system is still incomplete (Gatesoupe, 1999).

Improve feed digestion efficiency and growth by feeding probiotic diets have been demonstrated in various groups of aquaculture species including shrimps (Rengpipat et al., 2003, Hai et al., 2009a), lobster (Daniels et al., 2010), fishes (Merrifield et al., 2010a, Dimitroglou et al., 2011) and molluscs (Macey and Coyne, 2005, Prado et al., 2010, Kesarcodi-Watson et al., 2012). Improved growth and feed digestion efficiency by probiotics

as the probiotic improves appetite (Newaj-Fyzul et al., 2014), modulate intestinal bacteria population (Merrifield et al., 2009, Dimitroglou et al., 2011) and improve GIT health status of aquatic animals (Dimitroglou et al., 2009, Merrifield et al., 2010c).

2.6. Approach to evaluate health status of marron

The marron health can ultimately be assessed by growth and accepted survival rates, however other physiological and immunological indicators including total haemocyte counts (THC), bacteraemia, haemolymph clotting time, glutathione peroxidase (GPx) enzyme activity, intestinal bacteria population, organosomatic indices (H_{iw}) can also be used to understand the underlying mechanism for marron health (Evans and Jussila, 1997, Fotedar, 1998, Jussila et al., 1999, Jussila and Evans, 1996, Fotedar, 2004, Morrissy, 1979, Sang et al., 2009, Sang and Fotedar, 2010a;b, Nugroho and Fotedar, 2013a). This section reviews only physiological and immunological parameters which are measured in most chapters namely THC, intestinal bacteria population, hepatosomatic indices, growth and survival.

- ***Total Haemocyte Count (THC)***

The total haemocytes count has been successfully used as immune indicators in various crustacean groups including shrimps (Hai et al., 2009a, Van de Braak et al., 2002), crabs (Matozzo and Marin, 2010, Lorenzon et al., 2008), lobsters (Jussila et al., 1997, Fotedar et al., 2001) and crayfish (Johansson et al., 2000, Soderhall et al., 1984) including marron (Jussila et al., 1999, Sang et al., 2009). The variations in haemocyte number are mainly regulated by release from the haematopoietic tissue, perhaps complemented by storage and release of haemocytes at other sites (Johansson et al., 2000).

In marron, various THC have been observed in relation to culture conditions where the lowest (2.1 – 4.0 million CFU/mL) were observed in farmed and acclimated for 14 days in an aquarium (Evans et al., 1999, Jussila, 1997b) while highest THC of marron (9.7 – 10.7 million CFU/mL) were detected in marron kept in communal tank (Jussila, 1997b).

- ***Intestinal bacteria population***

It is widely accepted that digestion and immunity are complicated physiological processes that have co-evolved (Cerezuela et al., 2013, Liu et al., 2013). The microflora of the aquatic animal digestive tract plays a crucial role in digestion and metabolism (Ganguly and Prasad,

2012) and a source of various enzymes (Ray et al., 2012) and thus can be considered as a metabolically active organ (Gaggìa et al., 2010). The microbial population of gastro intestinal track (GIT) also determine vulnerability of the host to diseases (Ghanbari et al., 2015), therefore a reduction in either diversity or quantity of the GIT commensal microbiota is likely to reduce the effective barrier mechanism (Denev et al., 2009, Ige, 2013) which provide opportunistic pathogens to become established (Lorenzon et al., 2001, Ringø et al., 2010). GIT is one of the three main entries of pathogen invasions in aquatic animals, in addition to gill and skin (Ringø et al., 2003).

Contribution of intestinal bacteria population to the digestive process is not well investigated (Lazado et al., 2012) while its contribution to overall health status of the animal has been underestimated (Gómez et al., 2007). Bacterial population and diversity in the GIT is an important health component for aquatic animals (Gomez and Balcazar, 2008, Gaggìa et al., 2010) significantly affected by acute stress (Olsen et al., 2005). Manipulation of the gut microbiota through probiotic supplementation is a novel approach from a nutritional point of view (Nayak, 2010, Dimitroglou et al., 2011, Pirarat et al., 2011, Tapia-Paniagua et al., 2011) and relevant to aquaculture practices (Ghanbari et al., 2015).

The density of bacterial population in the GIT approximately 10^5 in small intestine to 10^7 in large intestine in adult fish species has been noted (Ringø et al., 2003), whereas Pérez et al. (2010) suggested that generally the fish GIT contains bacteria with an estimated $10^7 – 10^8$ cfu/g gut. To date, no information available on marron intestinal bacteria population except study by Sang and Fotedar (2010b).

- ***Hepatosomatic indices (Hiw)***

Another crucial digestive organ which plays a significant role in nutrient digestion is hepatopancreas (Thompson et al., 2010) as a source of digestive enzymes which important for nutrient digestions and absorption (Hammer et al., 2000) and as a storage organ for a large amount of energy in the form of lipids (Wang et al., 2014). In addition to its role in digestion, the hepatopancreas especially its epithelial cells are major sources of immune molecules (Rőszer, 2014) and as an immune defence organ in crayfish as demonstrated in red swamp crayfish, *Procambarus clarkii* (Shi et al., 2010). It also contains antioxidant defence enzymes as in spiny cheek crayfish *Orconectes limosus* (Borković et al., 2008).

The hepatopancreas has been used as an indicator of nutritional status in various crustaceans including crab *Portunus trituberculatus* (Wang et al., 2014), freshwater prawn *Macrobrachium rosenbergii* (Sureshkumar and Kurup, 1999), red swamp crayfish *Procambarus clarkii*, (Shi et al., 2010) and marron *Cherax cainii* (Austin, 2002) (Jussila, 1997a, Fotedar, 1998, Sang et al., 2011). Various hepatosomatic indices of marron have been reported in several studies. Jussila (1999) compared hepatosomatic index of marron at molt and post-molt at different feeding status and found that the lowest (3.8±0.2 %) hepatosomatic index (H_{iw}) of marron was observed in non-fed post-molt marron, followed by fed post-molt marron (5.4±0.3%) and the highest (5.6±0.3%) in fed-intermolt stage marron. Sang and Fotedar (2010a) observed H_{iw} of marron fed β-1,3 glucan supplemented diet ranged between 6.35 – 7.17 %.

- ***Growth***

In crayfish, specific growth rate (SGR) is recommended for growth measurement since it is based on an exponential growth trend as an increase weight with time which typically occurs in juveniles and early adult crayfish (Evans and Jussila, 1997).

There are three main factors of culture conditions which strongly affect marron growth namely temperature, nutrition and stocking density (Lawrence and Jones, 2002). Jussila and Evans (1996) examined factors affecting marron growth in intensive culture concluded that there was a positive correlation between SGR and sessile organisms production, dissolved oxygen and temperature but negative correlation between SGR, stocking density and diurnal temperature variation. In addition, in terms of diets indicated that low protein content (23%) in diets was not a limiting factor for growth and survival when compared to diets containing high (43.6%) protein, but water stable diets produced significantly higher specific growth rate than unstable diets (Jussila and Evans, 1998).

In ponds, marron may grow to between 60 to 150g within 12 months and between 100 to 300g within 24 months (Lawrence and Jones, 2002, Morrissy et al., 1990). Jussila (1997b) observed similar growth rates of marron intensive crayfish culture system as in semi-intensive marron ponds and experimental tanks (SGRs from 0.4 to 0.7).

- ***Survival***

The maximum production (a combination fast growth and high survival) is normally obtained in lower temperature than maximum growth (Jussila, 1997b). In marron, survival rate is strongly affected by stocking density and water temperature. Morrissy (1979) observed survival rate of marron ranged between 30.7-100% (average 80.5 %) over 4 months and was independent of density in the range 2-15/m^2. In outdoor tanks, survival rate of marron ranged from 12 to 31 % stocked at density of 4, 8 and 12/m2 (Kartamulia and Rouse, 1992).

In laboratory conditions, survival of marron fed with diets supplemented with β - 1,3 - glucan ranged from 65 to 90 % (Sang and Fotedar, 2010a), whereas survival of marron fed diets supplemented with organic selenium (OS) ranged from 70 to 90 % (Nugroho and Fotedar, 2013b).

CHAPTER 3. Isolation and screening of probiotic candidates from marron, *Cherax cainii* (Austin, 2002) gastrointestinal tract (GIT) and commercial probiotic products for the use in marron culture.

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3.1. Introduction

As antibiotic use in aquaculture has been widely criticized for negative impacts, including the potential to spread antibiotic resistance genes and undesirable antimicrobial residue in aquaculture products sold for human consumption, the use of probiotics is highly recommended as an alternative for disease control, and the beneficial health effects and safety for sustainable aquaculture have been reviewed by many authors (Gatesoupe, 1999; Verschuere et al., 2000; Irianto & Austin, 2002a; Austin, 2006; Balcázar et al., 2006; Gómez et al., 2007; Das et al., 2008; Sahu et al., 2008; Denev et al., 2009; Lara-Flores, 2011; Cruz et al., 2012; Pandiyana et al., 2013; Newaj-Fyzul et al., 2014) in particular animal groups such as shrimp (Bachere, 2000; Farzanfar, 2006; Ninawe & Selvin, 2009; Lakshmi et al., 2013), salmonids (Ringø et al., 1995; Merrifield et al., 2010), fish (Ringo, 1998; Austin, 2006; S. K. Nayak, 2010), bivalve (Prado et al., 2010), Atlantic cod (Lazado et al., 2014) in countries including China (Qi et al., 2009), Nigeria (Kolndadacha et al., 2011) and Egypt (Ibrahem, 2013).

Evaluation of the safety profile of a potential probiotic strain is of critical importance in the selection process; particularly resistance to common classes of antibiotics (Kesarcodi-Watson et al., 2008; Wang et al., 2008), as the cross-resistance to antimicrobials used in human medicine could pose a significant hazard to human health (Moriarty, 1997; Verschuere et al., 2000; Gómez et al., 2007).

The main purpose of probiotic application currently remains disease prevention (Kesarcodi-Watson et al., 2012), thus antagonism ability towards pathogens (Chythanya et al., 2002; Seehanat, 2005; Hai et al., 2007; Leyva-Madrigal et al., 2011; Ariole & Oha, 2013) is the main indicator when screening probiotics for aquaculture (Vine et al., 2004). *Vibrio mimicus* and *V. cholerae* non-01 are opportunistic bacteria that cause septicaemia in crayfish and tail blister in marron (Eaves & Ketterer, 1994; Wong et al., 1995; Evans & Edgerton, 2002),

therefore antagonistic ability towards these strains is important when screening a probiotic candidate for marron.

Merrifield et al., (2010) classify the probiotic screening criteria into essential criteria (non-pathogenic, antibiotic non-resistance, resistant to bile salts and low pH) and favourable criteria (antagonistic ability towards pathogens, enzyme production, and indigenous to the host). In spite of antagonism ability, Vine et al., (2004) suggested that other important criteria such as production of digestive enzymes (Ramirez & Dixon, 2003; Ziaei-Nejad et al., 2006; Lazado et al., 2012; Ray et al., 2012) should also be considered. In the present study, the probiotic candidates were evaluated for their susceptibility to antibiotics commonly used in aquaculture, their antagonism ability towards pathogenic bacteria in marron, and their ability to produce digestive enzymes.

3.2. Materials and Methods

3.2.1. Isolation, purification, identification and storage

Probiotic candidates used in this study were isolated from marron and commercial probiotic products. A number of healthy farmed marron were collected from farms and suppliers in Manjimup and Northcliffe, Western Australia, while commercial probiotics were supplied by Aquasonic Pty. Ltd and by Enviroplus Pty Ltd., Perth, Australia.

The intestine and hepatopancreas were removed aseptically, placed in a sterilized mortar and homogenised. Homogenised intestine and hepatopancreas were serially diluted with sterile normal saline and lawn inoculated on 5% horse blood agar (BA) plates and incubated for 24 h at 25^{o}C according to routine culture methods (Buller, 2004). Two predominant bacterial colonies from healthy marron were replated three times to ensure pure cultures were obtained.

All isolates were identified by the Bacteriology Laboratory, Animal Health Laboratories, Department of Agriculture and Food, Western Australia using matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometer (Bruker Bioscience Corporation), Vitek Compact II (Biomerieux) and conventional biochemical methods according to standard procedures and identification methods (Buller, 2004). After identification, all strains were suspended individually into 1 mL aliquots of GLL (Glycerol Lab Lemco broth) and stored at −80 °C for later use.

3.2.2. Antibiotic susceptibility test

The antibiotic susceptibility test was conducted according to Clinical and Laboratory Standard Procedure (CLSI) guidelines (CLSI M31-A3, 2008). In brief, 5 – 10 colonies of the 24 h BA grown strains were suspended in Lab Lemco solution prepared to a cell density of McFarland tube 1 opacity standard and lawn inoculated on to Mueller Hinton agar plates. The antibiotic disks were placed onto the plates and incubated at 25^{o}C for 24 h. The size of the zone (mm) of growth inhibition was measured and recorded to determine the susceptibility of the strains.

Nine antibiotics were used to evaluate the strains susceptibility: amoxycillin AML25, ampicillin AMP10, erythromycin E15, oxolinic acid OA2, oxytetracycline OT30, sulphamethoxazole RL100, compound sulphonamides S3 300, tetracycline TE30 and trimethaprim/sulphamethoxazole SXT25.

3.2.3. Pathogenicity feeding test

To determine the pathogenicity of the probiotic candidate on marron, a challenge test was performed in the laboratory by feeding the marron with the probiotic-supplemented diets. Seven diets were evaluated; a basal diet as a control and six diets supplemented with each probiotic candidate. Two 250 L recirculated culture system tanks used for each test diet were stocked with seven healthy marron (59.04 ±1.64 g). Basal marron diet contained 34% crude protein, 8% crude lipid, 6% ash supplemented with each strain of probiotic candidate as per described method (Hai & Fotedar, 2009; Ambas et al., 2013) at 10^{8} cfu/g (colony forming unit per gram) of feed and fed once per day at 2.5 % of the total biomass. Mortality and disease symptoms such as tail blister and shell colour were observed in each tank for up to 2 weeks. Marron were tested for response to stimuli to indicate morbidity.

3.2.4. Antagonism assay

Two antagonism assays performed in this study were the Well Diffusion Antagonism Assay (Schillinger & Lucke, 1989) and Spot on Lawn antagonism assay (Fleming et al., 1975).

- ***Well diffusion antagonism assay***

The well diffusion antagonism test was performed according to an established method described by Schillinger & Lucke (1989). In brief, isolates were grown in TSB for 24 and 72 h. The broths were centrifuged and filter sterilized. Wells 3 mm in diameter were punched into the agar plates, and 50 uL of the sterile filtrate was added into each well. A semisolid overlay was prepared from the bacterial pathogen cells in semisolid agar. *V. mimicus* and *V. cholerae* non-01 were suspended in sterile normal saline to a concentration of MacFarland standard tube 2 and 1 mL was added to 10 mL sterile semisolid agar, mixed thoroughly then overlaid onto the agar plates, and incubated overnight at 25^{o}C. A clear zone of growth inhibition surrounding the sterile filtrate spot indicated antagonism towards or growth inhibition of the pathogen.

- ***Spot on lawn antagonism assay***

The probiotic isolates were grown on BA plates for 24 h at 25^{o}C, and a suspension using sterile normal saline was prepared as per the well diffusion assay. Individual spots of 5 uL suspension were dispensed onto a nutrient agar (NA) plate then incubated at 25^{o}C for 24 h and 72 h. A layer of semisolid agar containing the pathogen as described for the well diffusion assay was applied as per the well diffusion assay. A clear zone surrounding the spot indicated antagonism towards the pathogen.

3.2.5. Digestive enzymes

- ***Production of protease***

The following substrates were prepared in agar plate media for the evaluation for production of protease: 1.5% insoluble elastin (EA), 1.5% soluble elastin (EAS) as per EA but using soluble bovine neck ligament (Sigma) as a source of soluble elastin, keratinase prepared with 1.5 g/L dried and ground chicken feathers prepared in agar, FMEAL (feather meal agar), gelatinase using agar containing 1.5% gelatine (GEL), and caseinase prepared with 0.75% skim milk powder (TASK plates).

Each isolate was grown on BA plates for 24h at 25^{0}C. A single colony from each isolate was diluted in a sterile tube filled with 3 mL of sterile distilled water. An aliquot (5 uL) was spotted onto each media plates, and incubated overnight at 25^{o}C before each plate was evaluated for the growth and a clear zone of lysis.

- ***Production of chitinase and amylase***

Chitinase activity was determined by the rapid filter paper method (O'Brien & Colwell, 1987). In brief, colonies from 24 h growth were smeared onto Whatman no.1 filter paper, and 20 uL of 4-MUF.Glc.Nac-buffered working solution was added to each smear then incubated for 10 minutes. One drop of saturated sodium bicarbonate solution was added to the smear and exposed to ultraviolet light wavelength 366nm. A bright blue fluorescence indicated a positive result. *Hafnia alvei* P00-1599#4 and *Aeromonas hydrophila* NCTC 7810 were used as positive controls.

Amylase activity of the strains was detected using starch agar plates purchased from Pathwest Laboratory, Perth, Western Australia and conducted according to the method described by Lennette et al., (1974). Overnight growth of each probiotic candidate was cultured in a single streak line to a starch plate and incubated at 25^{o}C for 24 h. The bacterial growth was flooded with lugol's iodine and amylase production was observed as a clear zone around the growth. A blue-black colour indicated the starch was not hydrolysed.

- ***API ZYM test***

To identify enzymes produced by the probiotic candidate isolates, the API ZYM test (Biomerieux) was performed. The test was conducted according to the manufacturer's instructions. In brief, pure colonies from BA plates were prepared in suspension using sterile distilled water to turbidity 5 or 6 of McFarland standard. A 65 uL of the suspension was dispensed into each cupule, covered with the plastic lid and incubated for 4 h at 37 ^{o}C. After incubation, one drop of ZYM A and ZYM B was added to each cupule and the colour reaction was allowed to develop over 5-10 minutes. The intensity of colour development was recorded according to the supplied colour chart.

3.3. Results

3.3.1. Isolation, purification, identification and storage

Two predominant bacteria from healthy marron were identified as *Bacillus mycoides* (A10) and *Shewanella* sp. *(*A12*)*. Strains from Enviroplus Pty Ltd product were identified as *Bacillus* sp. *(*PM1), *Bacillus subtilis* (PM3) and *Bacillus* sp. *(*PM4). *Bacillus* sp. (AQ) was indentified from Aquasonic Pty Ltd product.

These six species of bacteria have not been reported as known pathogens of marron and were selected for further testing and growth inhibition capabilities against *V. mimicus* and *V. cholera* non-01.

3.3.2. Antibiotic susceptibility test

Of the nine antibiotics evaluated in this study only PM3 and PM4 were susceptible to all the antibiotics. A10, A12 and AQ were resistant to ampicillin and amoxycillin (penicillin class), whereas PM1 was resistance to amoxycillin (penicillin class) and erythromychin (macrolide class) as detailed in Table 3.1.

Table 3.1. Antibiotic susceptibility of the probiotic candidates

	A10	A12	PM1	PM3	PM4	AQ
Amoxycillin AML25	R	R	S	S	S	R
Ampicillin AMP10	R	R	R	S	S	R
Erythromycin E15	S	S	R	S	S	S
Oxolinic Acid OA2	S	S	S	S	S	S
Oxytetracycline OT30	S	S	S	S	S	S
Sulphamethoxazole RL100	S	S	S	S	S	S
Compound Sulphonamides S3 300	S	S	S	S	S	S
Tetracycline TE30	S	S	S	S	S	S
Trimeth/Sulphamethoxazole SXT25	S	S	S	S	S	S

R = resistant
S = sensitive

3.3.3. Pathogenicity feeding test

After feeding marron with feed supplemented with each probiotic candidate for up to two weeks in tanks, all marron remained alive and no disease symptoms were detected. Individual investigation of the marron shell, particularly the tail, also confirmed the absence of any tail blister among marron fed diets supplemented with these strains, and the marron fed only with the control diet. All marron in each tank responded normally to stimuli and were aggressive to touch, showed active response to the feed, produced a reasonable amount of pelleted faeces and showed normal escape behaviour when approached.

3.3.4. Antagonism assay

- ***Well diffusion test antagonism assay***

A strong inhibition ability was shown by probiotic candidates PM4 and PM3 isolated from commercial products at 24 and 72 h, with increased inhibition by PM4 at 72h growth. A strain of host origin A10 (*Bacillus mycoides*) had a small zone of inhibition towards *V.*

mimicus at 24 h, but a large increased zone of inhibition after 72 h incubation, whereas at 72 h the zone of inhibition remained small for *V. cholerae* non-01. In contrast, A12 showed strong antagonism against *V. cholerae* non-01 at 72 h incubation, but remained weak against *V. mimicus* after 72 h of growth. PM1 showed a very weak antagonism against both *V. mimicus* and *V. cholerae* non-01 up to 72 h incubation, whereas AQ (*Bacillus* sp.) did not show any growth inhibition ability.

- ***Spot on lawn antagonism assay***

Antagonism ability of the probiotic candidates against *V. mimicus and V. cholerae* non-01 was also evaluated using the spot on lawn method. Similar to the well-diffusion method, PM4 and PM3 exhibited antagonism ability towards *V. mimicus and V. cholerae* non-01 at 24 h of growth, but greater antagonism ability was shown by PM4 at 72 h of incubation. A10 showed strong inhibition toward *V. mimicus* after 72 h, however towards *V. cholerae* non-01, the inhibition was unclear. Another host origin strain (A12, *Shewanella* sp.), on the other hand, inhibited *V. cholerae* non-01 strongly at 72 h of growth. Moreover, PM1 showed a very weak antagonism against both *V. mimicus and V. cholerae* non-01 at 72 h of growth, while AQ did not show any antagonism ability to both pathogens tested.

3.3.5. Digestive enzymes

- ***Production of protease***

Isolates from healthy marron (A10 and A12) showed no protease enzyme activity after 24 h and up to 72 h incubation. PM3 and PM4 degraded soluble elastin, gelatin and casein after 24 h and insoluble elastin after 72 h. AQ produced proteases that degraded soluble elastin, gelatin and casein, whereas PM1 only produced protease capable of degrading gelatin only.

- ***Production of chitinase and amylase***

The control strains *Hafnia alvei* P00-1599#4 and *Aeromonas hydrophila* were positive for chitinase, whereas all probiotic candidates were negative for chitinase. All probiotic candidates were also negative for amylase production.

- ***API ZYM test***

All 19 enzymes were detected in all candidate probiotic bacteria. The enzyme profiles varied between each strain in terms of detectable quantity. There was little difference in terms of

enzyme profiles between isolates from marron GIT (A10 and A12) compared to isolates from commercial probiotic products (PM1, PM3, PM4 and AQ).

3.3.6. Summary of the tested criteria

Based on the results from all evaluated criteria (susceptible to antibiotics, non-pathogenic to marron, inhibitory to growth of two major bacterial pathogens, production of enzymes that may assist in digestion of feed), the probiotic candidates were rated for their suitability as probiotics in marron culture. The results for each criterion were rated low to high (+ to +++) or nil, indicating their potential as probiotics. Probiotics PM3 and PM4 ranked higher scores in more criteria than the other probiotic candidates. A summary of the criteria evaluated for the probiotic candidates tested in the present study is presented in the Table 3.2.

Table 3.2. Summary of the criteria evaluated for the probiotic candidates.

Test criteria	A10	A12	PM1	PM3	PM4	AQ
Antibiotic susceptibility test	++	+	-	+++	+++	+
Pathogenicity feeding test	+++	+++	+++	+++	+++	+++
Antagonism assay	++	+	+	+++	+++	-
Production of protease	-	-	+	+++	+++	+++
Production of chitinase and amylase	+	+	+	+	+	+
API ZYM test	+++	++	++	+	+	+++

+++ = High potential
++ = Reasonable potential
+ = Less potential
- = Nil

3.4. Discussion

The safety profile, including the safety to the host, the environment and to humans, is proposed as an essential criterion when screening a probiotic candidate (Gomez-Gil et al., 2000; Verschuere et al., 2000; Gómez et al., 2007; Kesarcodi-Watson et al., 2008; Sahu et al., 2008; Wang et al., 2008; Merrifield et al., 2010; Sihag & Sharma, 2012) although other favourable criteria, such as antagonism ability towards pathogens and ability to produce digestive enzymes are also important (Vine et al., 2004; Merrifield et al., 2010; Lazado et al., 2012; Ray et al., 2012).

In the present study, evaluation of the safety of the probiotic candidates was tested using antibiotic susceptibility and pathogenicity feeding test. The antibiotic susceptibility test results indicated that bacteria from commercial products, *B. subtilis* (PM3) and *Bacillus*

species (PM4) were sensitive to all of the antibiotics tested; however, bacteria indigenous to the host *B. mycoides* (A10) and *Shewanella* sp. (A12) and a *Bacillus* species (AQ) from a commercial product showed resistance to amoxycillin AML25 and ampicillin AMP10, whereas another *Bacillus* sp. (PM1) from a commercial product was resistant to ampicillin AMP10 and erythromycin E15. The resistance of the candidates, especially PM1, towards two classes of antibiotics (penicillins and macrolides) indicate that this strain has less potential to be considered as a probiotic in marron culture, as there is a possibility that this resistance may be transferred to other bacteria. It is important that the probiotic candidate be free of plasmid-encoded antibiotic resistance genes, a major resistance mechanism found in the β-lactam antibiotics in the penicillin class (Kesarcodi-Watson et al., 2008; Wang et al., 2008; Dimitroglou et al., 2011). Plasmids carrying resistance factors have been transferred from fish bacterial pathogens to human pathogens including *Escherichia coli* (Angulo, 2000; Gómez et al., 2007). Antibiotic resistant bacteria transfer their resistance genes via plasmids or bacteriophages (Gomez-Gil et al., 2000) to other bacteria that have never been exposed to the antibiotics (Verschuere et al., 2000).

Pathogenicity tests using supplementation of each strain onto marron feeds resulted in no mortalities and no disease symptoms from bacteria isolated from the host (*B. mycoides* and *Shewanella* sp.) and commercial products. *B. mycoides* and *Shewanella* sp. dominated the gut flora from a number of healthy hosts, and the feeding trial confirmed their non-pathogenic status. A bacterium of host origin (autochthonous) is an ideal candidate if it produces favourable probiotic properties (Gatesoupe, 1999; Verschuere et al., 2000; Rollo et al., 2006; Merrifield et al., 2010) as their efficacy is likely to be highest in the host (Verschuere et al., 2000; O'Sullivan, 2001). The application of host origin probiotic strains has been successfully demonstrated in some aquatic species (Rengpipat et al., 2000; Irianto & Austin, 2002b; Hai et al., 2009a; Dimitroglou et al., 2011).

Supplementation of feed with strains isolated from commercial products *Bacillus subtilis* and *Bacillus* sp also caused no pathogenicity symptoms or mortality of marron. A number of *Bacillus* species have been used extensively as probiotics in aquaculture in the last decade (Verschuere et al., 2000; Balcázar et al., 2006; Ziaei-Nejad et al., 2006) in different aquatic animals including tiger shrimp *P. monodon* (Rengpipat et al., 2000), kuruma shrimp *P. japonicus* (Zhang et al., 2011), indian white shrimp *Fenneropenaeus indicus* (Ziaei-Nejad et al., 2006), rainbow trout *Oncorhynchus mykiss* (Irianto & Austin, 2002b), grouper

Epinephelus coioides (Sun et al., 2010) and freshwater prawn *Macrobrachium rosenbergii* (Keysami et al., 2007; Rahiman et al., 2010; Keysami et al., 2012).

Disease outbreaks are a major constraint for the aquaculture industry and thus the majority of probiotic screening studies are focused on antagonism ability against pathogens (Kesarcodi-Watson et al., 2008, 2012). In this study, all strains (except AQ) showed antagonism ability as measured by growth inhibition activity against *V. mimicus* and *V. cholerae* non-01, after 24 h incubation, and all bacterial strains (with the exception of *Bacillus* sp. AQ2) showed increased activity by 72 h incubation. The growth inhibition activity was greater at 72 h compared to 24 h incubation, indicating more antimicrobial compound was produced. Hai et al., (2007) evaluated various inhibition test methods and suggested that administering a suitable concentration of probiotic candidate and allowing growth and production of antimicrobial compounds before the addition of pathogens produced the best inhibition results.

Many studies have demonstrated that *Bacillus* sp. and *Bacillus subtilis* in particular, have antagonistic activity towards pathogens such as *V. harveyi* (Rengpipat et al., 2003), *V alginolyticus* (Zhang et al., 2011) and enhance the immunity of shrimp, *Litopenaeus vannamei* (Jiqiu et al., 2009; Tseng et al., 2009; Liu et al., 2010), *P. japonicus* (Zhang et al., 2011), *P. monodon* (Rengpipat et al., 2000), grouper *Epinephelus coioides* (Liu, 2012) and sea bream *Sparus aurata* (Cerezuela et al., 2013), indicating that the strains are able to exert their probiotic potential in different aquatic animals. *B. subtilis* can grow at a wider range of temperatures, pH and salt concentrations, is non-haemolytic, non-antibiotic resistant, non-pathogenic and inhibited all the primary and secondary pathogens tested (Nayak & Mukherjee, 2011).

Another predominant host origin bacterium evaluated in our study was a *Shewanella* sp (A12). This strain demonstrated growth inhibition ability towards *V. cholerae* non-01 and *V. mimicus*. This indicates that the strain is believed to possess several other beneficial effects for the host when supplemented onto the host feed as this strain also showed comparable enzyme profiles similar to other strains tested. In abalone, *Haliotis hannai*, Jiang et al., (2013) observed that supplementation of probiotic *Shewanella colwelliana* WA64 and *S. olleyana* WA65 demonstrated ability to improve cellular and humoral immune responses,

notably increased haemocyte numbers, respiratory burst activity, serum lysozyme activity and total protein levels of abalone after one week administration.

Digestion enzyme producing bacteria have been recommended as an important criterion when selecting a probiotic strain (Gatesoupe, 1999; Ramirez & Dixon, 2003; Balcázar et al., 2006; Merrifield et al., 2010; Ganguly & Prasad, 2012; Ray et al., 2012); however, nutritional effects of probiont bacteria, especially the effects of the bacteria on digestive enzyme activity, have been studied to some extent in finfish species but less so in crustaceans (Ziaei-Nejad et al., 2006). Enzymes important for digestion in fish include proteases, lipases, amylase, chitinase and cellulase (Ray et al., 2012). The present study demonstrated that all probiotic candidates produce a number of enzymes but in different concentrations. The isolates exhibited lipase enzyme activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), and β-glucosidase, which is involved in cellulose hydrolysis, and other enzymes such as leucine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and N-A-β-glucosaminidase.

Proteases are involved in the hydrolysis of proteins into smaller peptides which facilitates their digestion in the animal intestine. In this study, production of proteases was variable amongst the probiotic candidate strains, but proteases with activity against gelatin, casein and soluble elastin was produced by the *Bacillus* species (PM3, PM4, AQ), but not from the bacteria from the host bacterial flora (A10, A12). These results are in agreement with Ramirez and Dixon (2003) who found that the enzyme patterns varied with species of bacteria, and concluded that, in general, the gram positive isolates produced peptidases, while the Gram negative isolates produced carbohydrases.

Chitinase and amylase are regarded as enzymes important in digestion in fish; however, in the present study none of the strains produced chitinase, or amylase. Chitinase production by probiotic bacteria in aquaculture of crustaceans may not be ideal as bacterial chitinase is considered a virulence factor for hosts that contain exoskeleton chitin (Frederiksen et al., 2013). Therefore, probiotics that do not produce chitinase may be more suitable for marron aquaculture. The fact that no chitinase-producing bacteria were dominant in the GIT flora of marron suggests chitinase may not be involved in digestion. Chitinase negative reaction of *B. subtilis* was also found by O'Brien & Colwell (1987) in their study, whereas a positive reaction was found in several *Aeromonas* and *Vibrio* species. None of the predominant

bacteria from the marron GIT flora studied for probiotic potential produced amylase, which is surprising given that gut microorganisms are involved in the synthesis of amylase and cellulase (Bairagi et al., 2002) and that in *Cherax albidus*, Coccia et al., (2011) found amylase as the highest of the carbohydratases detected.

3.5. Conclusion

Based on criteria evaluated in this study (Table 3.2) bacteria with the highest potential for use as probiotics in order of those meeting the most criteria to those meeting the least criteria were; A10, PM3, PM4, A12 and AQ followed by PM1. The bacteria isolated from commercial products, *Bacillus subtilis* (PM3) and an unidentified *Bacillus* species (PM4) had the most potential as they met safety criteria including susceptibility to antibiotics, were non-pathogenic, demonstrated antagonism ability towards marron pathogens, and produced possible digestion enzymes. However, further *in vivo* tests are required to evaluate their ability to colonize and remain predominant in marron GIT, and subsequently exert their health beneficial effects on the host.

Chapter 4. Immunological responses of customized probiotics-fed marron, *Cherax cainii* (Austin, 2002) when challenged with *Vibrio mimicus*

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4.1. Introduction

Since the abuse of antibiotics and other chemicals for disease management in many aquaculture facilities were uncovered, safety in seafood products has received public attention. The research has shown that mismanagement of antibiotic use can lead to the emergence of bacterial resistant species (Verschuere et al. 2000). Consequently, the research now is more focused in finding an alternative to antibiotics for disease management. Recently, probiotics have emerged as an alternative to antibiotics for disease management (Farzanfar 2006; Zhou et al. 2009). The increasing demand for environment-friendly aquaculture has also led to probiotics becoming more popular as prophylactic agents and providers of improved nutrition (Li et al. 2009; Ninawe & Selvin 2009).

In recent years, studies in shrimp aquaculture have demonstrated that probiotics are beneficial for enhancing growth (Gullian et al. 2004; Balcázar et al. 2006b; Ziaei-Nejad et al. 2006; Gómez & Shen 2008) and the immune system (Perdigon & Alvarez ; Rengpipat et al. 1998; Rengpipat et al. 2000; Rengpipat et al. 2003; Jiqiu et al. 2006; Sahu et al. 2008), by combating the pathogens through competitive exclusion mechanism (Purivirojkul et al. 2005; Hai et al. 2009; Castex et al. 2010; Sun et al. 2010; Thompson et al. 2010). The use of probiotics has also resulted in improving water and sediment quality (Moriarty 1998; Wang et al. 2005; Balcázar et al. 2006a; Jiqiu et al. 2006) and thus, leading to higher survival rates of healthier animals (Gomez-Gil et al. 2000; Verschuere et al. 2000; Irianto & Austin 2002; Far et al. 2009). Some species of probiotics have the ability to protect against viruses, although the mechanism of combating the virus is not fully understood (Girones et al. 1989; Direkbusarakom et al. 1998; Balcázar et al. 2006b; Hai & Fotedar 2009). In aquaculture, probiotics can be applied either as a food supplement or as a water additive.

Marron, *Cherax cainii* (Austin, 2002) is one of the important freshwater crayfish species native to Western Australia. Marron have attracted global interest as a potential aquaculture species due to their positive attributes, such as large harvest-size (up to 2 kg), high price, non-burrowing behaviour, simple life cycle and ease of live transport (Morrissy 1979; Morrissy et al. 1990; Rouse & Kartamulia 1992; Lawrence & Jones 2002).

Though fungus and parasites are dominant disease causing agents in marron aquaculture, yet prevalence of infection and incidence of mortality are relatively low (Herbert 1987; Langdon 1991a). *Epistylis* and *Temnocephala* are two epibionts which are commonly found in marron, caused by poor water quality, particularly in unaerated ponds containing excessive organic matter (Lawrence & Jones 2002). These epibionts can decrease growth rates and reduce consumer appeal (Morrissy et al. 1990). Although there is no current report on the losses in marron aquaculture caused by bacterial infection, the threat of marron getting bacterial infection is likely as the industry grows and expands. *Vibrio mimicus* has emerged as a dominant bacterial pathogen of freshwater crayfish in aquaculture (Wong et al. 1995; Evans & Edgerton 2002).

The marron's health can ultimately be assessed by growth and accepted survival rates, however, other physiological indicators, such as organosomatic indices, moisture content and osmoregulatory capacity, total haemocyte counts (THC), proportion of granular cells, bacteraemia and haemolymph clotting time (Sang & Fotedar 2010), can also be used to understand the underlying mechanism for marron health. There has been no study on the effect of supplementation of probiotics in marron diets, therefore it is important to evaluate the effectiveness of dietary probiotics for the benefit of marron health under aquaculture environment.

The present study was designed to examine the effects of different sources of customized probiotic-supplemented feeds on the growth, survival, intermoult period, physiological, immune responses and bacteria load in the intestine of marron.

4.2. Materials and Methods

An experiment with two continuous phases was conducted. During the first phase, marron were fed different sources of probiotic supplemented diets and the second phase, involved the challenge test wherein, marron were injected with pathogenic bacteria *V. mimicus* under the laboratory conditions.

4.2.1. Experimental system

The experimental system was setup in a purpose-built laboratory designed for aquaculture research in the indoor aquarium facility of the Curtin Aquatic Research Laboratory (CARL), Curtin University, Perth, Western Australia. The experimental system consisted of three standing units of steel racks with three shelves in each unit. Each rack held six experimental units. The experimental units were cylindrical plastic tanks (80 cm diameter and 50 cm high and 250 L in capacity). The tanks were filled up with freshwater and supplied with constant aeration. Each tank was equipped with a submersible thermostat set to 24^{o}C and a re-circulating biological filtration system (Fluval 205, Askoll, Italy). The water in the tank was running continuously, at a rate of approximately 3 L/min. The tanks were also provided with sufficient marron shelters in the form PVC pipes of appropriate diameters.

4.2.2 Experimental animals

The marron juveniles (33 – 65 g) were purchased from Aquatic Resource Management Pty Ltd., Manjimup, Western Australia. Before commencement of the experiments, all juvenile healthy marron were kept for two weeks in holding tanks at CARL for acclimation. The holding tanks were provided with aerated recirculating filtered freshwater. A commercial pelleted diet (26% protein, 47- 50% carbohydrate, 9% fats and 8.9% ash) from Enviroplus Pty Ltd., Perth Australia was fed to marron, at a rate of 3% body weight on alternative days.

4.2.3. Test diets

During the experimental period, the marron were fed a basal diet (34% crude protein, 8% crude lipid, 6% ash), formulated at CARL. The feed ingredients were passed through a 100 µm mesh sieve and thoroughly mixed to obtain uniform particle size. The largest proportions of ingredients were mixed first before the smaller ones, to ensure all of the ingredients were mixed well. A mince mixer was then used to make pellets. The pellets were air dried, packed and stored at 4 ^{o}C until used.

Five species of probiotic bacteria isolated from the various sources were selected for their growth inhibition capabilities againts *Vibrio mimicus* and *V. cholera* non-01 and then tested for pathogenicity by feeding to marron in a tank trial (unpublished results). *Bacillus mycoides* (A10) and *Shewanella* sp. (A12) were selected from a number of healthy farmed marron intestines, *Bacillus* sp. (AQ2) was selected as a commercial product from Aquasonic Pty. Ltd

NSW Australia and finally *Bacillus subtilis* (PM3) and *Bacillus* sp. (PM4) were selected from another commercial probiotic product supplied by Enviroplus Pty Ltd., Perth Australia. All probiotics were identified by the Bacteriology Laboratory, Animal Health Laboratories, Department of Agriculture and Food, Western Australia. These selected probiotics have not been reported as known pathogens of marron. A basal diet without any probiotic supplementation was used as a control diet.

The probiotics were supplemented to the basal diet using a described procedure (Hai & Fotedar 2009), with some modifications. The isolated probiotics from stock culture were re-grown onto a new blood agar plate. After overnight incubation at 25^{o}C, an appropriate inoculum of each probiotic species was diluted into 20 mL of sterilized normal saline. Before being sprayed onto the basal diet, all feeds were coated with fish oil blend (Bait mate®, Western Australia) at 20 mL per kg basal diet. A probiotic concentration of 10^8 cfu/g of feed was selected following the previous studies (Keysami et al. 2007; Zhang et al. 2011; Keysami et al. 2012; Liu 2012). The probiotic species were sprayed onto 1 kg of basal diet (10^8 cfu/g feed) and then immediately covered with aluminium foil and stored in a refrigerator at 4^{o}C to avoid bacterial growth. The concentration (cfu/mL) of each probiotic bacterium sprayed onto the feed was determined using an established method (Hai et al. 2007) where optical density (Spectrophotometer, BOECO S-20, Hamburg, Germany) correlates to the bacterial concentration (cfu/mL). The concentration sprayed onto the feed was confirmed by performing a total bacterial count using blood agar plates and an overnight incubation at 25^{o}C.

4.2.4 Feeding the marron with different probiotics supplemented diets – *phase 1*

Eighteen 250 L cylindrical plastic tanks were used to culture marron in a laboratory scale experiment. Each tank was stocked with 7 healthy juvenile marron which were cultured for 70 days, a time considered optimal for studying the marron growth and effects of probiotics. The marron were fed with a basal (control) diet and probiotic-supplemented diets at a rate of 1.5% / body weight every alternate day during the experimental period. Each treatment was set up in triplicate. The effect of feeding treatments were measured in terms of growth, survival, intermoult period, physiological response (organosomatic indices), immune responses (total haemocyte count, differential haemocyte count and bacteraemia), bacteria load in the intestine and water quality parameters.

4.2.5 Challenge test with *Vibrio mimicus – phase 2*

The marron from phase 1 were used in the challenge test (phase 2). In six culture tanks, each tank was stocked with 3 marron and were fed at a rate of 1.5% / body weight every day during the challenge test. Uneaten food and faeces were removed before feeding.

Pathogen bacteria *Vibrio mimicus* (isolated from blisters of dead yabbies, *Cherax albidus*) was obtained from the Department of Agriculture and Food, Western Australia. A stock solution of 2.04 x 10^8 cfu/mL was prepared for the injection. All marron in all tanks were injected through the base of the fifth thoracic leg with 50 µl of *V. mimicus*. Three marron were injected with 50 µl of artificial saline water as a mock challenge test (control). The injected marron were kept in separate tanks before being put back into their original tanks to avoid repeating sampling. The infected marron were monitored for survival, total haemocyte count, differential haemocyte count and bacteraemia at 24, 48 and 96 h post- injection.

4.2.6. Data collection

- ***Feeding the marron with different probiotics supplemented diets – phase 1***

- Survival rate of marron was determined by the formula:
  Survival rate (%) = $(N_t/N_o)\times 100$,
  where N_t and N_o are the number of marron at the end (t) of the experiment and at the commencement (0), respectively.
- Marron biomass increment (BI) was measured by the following formula:
  BI (%) = [(final biomass – initial biomass)/initial biomass] x 100
- Specific Growth Rate (SGR) was measured by the following formula:
  SGR (%)/g/day = $100\times \ln W_t - \ln W_o/t$, where SGR is the specific growth rate in weight (% g/day), and Wt and Wo are the weight of marron at current time (t) and at the commencement of the experiment (0), respectively

Marron survival rate, specific growth rate (SGR) and biomass increment were determined after every 14 days. The presence of moults in tanks were checked daily and removed immediately. Intermoult period was determined as per established equation (Jussila & Evans 1996b; Reynolds 2002).

T_{im} (day) = T_{n+1} - T_n

Where T_n = date of n moult; and T_{n+1} = date of $_{n+1}$ moult

The wet hepatosomatic index (H_{iW}), tail muscle to wet body ratio (T/B_W), the percentage moisture of the hepatopancreas (HM%) and tail muscle (TM%), dry hepatopancreas index (Hid), wet tail muscle to wet body weight ratios (T/Bw) and dry tail muscle to wet body weight ratios (T/Bd) were calculated as per established equations (Jussila 1997; Fotedar 1998). Moisture content and organosomatic indices assessments were conducted at day 0, 35 and 70.

The immune parameters of marron, such as total haemocyte count (THC) and differential haemocyte counts (DHC) were determined following the established protocol (Jussila 1997; Sang et al. 2009). The haemolymph bacteria (bacteraemia) were assessed by using the established method (Hai & Fotedar 2009). All of the immune parameters were determined at commencement of the experiment (0 day), in the middle of the experiment (35^{th} day) and at the end of the experiment (70^{th} day).

The haemolymph bacteria (bacteraemia) were assessed using total bacterial counts on blood agar plates. Individual drops of the haemolymph 0.5 uL aliquot were placed onto blood agar plates and the lawn inoculated. For each marron, 3 drops of haemolymph were tested for bacteraemia rank. The plates were then incubated for 24 hours at 25°C and CFUs were determined for each drop. Total cfu/mL haemolymph was calculated on the basis of a total volume of 50 µL for each drop. The bacterial loads in the haemolymph were ranked from 1 (0 – 19 cfu/mL) to 10 (180-199 cfu/mL) and the rank 11 was used for "too numerous for an accurate count".

The bacterial load in the intestine (IBL) was determined following the protocol of Li et al., (2009), with some modification, on the initial (0 day), middle (35^{th} day) and at the end of the experiment (70^{th} day). The marron samples were selected and rinsed in distilled water, quickly washed with 70% alcohol and then rinsed again in sterilised distilled water to remove the external bacteria. The intestinal tract of each marron was removed and weighed. A sterilised mortar was used to homogenise the intestine, then diluted serially with sterilised normal saline water, and was lawn inoculated to blood agar plates. The plates were incubated for 24 hours at 25 °C. The bacterial loads were ranked from1 (1 – 250 cfu/mL) to 10 (2501-3000 cfu/mL) and the rank 11 was used for "too numerous for an accurate count".

To maintain good water quality in every tank, water exchange at a rate of 10-15 % of the total water volume was performed twice a week, after siphoning out the faeces and uneaten feed. Water quality parameters were measured weekly which included: total ammonia and nitrite which were measured using Calorimeter PR 1890, USA; temperature and pH using a digital pH/mV/^{o}C meter, Cyberscan pH300, Eutech instruments Singapore; and dissolved oxygen was measured using a digital DO meter SM600, Milwaukee, Romania.

- ***Challenge test with Vibrio mimicus – phase 2***

Total haemocyte count (THC) and differential haemocyte count (DHC) of the marron were determined before the *V. mimicus* injection and at 24 hours, 48 hours and 96 hours after the injection. Bacteria in marron haemolymph (bacteraemia) were counted before the *V. mimicus* injection and at 24 hours, 48 hours and 96 hours after the injection. The blood agar plates were used for culture of haemolymph bacteria. The bacterial loads were ranked from 1 (0 – 49 cfu/mL) to 10 (450 - 499 cfu/mL) and the rank 11 was used for "too numerous for an accurate count. The number of dead marron was recorded daily after the injection to determine accumulative mortality rate.

4.2.7 Data analysis

All collected data were analysed with SPSS statistical package version 18.0 (PASW version 18.0) for Windows and Microsoft Excel. The results were presented as means ± SE (standard error) and significant differences among treatment means were determined using one way ANOVA (analysis of variance) and Independent-Samples T test. The Tukey post hoc test was used for multiple mean comparisons when the *P* value showed significance. For ranking data (bacteraemia) and where the data were not normally distributed, the Kruskal-Wallis test and Mann Whitney U test were used. All significant tests were performed at $P<0.05$ level.

4.3. Results

4.3.1. Feeding the marron with different probiotics supplemented diets – *phase 1*

- *Intestinal bacteria load (IBL)*

The mean intestinal bacteria load (IBL) of marron fed different probiotic test diets were the same ($P > 0.05$) at the commencement of phase-1 (Fig 4.1). At day 35, the IBL was significantly different among the treatments ($P < 0.05$): the highest IBL was in the marron fed A10, while the lowest were in the control and PM4 dietary treatments. At the end of the trial,

means IBL were also significantly different (P<0.05) among marron fed different diets but, the marron fed A10 had the highest IBL and the marron fed the basal diet had the lowest rank.

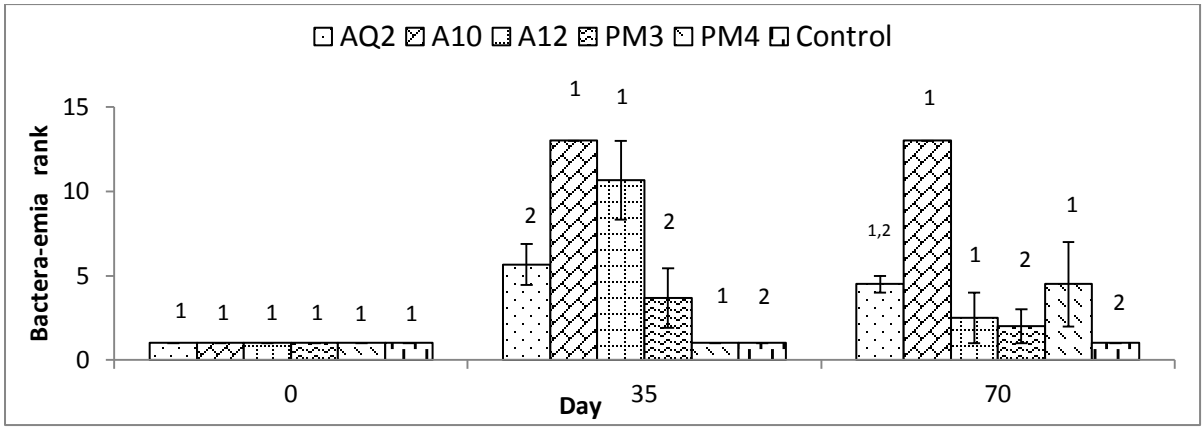

Fig.4.1. Mean ± SE of intestine bacteria load (IBL) of marron fed on different probiotic supplementation diets. Different numbers ($_{1, 2}$) over bars indicating significantly different means at different times at $P \leq 0.05$. Note: AQ2 (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); PM4 (*Bacillus* sp)

- ***Immune responses***

The mean THC, the mean proportion of granular cell (GC) and the mean haemolymph bacteria ranks (bacteraemia) of the marron fed different probiotic diets were not significantly different (P>0.05) until day 35 of feeding (Table 4.1). However, at the end of phase-1, the mean THC in all marron fed probiotic diets was significantly higher (P<0.05) than the control diet. The highest mean THC was for the A10 diet and the lowest was for the marron fed the control diet, while other treatments had similar levels of THC. At the end of day 70, the mean GC was significantly higher (P < 0.05) in the marron fed AQ2, A10 and PM4 supplemented diets. The highest proportion of GC was associated with the PM4 supplemented diet (35.54± 4.79 %) and the lowest one was with the control diet (10.64±0.60%). At the end of phase-1, the mean bacteraemia was significantly lower (P<0.05) in the marron fed probiotic supplemented (PM3, A12, PM4, AQ2) and control diet compared to marron fed A10 diet. The bacteraemia of PM3-treated marron decreased gradually over the culture period, while bacteraemia of marron fed other diets fluctuated during phase 1 of the trial.

Table 4.1. Mean ± SE of marron immune parameters fed probiotic supplemented diets.

Immune parameter	Day	Treatment					
		AQ2	A10	A12	PM3	PM4	Control
Total Haemocyte Count	0	$_{1}$0.84±0.00^{a}	$_{1}$0.84±0.00^{a}	$_{1}$0.84±0.00^{a}	$_{1}$0.84±0.00^{a}	$_{1}$0.84±0.00^{a}	$_{1}$0.84±0.00^{a}
	35	$_{2}$10.17±3.57^{a}	$_{2}$12.25±2.46^{a}	$_{2}$11.79±2.81^{a}	$_{2}$10.65±1.45^{a}	$_{2}$10.13±1.55^{a}	$_{1}$7.96±0.88^{a}
	70	$_{2}$14.47±0.46^{ab}	$_{2}$15.80±1.30^{b}	$_{2}$9.81±1.32^{ab}	$_{2}$11.43±0.78^{ab}	$_{2}$11.06±1.72^{ab}	$_{1}$7.73±3.097^{a}
Granulocyte Cell (%)	0	$_{3}$49.96±4.77^{a}	$_{3}$49.96±4.77^{a}	$_{3}$49.96±4.77^{a}	$_{3}$49.96±4.77^{a}	$_{3}$49.96±4.77^{a}	$_{3}$49.96±4.77^{a}
	35	$_{3}$49.96±4.77^{a}	$_{12}$37.10±8.60^{a}	$_{12}$31.16±7.02^{a}	$_{2}$31.44±3.68^{a}	$_{1}$37.77±2.28^{a}	$_{2}$23.31±1.73^{a}
	70	$_{1}$20.14±4.22^{ab}	$_{1}$24.85.99±5.49^{ab}	$_{1}$14.19±5.25^{a}	$_{1}$13.39±2.70^{a}	$_{1}$35.54±4.79^{b}	$_{1}$10.64±0.60^{a}
Haemolymph bacteria (Bacteraemia)	0	$_{2}$5.00±0.0 0^{a}	$_{1}$5.00±0.0 0^{a}	$_{1}$5.00±0.0 0^{a}	$_{2}$5.00±0.0 0^{a}	$_{2}$5.00±0.0 0^{a}	$_{1}$5.00±0.0 0^{a}
	35	$_{1}$5.67±1.20^{a}	$_{1}$13.00±0.0 0^{b}	$_{1}$10.67±2.3^{b}	$_{1}$3.67±1..76^{a}	$_{2}$1.00±0.00^{a}	$_{1}$1.00±0.00^{a}
	70	$_{1}$4.50±0.50^{a}	$_{1}$13.00±0.00^{b}	$_{1}$2.50±1.50^{a}	$_{1}$2.00±1.00^{a}	$_{1}$4.50±2.50^{a}	$_{1}$1.00±0.00^{a}

Data in the same column within an index having different subscript numbers (1, 2) are significantly different at α level of 0.05. Data in the same row having the same superscript letters (a,b) indicate a similar mean which is not significantly different at α level of 0.05. Note: AQ2 (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); PM4 (*Bacillus* sp).

- ***Organosomatic indices***

There were no significant differences ($P > 0.05$) in HM%, TM%, H_{iw}, H_{id}, T_{iw} and T_{id} among any marron fed probiotic supplemented diets over any sampling periods (Table 4.2). However, marron fed AQ2 showed a significantly higher ($P<0.05$) in T_{iw} and T_{id} at day 35 than day 0.

Table 4.2. Hepatopancreas moisture content (HM%), tail muscle moisture content (TM%), wet hepatosomatic index (H_{iw}), dry hepatosomatic index (H_{id}), wet tail muscle indices (T_{iw}) and dry tail muscle indices (T_{id}) of marron with different species of probiotic bacteria supplementation .

Index	Day	Treatment					
		AQ2	A10	A12	PM3	PM4	Control
HM%	0	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16
	35	$_{1}$62.53±1.46^{a}	$_{1}$62.05±5.38^{a}	$_{1}$65.33±6.48^{a}	$_{1}$62.76±5.61^{a}	$_{1}$68.42±5.54^{a}	$_{1}$75.16±7.07^{a}
	70	$_{1}$58.44±1.30^{a}	$_{1}$63.16±5.40^{a}	$_{1}$64.17±3.28^{a}	$_{1}$65.11±3.92^{a}	$_{1}$61.44±1.38^{a}	$_{1}$70.79±6.26^{a}
TM%	0	$_{2}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30
	35	$_{1}$79.13±0.26^{a}	$_{1}$82.00±2.193^{a}	$_{1}$79.55±0.88^{a}	$_{1}$79.47±0.90^{a}	$_{1}$80.57±0.727^{a}	$_{1}$80.82±0.41^{a}
	70	$_{1}$78.74±0.19^{a}	$_{1}$79.34±0.83^{a}	$_{1}$80.36±1.13^{a}	$_{1}$86.46±6.36^{a}	$_{1}$79.15±0.09^{a}	$_{1}$80.67±0.94^{a}
Hiw	0	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{12}$6.06±1.10
	35	$_{1}$7.83±0.87^{a}	$_{1}$6.99±0.64^{a}	$_{1}$5.87±0.85^{a}	$_{1}$6.73±1.29^{a}	$_{1}$6.70±1.27^{a}	$_{1}$5.50±0.51^{a}
	70	$_{1}$5.86±0.68^{a}	$_{1}$6.30±0.66^{a}	$_{1}$6.17±0.65^{a}	$_{1}$6.72±0.50^{a}	$_{1}$6.44±0.81^{a}	$_{1}$6.15±0.91^{a}
Hid	0	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49
	35	$_{1}$2.96±0.43^{a}	$_{1}$2.72±0.63^{a}	$_{1}$1.94±0.223^{a}	$_{1}$2.65±0.81^{a}	$_{1}$2.26±0.84^{a}	$_{1}$1.39±0.48^{a}
	70	$_{1}$2.76±0.17^{a}	$_{1}$2.34±0.46^{a}	$_{1}$2.19±0.19^{a}	$_{1}$2.32±0.13^{a}	$_{1}$2.50±0.29^{a}	$_{1}$2.29±0.88^{a}
Tiw	0	$_{1,2}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35
	35	$_{2}$29.69±0.91^{a}	$_{1}$35.32±6.51^{a}	$_{1}$28.60±0.77^{a}	$_{1}$28.04±0.54^{a}	$_{1}$27.21±1.51^{a}	$_{1}$27.43±1.21^{a}
	70	$_{1}$25.80±1.04^{a}	$_{1}$25.42±1.07^{a}	$_{1}$28.58±2.55^{a}	$_{1}$24.90±3.00^{a}	$_{1}$27.96±0.94^{a}	$_{1}$27.64±1.09^{a}
Tid	0	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}
	35	$_{2}$6.20±0.26^{a}	$_{1}$6.07±0.26^{a}	$_{1}$5.84±0.17^{a}	$_{1}$5.75±0.23^{a}	$_{1}$5.27±0.24^{a}	$_{1}$5.27±0.32^{a}
	70	$_{12}$5.49±0.25^{a}	$_{1}$5.24±0.12^{a}	$_{1}$5.64±0.72^{a}	$_{1}$3.72±1.86^{a}	$_{1}$5.83±0.18^{a}	$_{1}$5.36±0.45^{a}

Data in the same column within an index having different subscript letters ($_{1, 2}$) are significantly different at α level of 0.05. Data in the same row having the same superscript letters (a,b) indicate not significantly different at α level of 0.05. AQ2 (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); PM4 (*Bacillus* sp).

- ***Survival rate, growth, intermoult period and water quality***

Survival rate of marron at the termination of the experiment ranged between 33.33 ± 4.76 % and 61.90 ± 9.52 %. The mean biomass increment (BI %) ranged between 4.79 ± 2.42 % and 9.68 ± 4.24 %, while specific growth rate (SGR) varied from 0.27 ± 0.09% to 0.51 ± 0.35%. There was no significant difference ($P > 0.05$) in the mean survival rates, mean biomass increment (BI %) and specific growth rate (SGR) in marron fed any diets. In addition, intermoult period (days) ranged between 25.25 ± 8.50 to 43.00 ± 1.00 and no significant difference ($P > 0.05$) between marron fed different diet. Water quality parameters in all tanks were within the range for optimum growth of marron.

4.3.2 Challenge test with *Vibrio mimicus – phase 2*

- ***Bacteraemia***

The mean bacteraemia ranks of the marron fed probiotic-supplemented diets were significantly lower than the marron fed control diet. At 24 h post-injection with *V. mimicus*, there were significant increases ($P <0.05$) of bacteraemia ranks in all treatments. However, there was no significant difference ($P >0.05$) of bacteraemia 24 h post *V. mimicus* injection. These bacteraemia values remained similar to 48 h post-injection.

After 96 h, there was a significant decrease ($P <0.05$) of the bacteraemia ranks of marron fed AQ2, A10 and PM4. There was also a significant difference ($P <0.05$) in bacteraemia ranks among treatments, of which the lowest bacteraemia was observed in marron fed A10 and PM4 supplemented diets. However, AQ2 and PM3 diets produced similar ranks as the control diet.

- ***Total haemocyte count (THC)***

The mean THC of marron were not significantly different among any dietary treatments before the *V. mimicus* challenge. After *V. mimicus* injection, there were significant decreases ($P <0.05$) in the mean THC in all treatments at 24 hours, 48 hours and 96 hours. At 24 hours post injection, the THC of marron fed probiotic-supplemented diets were significantly higher ($P<0.05$) than those fed the control diet. The marron fed the diet supplemented with PM3 showed the highest THC ($1.53\pm0.35 \cdot \times 10^6$ cells/mL), whereas the marron fed the control diet had the lowest THC ($0.36\pm0.07 \times 10^6$ cells/mL). After 48 hours into the challenge, the THC of marron fed probiotic-supplemented diets were significantly higher ($P<0.05$) in marron fed

A10, A12 and PM4, whereas the other dietary treatments showed similar values as the control. After 96 hours of *V. mimicus* injection, A10, PM3 and PM4 treatments showed significant differences (P <0.05) than the control. Whereas, other probiotic fed marron had similar mean THC to those of the control.

- ***Differential Haemocyte Count (DHC)***

Before the *V. mimicus* was injected, the mean proportions of granular cells (GC) of marron fed the probiotic-supplemented diets were significantly different (P < 0.05) than the control. The PM4 treatment produced the highest GC proportion, followed by the A10 treatment (Table 4.3). Other treatments had a similar proportion of GC as the control. After *V. mimicus* injection, marron exhibited a declining trend (P <0.05) in the mean GC proportions in all treatments. At 24 hours post-injection, there was a significant difference (P<0.05) among treatments, with the PM3 producing the highest GC proportion and the control showing the lowest. At 48 hours into the challenge, there was a significantly higher (P <0.05) GC proportion for A12 and PM4 supplemented diets than others. At 96 hours, the GC proportion of marron fed probiotic-supplemented diets were significantly higher (P<0.05) than the marron fed control diet.

Table 4.3. Mean ± SE of marron immune parameters when challenged with *V. mimicus*

Immune parameters	Hour	Treatment					
		AQ2	A10	A12	PM3	PM4	Control
Total Haemocyte Count (million cfu/mL)	0	$_{1}$10.10±3.62 ^{a}	$_{1}$11.10±2.25 ^{a}	$_{1}$10.54±2.46 ^{a}	$_{1}$10.31±1.35 ^{a}	$_{1}$9.77±1.49 ^{a}	$_{1}$8.04±1.67 ^{a}
	24	$_{2}$1.25±0.10 ^{ab}	$_{2}$0.87±0.01 ^{ab}	$_{2}$1.09±0.08 ^{ab}	$_{2}$1.53±0.35 ^{b}	$_{2}$1.10±0.08 ^{ab}	$_{2}$0.36±007 ^{a}
	48	$_{2}$1.19±0.35 ^{ab}	$_{2}$0.82±0.18 ^{ab}	$_{2}$0.87±0.13 ^{ab}	$_{2}$1.36±0.40 ^{b}	$_{2}$1.00±0.05 ^{ab}	$_{2}$0.22±0.05 ^{a}
	96	$_{2}$1.19±0.19 ^{ab}	$_{2}$0.61±0.01 ^{a}	$_{2}$0.81±0.16 ^{ab}	$_{2}$1.34±0.15 ^{b}	$_{2}$1.49±0. 07 ^{b}	$_{2}$0.53±0.07 ^{a}
Granulocyte Cell (%)	0	$_{1}$20.14±4.22 ^{ab}	$_{1}$24.65±5.58 ^{ab}	$_{1}$14.19±5.24 ^{a}	$_{12}$13.39±4.67 ^{a}	$_{2}$35.54±4.79 ^{b}	$_{1}$10.64±0.60 ^{a}
	24	$_{1}$15.98±4.91 ^{a}	$_{1}$18.35±0.21 ^{a}	$_{1}$17.90±3.31 ^{a}	$_{2}$26.10±0.66 ^{a}	$_{12}$24.93±6.53 ^{a}	$_{1}$9.21±0.12 ^{a}
	48	$_{1}$7.98±4.36 ^{ab}	$_{1}$8.37±2.09 ^{ab}	$_{1}$9.52±0.78 ^{ab}	$_{1}$5.65±1.17 ^{ab}	$_{12}$16.73±3.10 ^{b}	$_{1}$3.06±0.19 ^{a}
	96	$_{1}$10.95±1.64 ^{b}	$_{1}$6.46±0.12 ^{ab}	$_{1}$9.36±1.25 ^{b}	$_{1}$8.98±2.04 ^{b}	$_{1}$8.13±0.57 ^{ab}	$_{1}$1.59±0.43 ^{a}
Haemolymph Bacteria (Bacteraemia)	0	$_{1}$1.00±0.00 ^{a}	$_{1}$1.33±0.57 ^{a}	$_{1}$1.67±0.33 ^{a}	$_{1}$1.00±0.00 ^{a}	$_{1}$1.00±0.00 ^{a}	$_{1}$3.67±0.67 ^{b}
	24	$_{3}$10.00±1.00 ^{a}	$_{3}$9.50±1.50 ^{a}	$_{2}$11.00±0.00 ^{a}	$_{3}$11.00±0.00 ^{a}	$_{1}$6.50±4.50 ^{a}	$_{2}$11.00±0.00 ^{a}
	48	$_{3}$10.00±1.00 ^{a}	$_{23}$6.00±1.00 ^{a}	$_{2}$11.00±0.00 ^{a}	$_{2}$8.00±1.00 ^{a}	$_{1}$7.50±1.50 ^{a}	$_{2}$9.50±0.50 ^{a}
	96	$_{2}$6.00±1.00 ^{ab}	$_{12}$2.50±0.50 ^{a}	$_{2}$10.00±1.00 ^{b}	$_{2}$7.50±0.50 ^{b}	$_{1}$2.50±0.50 ^{a}	$_{2}$10.00±1.00 ^{b}

Data in the same column within an index having different subscript letters ($_{1, 2}$) are significantly different at α level of 0.05. Data in the same row having the same superscript letter indicate a similar mean which is not significantly different at α level of 0.05. Note: AQ2 (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); PM4 (*Bacillus* sp)

- ***Survival Rate***

At 96 h post-injection, all marron injected with *V. mimicus* and those injected only with normal saline water remained alive and started to respond to the feed given on day 4. The

normal saline injected marron, however, remained actively responsive to the feed given earlier than bacteria injected marron.

4.4. Discussion

Hepatopancreas and tail muscle indices have been widely used as tools to monitor the effects of different culture environments and diets on penaeid (Sang & Fotedar 2004; Tantulo & Fotedar 2006; Prangnell & Fotedar 2006a, 2006b; Tantulo & Fotedar 2007) and non-penaeid shrimps (Jussila 1997; Fotedar 1998). The present study showed that probiotic supplementation of *Bacillus sp.* on a marron diet did improve the physiological condition of marron, as shown by the changes in dry tail index (T_{id}). The significantly higher T_{id} observed in marron fed a diet with *Bacillus* sp. indicates that this probiotic bacteria helped the marron to absorb nutrients more efficiently and thus, they could store more energy in their tail muscle (Gullian *et al.* 2004). Similarly, probiotic bacteria in the formulated shrimp diet, act as a facilitator to digest all of the protein components, which in turn synthesises required enzymes responsible for increasing the shrimp's protease activity and food digestibility (Ochoa-Solano & Olmos-Soto 2006).

Haemocytes perform a key role in the host-defence mechanism of crustaceans by destroying micro-organic invaders (Langdon 1991a; Soderhall & Soderhall 2002). Environmental conditions, such as the presence of stressors and disease, have a relationship with the number of circulating haemocytes in crayfish (Soderhall et al. 1988). A poor health condition in crayfish can be reflected by a low number of total haemocyte counts in their haemolymph (Jussila 1997). The results from this study showed that marron fed probiotic diets had higher THC counts than those fed the control diet; this indicates that marron are healthier when they are fed a probiotic supplemented diet. The THC of the western king prawn, *Penaeus latisulcatus* also increased gradually when they were treated with two selected probiotics (*Pseudomonas synxantha* and *Pseudomonas aeruginosa*) (Hai et al. 2009b). Administration of *Bacillus licheniformis* in the *Litopenaeus vannamei* diet enabled an increase in the THC (Li et al. 2007). In the present study, *Bacillus mycoides* produced the highest THC, demonstrating its higher effectiveness than other probiotics.

After being injected with *V. mimicus*, the THC decreased sharply. Similar results has been reported in Chinese shrimp *Fenneropenaeus chinensis* injected with *Vibrio anguillarum* (Yao

C-L et al. 2008). Another study also found the THC of *V. mimicus* injected marron declined (Sang et al. 2009). Environmental stressors and diseases can influence the number of circulating haemocytes (Soderhall et al. 1988; Jussila 1997; Le Moullac G & Haffner 2000). The decrease in THC after *V. mimicus* injection, could be associated with an inflammatory response of the haemocytes leaving the circulation and migrating to the site of the injection (Omori et al. 1989; Van de Braak et al. 2002). However, the THC of marron fed probiotic diets remained higher than those fed the probiotic-free diet during the challenge test. Similar results have been reported in white shrimp *Litopenaeus vannamei* when challenged with *V. harveyi,* after administration of probiotic bacteria (*Lactobacillus plantarum*) (Vieira et al. 2008). The higher THC of the marron fed a probiotic-supplemented diet in the present study indicates the greater ability of the marron haemocytes in the degranulation process to fight against the foreign substance. Among the probiotics diet, PM4 (*Bacillus* sp.) resulted in the highest THC. *Bacillus* sp. is known as an effective probiotic bacteria in shrimp aquaculture and has been studied for its effectiveness by many researchers, under both laboratory and commercial trials (Rengpipat *et al.* 1998; Gomez-Gil *et al.* 2000; Balcázar *et al.* 2006b; Li *et al.* 2007; Rahiman *et al.* 2010)

In the present study, the marron diets with probiotic supplementation of AQ2 (*Bacillus* sp.), A10 (*Bacillus mycoides*) and PM4 (*Bacillus* sp.) produced significantly higher proportions of granular cell in marron haemolymph than the control diet. Therefore, indicating a positive effect of the probiotic, *Bacillus spp* in enhancing the health condition of these marron (Jussila 1997; Fotedar S et al. 2001). After *V. mimicus* injection, the proportions of granular cells in marron were decreased in all treatments. Similar results were also reported where an injection of 20 uL of 0.53 ± 10^6 cfu/mL *V. mimicus* reduced the proportion of GC (Sang et al. 2009). In other species the reduction of GC proportion have been reported in black tiger shrimp (*Penaeus monodon*) (Van de Braak et al. 2002) and Chinese shrimp (*Fenneropenaeus chinensis*) (Yao C-L et al. 2008) when they were injected with *Vibrio anguillarum*. A decreasing GC proportion suggests that GC degranulates at first, followed by lysis of the lysosomes (Yao C-L et al. 2008). Nevertheless, the proportion of GC of marron fed with probiotic diets remained higher than GC of marron fed probiotic-free diet (control) during the challenge test. Similar results have been reported for western king prawn *(Penaeus latisulcatus)* when challenged with *Vibrio harveyi* at 10^3, 10^5, and 10^7 cfu/mL (Hai et al. 2010). The marron fed the diets supplemented with genus Bacillus (AQ2, PM3 and PM4) and

then challenged with high dosage of *V. mimicus* had higher GC proportions than other treatments. Similar to shrimps, Bacillus surface antigens, or their metabolites, act as immunogens by stimulating phagocytic activity of granulocytes (Itami et al. 1998).

The bacteraemia of marron fed probiotic supplementation in their diets was lower than the marron fed control diet. The probiotics act as immunostimulants to stimulate the non-specific immune system against bacterial infection (Rengpipat *et al.* 2000; Balcázar *et al.* 2006b; Kumar R *et al.* 2008). PM3 (*Bacillus subtilis*) and PM4 (*Bacillus* sp) supplementations had the lowest haemolymph bacteraemia. Probiotic bacteria *Bacillus,* not only competes for nutrients and thus inhibits other bacteria from growing rapidly (Ninawe & Selvin 2009), they naturally are also able to produce many different antibiotic compounds (Moriarty 1998). At 24 and 48 h post injection with *V. mimicus,* the bacteraemia values increased sharply in all treatments. Similar results has been reported wherein bacteraemia of marron fed the control diets (without immunostimulant Bio-Mos^{T}) increased significantly 24 and 48 h post *V. mimicus* injection (Sang et al. 2009). However, the bacteraemia of marron fed probiotic-supplemented diets had lower levels of bacteraemia than the control diet after 96 hours. This indicates that the probiotics were effective in reducing the bacterial load in the marron haemolymph. In western king prawn, administration of two combined probiotics, *Pseudomonas synxantha* and *Pseudomonas aeruginosa* (10^5 cfu/mL), at 20 mL/kg feed, reduced the number of bacteria in the haemolymph (Hai & Fotedar 2009; Hai et al. 2009b). The increase bacteraemia levels in haemolymph indicates that the immune capacity of the animal has declined and thus, possibly can result in increased susceptibility to infections (Fotedar et al. 2001).

The average survival rate of the marron in the feeding trial (phase 1) was 46.03% of the initial stocking biomass of approximately 103.82 g/m^2. There was no evidence to prove that supplementation of the probiotics in marron diets improved marron survival rate. However, this rate is still higher than marron cultured in the earthen ponds, where the survival of marron ranges from 13.82% to 34.66% with a lower stocking density (4.01 ± 0.28 g/m^2) (Sang & Fotedar 2004). When beta 1,3 β glucan is used as an immunostimulant, more than 65% of the initial stocking biomass (16.83 g/m^2) of marron can be achieved (Sang & Fotedar 2010). In an intensive battery culture system the survival rate can also be higher (71%) than the present trial, due to absence of cannibalism (Jussila & Evans 1996a). In the present study,

the average survival was relatively low as marron mortality was mainly caused by cannibalism. Cannibalism frequently occurs when a population of crayfish is at high density and it is often triggered by moulting individuals (Nystrom 2002).

In shrimp aquaculture, the application of probiotics, either through mixing into the water or supplementing with feed, has been reported to increase the survival rate (Rengpipat et al. 1998; Balcázar et al. 2006b; Ziaei-Nejad et al. 2006; Gómez & Shen 2008; Hai & Fotedar 2009; Zhou et al. 2009; Hai et al. 2009b; Rahiman et al. 2010). The injection of V. mimicus did not affect the survival of marron (59.04 ± 1.64 g initial mean weight). However, it took a few days for marron to recover and resume its response to the feed given. Different results have been reported where 20 uL of 0.53 x 10^6 cfu/mL of *V. mimicus* stock solution injected into marron (10.44±0.20 g initial mean weight) resulted in 100% mortalities in marron (Sang *et al.* 2009). The different results in the present experiment could be attributed to the low dosage of injected *V. mimicus* per unit weight of the marron, as the mean weight of the marron was five times higher than those used by other authors (Sang et al. 2009).

Injection of *V. alginolyticus* into white shrimp (*Litopenaeus vannamei*) at dosages of 10^5 and 10^6 cfu/shrimp could produce survival rate of 40 – 50% (Liu C-H et al. 2004; Hsieh S-L et al. 2008). *P. monodon* mean weight of 18g survived for 6 days after challenged by bath exposure to 10^7 cfu/mL of *V. harveyi* 1526 in aquarium water (Rengpipat et al. 2003), whereas, when challenged with 40 – 200 x 10^6 cfu of *V. anguillarum* at body weight of 2 – 4 g, *P. monodon* survived for a week (Van de Braak et al. 2002). Furthermore, the isolates of *V. mimicus a*t a dosage of 10^5 cfu, can cause high mortalities in *Cherax albidus* (Wong et al. 1995). This comparison shows that the survival of injected animals is dependent on species of the pathogenic bacteria, the mean weight of the host species and the virulent effects of the pathogen, rather than numbers of bacteria. Hence, in considering the resistance of marron against *V. mimicus*, the virulent effect and the size of marron need to be considered to explain the effects of a probiotic on marron survival.

Marron fed probiotic-supplemented diet in the present study did not show any growth improvement or biomass increment, although supplementation has been reported to increase the growth in other cultured shrimps (Ziaei-Nejad et al. 2006; Balcázar et al. 2007; Wang Y-B 2007; Gómez & Shen 2008). The average SGR in the present experiment ranged from 0.13

to 0.66, with moult interval ranging between 25.5 to 43 days. The average SGR of marron was different in various culture conditions; pond culture studies range from 0.5 – 2.1 and intensive crayfish culture system studies range from 0.4 – 1.1 (Evans & Jussila 1997). The intermoult period in juvenile marron ranged between 15 to 45 days (Morrissy 1984; Jussila 1997). The presence of probiotic bacteria in shrimp intestine can increase the health status of the host by providing competitive exclusion in the shrimp's gut (Rengpipat et al. 1998; Rengpipat et al. 2000). Moreover, probiotics may synthesise the vitamins that can lead to increased digestive activity or improve enzymatic activities; therefore, this could be responsible for weight increase, improved digestion and/or nutrient absorption (Gullian et al. 2004).

The present study showed that marron fed probiotics had higher intestinal bacteria ranks than those fed the control diet, especially diet supplemented with *Bacillus mycoides*. Though our studies could not confirm whether higher bacterial load in the intestine of marron are due to increased densities of probiotic species, other studies have reported that the administration of probiotic *Bacillus licheniformis* can improve the white shrimp's intestinal micro-flora and its immunity by colonizing and replacing pathogenic bacteria in their intestine (Rengpipat et al. 1998; Li et al. 2007).

Table 4.4. Matrix of comparison between five probiotic diets using various parameters

Parameters	*AQ*	*A10*	*A12*	*PM3*	*PM4*
Organosomatic index	+	-	-	-	-
Biomass increment (%)	-	-	-	-	-
Specific Growth Rate	-	-	-	-	-
Intermoult period (days)	-	-	-	-	-
Survival rate (%)	-	-	-	-	-
Immune parameter:					
Total haemocyte count	+	+	+	+	+
Granular cell proportion (%)	+	+	-	-	+
Haemolymph bacterial rank	+	+	-	+	+
Bacterial assessment:					
Intestinal bacteria rank	-	+	-	-	-
Total water bacteria	-	-	-	-	-
Challenge test:					
Total haemocyte count	-	+	-	+	+
Granular cell proportion (%)	+	+	+	+	+
Survival rate	-	-	-	-	-
Haemolymph bacterial rank	-	+	-	-	+
Water quality	-	+	-	-	-

Note: AQ2 (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); PM4 (*Bacillus* sp), (+) = Significant (1 point), (-) = Not significant (0 point)

In a shrimp's digestive tract, *Bacillus* enhance the specific activity of lipase, protease and amylase (Ziaei-Nejad et al. 2006). In the current study, most species of probiotic bacteria for the trial came from the genus *Bacillus*, as the enzymes of *Bacillus* are very efficient in digesting a large variety of carbohydrates, lipids and proteins into smaller units (Ninawe & Selvin 2009). In shrimp culture within ponds, *Bacillus* species also degrade organic materials (Rengpipat *et al.* 1998; Verschuere *et al.* 2000), thus improving the water quality.

4.5. Conclusion

Using the matrix (Table 4.4) results from phase 1 and 2 showed that A10 can achieve the highest score, followed by PM4, AQ2, PM3, and A12. Furthermore, A10 (*Bacillus mycoides*) and PM4 (*Bacillus* sp.) are most effective probiotic supplements in the marron diets. In the present study, marron fed probiotic supplemented diets produced beneficial outcomes in terms of physiological condition (organosomatic indices), immune parameters (THC, DHC, GC proportion and bacteria load in haemolymph) and level of the bacteria load in the intestine than control diet. However, further research is required to investigate the effects of supplementing two or three different species in marron diets under a commercial farming environment.

CHAPTER 5. *Bacillus mycoides* improves health of gastrointestinal tract in marron *Cherax cainii* (Austin, 2002)

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5.1 Introduction

It is widely established that in addition to skin and gills, the gastrointestinal tract (GIT) is considered one of the major routes for pathogenic invasion in aquatic animals (Ringø and Birkbeck, 1999, Ringø et al., 2007a, Dimitroglou et al., 2011, Cerezuela et al., 2013). Therefore, study of the GIT of aquatic animals as a physical and immunological barrier is increasingly important, and it is accepted that digestion and immunity are complicated physiological processes that have co-evolved (Cerezuela et al., 2013, Liu et al., 2013).

The GIT of aquatic animals plays an important role in non-specific immune defences, as it provides an initial barrier to pathogen entry (Sugita et al., 1988, Verschuere et al., 2000, Ramirez and Dixon, 2003, Ringø et al., 2003, Rollo et al., 2006, Liu et al., 2013). The first step in bacterial invasion of the intestine is mediated by adhesion of pathogenic bacteria to mucosal surfaces and disruption of the microbial balance (Verschuere et al., 2000, Ige, 2013). In fish hatcheries, intestinal microbial disorders caused by bacterial disease are considered to be a major cause of mortality, thus stability of the intestinal microbes and gut health are essential for the health of an organism (Rollo et al., 2006).As a result, much attention has been focused on the development of probiotics in order to maintain a stable, beneficial gut microbial population(Merrifield et al., 2010a).

Study of morphology and intestinal health of aquatic animals using prebiotics has been evaluated in red drum, *Sciaenops ocellatus* (Zhou et al., 2010, Cheng et al., 2011b), rainbow trout, *Oncorhynchus mykiss* (Dimitroglou et al., 2009), gilthead sea bream, *Sparus aurata* (Dimitroglou et al., 2010), nile tilapia, *Oreochromis niloticus* (Merrifield et al., 2011), channel catfish, *Ictalurus punctatus* (Zhu et al., 2012), Pacific white shrimp, *Litopenaeus vannamei* (Zhang et al., 2012), marron *Cherax tenuimanus* (Sang and Fotedar, 2010b) and its combination with probiotic (Hai and Fotedar, 2009, Ganguly et al., 2010, Merrifield et al., 2010b, Daniels et al., 2010, Cerezuela et al.,

2013). Although the use of probiotics in aquatic animals has been reviewed by many authors (Gatesoupe, 1999, Verschuere et al., 2000, Irianto and Austin, 2002, Balcázar et al., 2006, Sahu et al., 2008, Nayak, 2010, Newaj-Fyzul et al., 2014), information for probiotic effect on intestinal health and morphology is extremely limited (Cerezuela et al., 2012, Zhu et al., 2012).To date, probiotic effects on intestinal health and morphology have been studied only in nile tilapia, *O. niloticus* (Pirarat et al., 2011), seabream *Sparus aurata* L. (Cerezuela et al., 2012), and beluga *Huso huso* (Salma et al., 2011).

Bacillus mycoides is a bacterium found in marron and the environment that has favourable probiotic properties including growth inhibition of *V. mimicus* and *V. cholerae* non-01,is susceptible to a majority of antibiotics, non-pathogenic to marron, produces a wide range of enzymes(Ambas et al., 2014) and improves the immunity and health of marron (Ambas et al., 2013).The aim of the present study was to examine the effects of *Bacillus mycoides* on intestinal health and morphology in marron as determined by bacterial density, hepatopancreas indices including moisture content, microvilli density and length, and histological examination of intestinal cells.

5.2. Materials and Methods

5.2.1 Culture system, experimental animal and feed preparation

The experimental units were cylindrical plastic tanks (80 cm diameter, 50 cm high and 250 L in capacity). The tanks were filled with freshwater and supplied with constant aeration, and sufficient number of marron shelters of PVC pipes with appropriate diameters. Each tank was also equipped with a submersible thermostat set to 24^{o}C and a recirculating biological filtration system. The water in the tank was recirculated continuously at a rate of approximately 3 L/min. To maintain good water quality in the tanks, water exchange at a rate of 10-15% of the total water volume was performed twice a week, after siphoning out the faeces and uneaten feed.

Marron (weight 33-65 g) were obtained from the Marron Growers Association (MGA) in Northcliffe and Manjimup, Western Australia. The 250 L tanks were stocked with marron at a density of 12 marron/tank. Before commencement of the experiment, marron were kept for two weeks in the experimental tanks for acclimation. During the

two month experimental period, a commercial pelleted diet (Tabel 5.1) supplied by Specialty Feeds Pty Ltd WA, Australia was fed to marron at a rate of 1.5 % body weight per day.

Bacillus mycoides was isolated from a number of healthy marron. The isolate was identified by the Bacteriology Laboratory, Animal Health Laboratories, Department of Agriculture and Food, Western Australia, using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Bioscience Corporation), Vitek Compact II (Biomerieux) and conventional biochemical methods according to standard procedures and identification methods (Buller, 2004). Subsequently, the strain was suspended into 1 mL aliquots of GLL (Glycerol Lab Lemco broth) and stored at -80 °C.

Table 5. 1. Ingredients of the basal diet

Ingredients	Percentage (%)
Wheat flour	49.35
Fish meal[a]	33.78
Soybean meal	10.15
Fish oil[b]	3.2
Wheat starch	1.85
Betaine[c]	1.20
Cholesterol	0.25
Premix[d]	0.15
Ascorbic acid	0.05
Calcium carbonate	0.02
Total	100

All ingredients were supplied by Specialty Feeds Pty Ltd WA, Australia.[a] Peruvian fishmeal, 56 % CP; [b] Cod liver oil ; [c] Betaine anhydrous 97%; [d] Commercial vitamin and mineral premix for trout

Supplementation using the probiotic strain was performed as per Hai and Fotedar (2009). In brief, prior to probiotic supplementation of the experimental diet, a pure culture of *B. mycoides* was grown on 5% horse blood agar (BA) plates for 24h at 25^{o}C. Colonies in logarithmic phase of growth were emulsified in sterilised distilled water and serially diluted. The optical reading of each serial dilution was recorded, and a viable count performed to obtain a standard curve for inoculum density. From the standard curve, the amount of the diluted probiotic was calculated to achieve the desirable

supplementation density of 10^8 colony forming unit (cfu) per gram of feed. The pellets were air dried, packed and stored at 4^{o}C until used.

5.2.2 Data collection

At the termination of the experiment, the GIT health status of marron was determined through analysis of bacterial density, microvilli length and density, histological assessment of GIT epithelium, and moisture content and weight of the hepatopancreas. All animals used for analysis from both treatment groups were of equal in weight or length size in order to minimise misinterpretations due to size variations.

- ***Intestinal bacterial density***

The bacterial density of marron GIT after feeding with probiotic supplemented feed was measured at the beginning and end of the experiment. Ten marron from each treatment group were sacrificed by placing them at -20^{o}C for 5 minutes before aseptic removal of the GIT. The marron dorsal shell was cut-off horizontally from tail to head until the hepatopancreas and intestine were exposed. The hepatopancreas was removed, placed in a sterilised pestle, weighed and then homogenised. Similarly, the intestine from individual animals was collected aseptically and homogenised with a micro pestle in a 1.5 ml microfuge tube. The homogenised hepatopancreas, and the homogenised intestine were serially (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) diluted. Fifty microliter of each serial dilution was inoculated onto a BA plate and incubated overnight in a CO_2 incubator at 25^{o}C. A colony count was performed for each dilution to determine the total number of aerobic bacteria.

- ***GIT microvilli assessment by micrograph***

The distal part of the marron intestine was observed using a scanning electron microscope (SEM) following an established method (Dunlap and Adaskaveg, 1997, Sang and Fotedar, 2010b). The intestinal tract of five marron from each treatment group was dissected and immersed in 3% glutaraldehyde in 0.1M cacodylate buffer overnight. Following overnight immersion, the GIT was washed in 3 changes of the cacodylate buffer and 3 changes in distilled water for 5 min per change. The intestine was immersed in 2% OsO4 for 2h followed by 3 washes in distilled water for 5 min

per wash. Dehydration of the sample was performed through solutions of 50%,75%,95% ethanol for 5 min per solution and finally 3 times in 100% ethanol for 5 min per change followed by chemical drying by washing in a series of 50%,75% and 100% (twice) hexamethyldisilizane (HMDS) in ethanol solutions for 5 min per change. The final stage involved drying the samples at room temperature, mounting on a stub using carbon tape and then coating with gold before viewing the samples under a pressure scanning electron microscope (LX30). The images obtained from SEM were used to describe villous height and density (number per group surface area) in the GIT.

The height of microvilli (µm) was measured following established methods (Pirarat et al., 2011, Cerezuela et al., 2012). At least 10 villi per section were randomly selected and measured using a computerised morphometric technique. The height of each villous was measured from the villous bottom to the tip, and the average height of these 10 villi was expressed as the mean villous height. Villous density (villous/100 µm surface area) and villi per group (number of villi in a row) were counted according to Sang and Fotedar (2010b).

- ***Histological assessment of the intestine***

Histological preparation and assessment of marron GIT post-feeding with probiotic and basal diets were prepared by Animal Health Laboratories, Department of Agriculture and Food Western Australia. Five marron GIT from each treatment group were dissected and fixed in 10% buffered formalin for 24h. Dehydration of the tissue was performed by passing through a series of 70%, 85% and 98% alcohol solutions. The samples were vacuum embedded in paraffin. The histological sections of 4–5µm were stained with hematoxylin and eosin (H&E). The sections were examined and photographed using an Olympus BX50 microscope.

- ***Hepatosomatic indices* (Hiw)**

The hepatosomatic indices (Hiw) of marron fed with probiotic supplemented diet and basal diet were calculated as per established equations (Jussila, 1997, Fotedar, 1998). In brief, the hepatopancreas of ten marron from each treatment group were removed, placed in foil and weighed. For hepatopancreas moisture content, the hepatopancreas

was dried at 110^{o}C for 24 h. The results, expressed as wet hepatosomatic indices (Hiw), dry hepatosomatic indices (Hid) and hepatopancreas moisture content (HM) were calculated as follows;

$Hiw = W_{wh} \times 100\ W_t^{-1}$

$Hid = W_{dh} \times 100\ Wt^{-1}$, and

$HM = (W_{wh} - W_{dh}) \times 100\ W_{wh}^{-1}$

Where;
Hiw = Wet hepatosomatic indices (%)
Hid = Dry hepatosomatic indices (%)
Wwh = Weight of wet hepatopancreas (g)
Wdh = Weight of dry hepatopancreas
W_t = Total weight of marron (g)
HM = Hepatopancreas moisture content (%)

5.2.3 Data analysis

Data were analysed using SPSS statistical program version 22. Comparison of the mean values using T-test was performed to determine significance and the results were presented in tables and graphs.

5.3. Results

5.3.1 Intestinal bacteria density

The mean bacterial density in marron intestine fed probiotic supplemented diet was significantly greater (4007±121 million cfu/g of GIT) compared to the bacterial density in the gut of basal diet fed marron (723.7±45.2 million cfu/g of GIT).

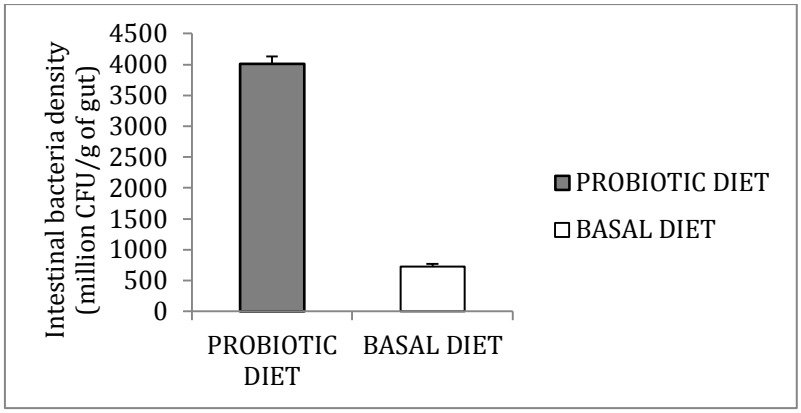

Figure 5.1. Intestinal bacteria population (million CFU/g of gut) of marron fed basal and probiotic diet.

The diversity of the bacterial population was greater in the GIT of probiotic supplemented diet fed marron compared to basal diet fed, as observed by colonial morphology on BA plates after 24h incubation at 25^{o}C.

5.3.2. GIT microvilli assessment by micrograph

The morphology of marron intestines after feeding with probiotic supplemented diet compared to basal diet is shown in Figure 5.2.

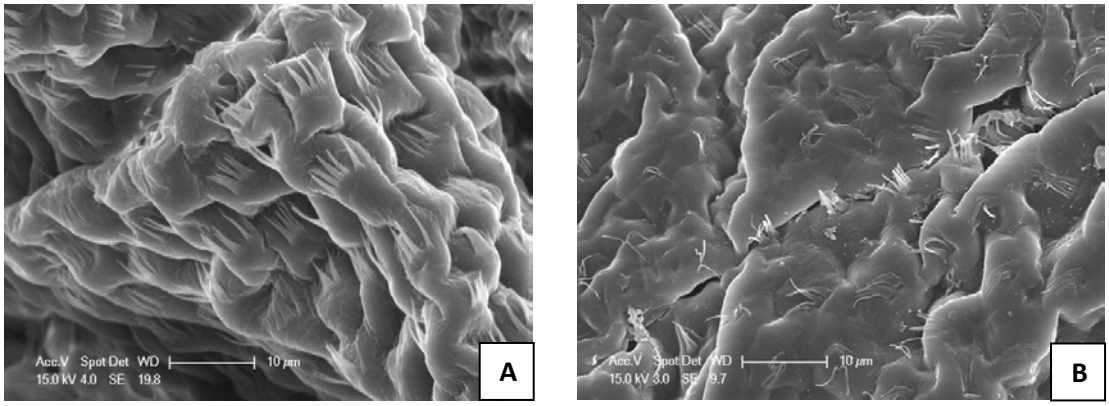

Figure 5.2. Scanning electron microscopy micrograph of marron hindgut fed probiotic supplemented diet (A) and basal diet (B). (x=2500. Bar=10 µm). In the probiotics fed marron there is an increase in folds and the villi are longer and more numerous.

The density and length of the microvilli per GIT surface area was significantly higher in marron fed *B. mycoides* supplemented diet (A) than microvilli of basal diet fed marron (B). The average density of microvilli per group (number of villous in a row) of marron fed the probiotic diet was 10.50 ± 0.94 compared to 5.71 ± 0.91 in basal diet fed marron.

Table 5.2. Mean +SE of villous height (µm), number per group and villous density (per 100 µm^2) of marron hindgut fed probiotic supplemented diet and basal diet.

Parameters	Probiotic diet	Basal diet
Villous height	4.93±0.11^{b}	3.91±0.18^{a}
Villous per group	10.50± 0.25^{b}	5.71±0.24^{a}
Villous density	20.28±0.70^{b}	13.93±0.41^{a}

*Value in the same row having different superscript indicates significantly different at $P < 0.05$.

5.3.3 Histological assessment of intestine

Histologically there were no major differences seen in the hepatopancreas of marron from the different treatment groups (Fig.3). However, the foregut and hindgut of the probiotic fed marron had more folds and fewer haemocytes compared to the marron fed the control diet without probiotics.

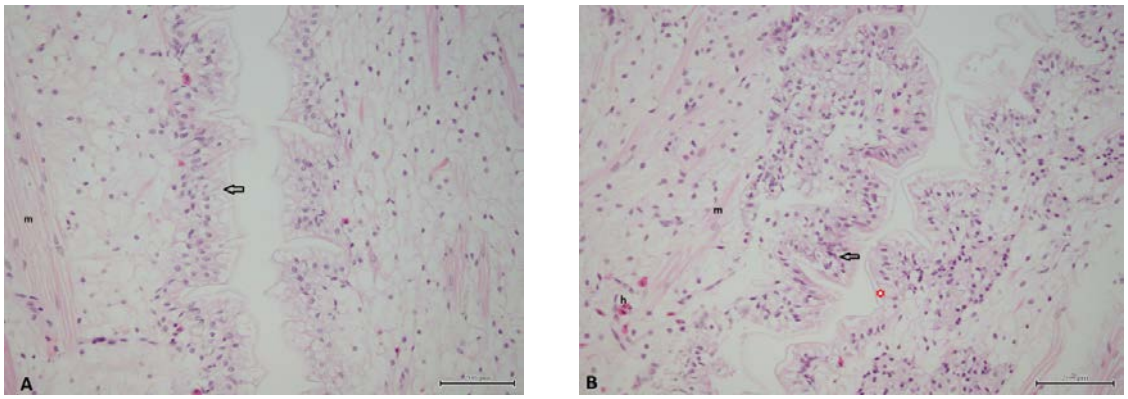

Figure 5.3. Histological sections of the hindgut from marron fed the control diet (A) and the probiotic supplemented diet (B). The epidermal cells (open arrow) in the probiotic supplemented group are larger and have a more foamy appearance. In both treatments there is some shrinkage artefact of the cuticle from the epithelial cells (red star). Muscle cells (m) and mixed populations of haemocytes (h) can be seen in the lamina propria in both the control and probiotic supplemented animals. Bars indicate 200µm.

5.3.4. Hepatopancreas indices

Hepatosomatic indices (Hiw) of probiotic fed marron were significantly higher (7.11 ± 0.34) compared to basal diet fed marron (6.20±0.02). In addition, hepatopancreas moisture content (HM%) was lower (53.43±1.68 %) in probiotic fed marron whereas in basal diet fed marron it was 62.16±1.09%.

Table 5.3. Mean ± SE of hepatosomatic indices (%) and moisture content (%) of marron fed basal and probiotic supplemented diet.

Treatment	Hiw (%)	Hid (%)	HM (%)
Basal diet	6.20 ± 0.02^{a}	2.71 ± 0.06^{a}	62.16 ± 1.09^{a}
Probiotic diet	7.11 ± 0.34^{b}	3.32 ± 0.25^{b}	53.43 ± 1.68^{b}

*Mean values in the same column with different superscript indicate significantly different (P< 0.05)
Hiw = hepatosomatic indices (wet)
Hid = hepatosomatic indices (dry)
HM = Hepatopancreas moisture content

5.4. Discussion

Balcazar et al (2007); Zhou and Wang (2012) suggested that probiotics act in several ways: firstly by maintaining and restoring normal intestinal microbiota and gut homeostasis; secondly by contributing to the competitive exclusion of bacteria (Vine et al., 2004, Seehanat, 2005, Hai et al., 2007, Ariole and Oha, 2013) and thirdly by acting as a source of nutrients and enzymes (Verschuere et al., 2000, Bairagi et al., 2002, Ramirez and Dixon, 2003, Ray et al., 2010, Ray et al., 2012, Lazado et al., 2012). Most authors suggest that probiotics of host origin are more favourable compared to other sources as it is believed that autochthonous bacteria are able to colonise, multiply and remain predominant in the same host (Gatesoupe, 1999, Verschuere et al., 2000, Rollo et al., 2006, Hai et al., 2009b).

The health status of the GIT is most likely determined by the microbial balance of indigenous microbiota (Ramirez and Dixon, 2003, Denev et al., 2009) with the density and diversity of bacteria in the intestine having the most impact on intestinal health (Ringø et al., 2007b).The main parameters commonly used to assess GIT health in aquatic animals are intestinal bacterial density and diversity, microvilli height and number, gut epithelium, hepatopancreas size and digestive enzyme activity (Dimitroglou et al., 2009, Cheng et al., 2011a, Geda et al., 2012, Cerezuela et al., 2012, Cerezuela et al., 2013, Sang and Fotedar, 2010b, Nugroho and Fotedar, 2013).

In the present study marron fed a diet supplemented with *Bacillus mycoides* had improved intestinal morphology, increased density of bacteria in the intestine and a larger hepatopancreas. All of these features suggest that the marron benefited from the probiotic supplementation. A number of studies show intestinal bacteria density increases after probiotic supplementation which is thought to be due to a probiotic of host origin providing a favourable environment for the indigenous intestinal bacteria. In shrimp *Penaeus monodon*, the number of intestinal bacteria increased by up to 803% after supplementation with three *Bacillus* species (*B. pumilus*, *B. sphaericus*, and *B. subtilis*) isolated from the host (Purivirojkul et al., 2005). In grouper *Epinephelus coioides* potentially beneficial bacteria were stimulated, whereas some potentially harmful strains such as *Staphylococcus saprophyticus*, were suppressed after supplementation with probiotic *Psychrobacter* sp. (Yang et al., 2011). Reduction of

either diversity or quantity of the indigenous microbiota is likely to reduce the effective barrier mechanism normally provided by the commensal microbiota (Denev et al., 2009, Ige, 2013).

Another feature of a healthy digestive system is the density and length of microvilli. In the present study, supplementation with *B. mycoides* significantly improved the height and number of villi. Other studies on probiotics had similar findings including one on marron using mannan-oligosaccharide (MOS) diet (Sang and Fotedar, 2010b), and others using *Lactobacillus* sp. in Nile tilapia *Oreochromis niloticus* (Pirarat et al., 2011), *Bacillus* sp. in European lobster *Homarus gammarus* L. (Daniels et al., 2010) and *Pedicoccus acidilactici* in rainbow trout (Merrifield et al., 2010c). Contrary to these findings, Merrifield et al., (2010c) observed no improvements in microvilli in rainbow trout fed with *Bacillus* sp. or *Enterococcus faecium* supplemented feeds, whereas Cerezuela et al., (2012), found shorter villi in gilthead seabream (*Sparus aurata*) fed diets containing *B. subtilis*, suggesting that the effect of probiotics on microvilli may not be consistent in all species.

In aquatic animals, longer intestinal villi provide greater absorption ability due to their increased surface area (Cerezuela et al., 2012, Caspary, 1992, Pirarat et al., 2011, Merrifield et al., 2009b). They also provide a larger surface area for bacterial colonisation as was reported for Arctic charr, *Salvelinus alpinus*. Ringo et al., (2001) observed large populations of bacteria associated with the villous brush borders, while Hellberg and Bjerkas(2000) detected the bacteria between the microvilli in common wolfish, *Anarhichas lupus* (L). In addition, Merrifield et al., (2009a) observed greater bacterial colonization between the folds of the mucosal surface because bacteria could become established and sustained more easily at the base of the villi and between the mucosal folds. Merrifield et al., (2009b) suggested that more dense and regular villi may also play a role in disease prevention by reducing exposure to enterocyte tight junctions in rainbow trout, *Oncorhynchus mykiss*.

Various hepatosomatic indices of marron have been reported in several studies. Jussila (1999) compared hepatosomatic indices of marron at molt and post-molt at different feeding status and found that the lowest (3.8±0.2 %) hepatosomatic indices (Hiw) of

marron was observed in non-fed post-molt marron, followed by fed post-molt marron (5.4±0.3%) and the highest (5.6±0.3%) in fed-intermolt stage marron. Sang and Fotedar (2010a) observed Hiw of marron fed β-1,3 glucan supplemented diet ranged between 6.35 – 7.17 %. In the present study Hiw of probiotic fed marron was 7.11 ± 0.34% compared to 6.20 ± 0.02% for basal diet fed marron.

The improved intestinal health in the probiotic fed marron most likely resulted in the higher hepatosomatic index of these marron. The hepatopancreas, as the main energy reserve in crustaceans and source of various enzymes, has been used as an indicator of crayfish condition (Jussila, 1997, Fotedar, 1998). A heavier hepatopancreas could be an indication of higher digestive enzyme activities (Hammer et al., 2000). Our previous study also indicated that marron fed *B. mycoides* had a larger hepatopancreas especially at day 35 (Ambas et al., 2013). *B. mycoides* produces a wide range of enzymes (Ambas et al., 2014), including many exo-enzymes important for digestion (Moriarty, 1998), and is a reason why *Bacillus* sp. have been used widely as probiotics (Ziaei-Nejad et al., 2006).

5.5. Conclusion

Overall, supplementation of host origin *B. mycoides* in marron feed improved the health of the marron gastrointestinal tract as indicated by an increase in bacterial density, increased and longer microvilli, thicker intestinal epithelium and higher hepatosomatic indices. Consequently the use of host origin (particularly mucosal inhabitants) strains of bacteria with probiotic properties is recommended as these bacteria are able to maintain microbial homeostasis, are well adapted to the host GIT environment and mucosal attachment, and can protect the epithelium layer from potential pathogens, which in turn preserve optimal function of the gastrointestinal tract.

CHAPTER 6. Survival and immunity of marron *Cherax cainii* (Austin, 2002) fed *Bacillus mycoides* supplemented diet under simulated transport.

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6.1. Introduction

Practices and methods used for crustacean handling and live trade may lead to serious physiological stress responses in aquatic animals (Lorenzon *et al.* 2007; 2008; Fotedar & Evans 2011) including marron (Morrissy *et al.* 1992; Jussila *et al.* 1999; Sang *et al.* 2009). Marron, *Cherax cainii* (Austin, 2002), is endemic to Western Australia and recently was introduced into South Africa, Zimbabwe, Japan, USA, China and the Caribbean as a commercial aquaculture species (Morrissy *et al.* 1990; David B Rouse & Izuddin Kartamulia 1992; Sang *et al.* 2009)

The reasons for live trade of crustaceans include: for consumption, grow-out, restocking, and the aquarium trade; hence survival and quality of the animals is extremely important for welfare and economic reasons. The duration of the stressors encountered in the live trade process leads to short and long term changes in immune parameters (Lorenzon *et al.* 2008), as stress response shifts from adaptive to maladaptive (Barton & Iwama 1991). Beyond this point the physiological stress response may reduce disease resistance and growth (Rollo *et al.* 2006; Ige 2013), reduced quality and eventually death (Lorenzon *et al.* 2008). Therefore, improving immunity, stress tolerance and optimising health conditions of crustaceans during storage and live transport is of fundamental importance (Fotedar & Evans 2011).

The successful culture and stocking of marron relies on better understanding the factors affecting their well-being during transport and their recovery afterwards (Jussila *et al.* 1999). Marron are an economically important aquaculture species in Western Australia and they show significant environmental stress tolerance post handling and live transport. Jussila *et al.* (1999) observed no mortality of marron post 24h handling and simulated transport, whereas Sang *et al.* (2009) detected no mortality of marron up to 36h under simulated transport. Moreover, marron may undergo live transport up to 72h without mortality, however longer periods of transportation resulted in an average dehydration of 4.5 % of body weight (Morrissy *et al.* 1992; Jussila *et al.* 1999).

Feed additives such as probiotics and prebiotics can improve stress tolerance and immunity of aquatic animals (Rollo *et al.* 2006; Lund *et al.* 2012; Soleimani *et al.* 2012; Zhang *et al.* 2012; Hoseinifar *et al.* 2015; Wongsasak *et al.* 2015) including marron (Sang *et al.* 2009). Our previous studies indicated that *Bacillus mycoides*, a marron origin customized probiotic, possessed a number of favourable probiotic properties (Ambas *et al.* 2015a), significantly improved gastrointestinal (GIT) health of marron (Ambas *et al.* 2015b) and improved immunity against the pathogenic bacterium *Vibrio mimicus* (Ambas *et al.* 2013), a dominant bacterial pathogen of freshwater crayfish in aquaculture (Eaves & Ketterer 1994; Wong *et al.* 1995; Evans & Edgerton 2002).

Cruz *et al.* (2012), suggested that aquatic animals should be treated with probiotics before exposure to transport and environmental stressors. To date, improved stress tolerance and immunity by feeding probiotics have been documented in fish (Rollo *et al.* 2006; Nayak 2010; Tapia-Paniagua *et al.* 2014), shrimps (Bachere 2000; Farzanfar 2006; Ninawe & Selvin 2009; Lakshmi *et al.* 2013) and molluscs (Kesarcodi-Watson 2009; 2012), however information on probiotic-fed marron under practical transport conditions is not available. The present study evaluated the effect of simulated transport conditions on marron fed the probiotic *B. mycoides* by measuring intestinal bacterial population, total haemocyte count (THC), bacteraemia, morbidity, dehydration and mortality.

6.2. Material and Methods

6.2.1. Acclimation and feeding trial

Marron juveniles were supplied by Blue Ridge Western Australia Pty Ltd and then acclimated to the culture tanks, fed using a basal diet for 2 weeks, and distributed into six experimental culture tanks at a density of 12 marron per tank.

The experimental system consisted of three standing units of steel racks with three shelves in each unit. The experimental units were cylindrical plastic tanks (80 cm diameter and 50 cm high and 250 L in capacity) filled with freshwater running continuously at a rate of approximately 3 L/min. using a recirculating biological filtration system (Fluval 205, Askoll, Italy). Each tank was supplied with constant aeration and equipped with a submersible thermostat set to 24^{o} C. PVC pipes of appropriate diameter were added to the tanks as shelters for the marron.

Prior to the simulated transport test, a feeding trial using basal and probiotic supplemented diet was conducted for 10 weeks. Each tank was stocked with 12 marron where each treatment consisted of five replicate tanks. The test diets were given to marron every day in the late afternoon at a rate of 1 % of the total biomass and adjusted weekly after determination of biomass at the end of each week.

6.2.2. Experimental diet and set up

The experimental diets used in this feeding trial were (1) basal diet of a marron commercial feed supplied by Specialty Feed Pty Ltd, WA and (2) the basal diet supplemented with customized probiotic *B. mycoides*. Before use, the pelleted diet was homogenised with a blender to obtain a desirable pellet size before supplementation with the *B. mycoides* at 10^8 cfu/g of feed. The density of *B. mycoides* was based on the density used in other *Bacillus* species studies (Keysami *et al.* 2007; Li *et al.* 2009; Zhang *et al.* 2011; 2012; Liu 2012) and from results of our previous studies (Ambas *et al.* 2013; Ambas *et al.* 2015b).

Supplementation of the probiotic followed established methods (Hai *et al.* 2010). In brief, the basal diet was placed on tray covered with sterilised aluminium foil and sprayed with 20 mL of fish oil per kg of feed to improve attachment of probiotic bacteria. The feed was wrapped in individual sterilised aluminium foil packs containing the amount adjusted to marron biomass for each tank, and stored at 4^oC until used. The diet was prepared each week and the feeding rate adjusted according to the marron weight.

6.2.3. Simulated live transport

After feeding with the test diet for 10 weeks, the animals were subjected to a simulated live transport following the "Code of Practice for the Harvest and the Post-Harvest Handling of Live Marron for Food" established by Department of Fishery Western Australia (Fisheries 2002) and the standard packing of marron commonly used by marron growers. Feeding ceased one day before the commencement of simulated transport trial.

In brief, healthy marron of equal size (12.3 ± 0.5 g) from probiotic fed and a control basal diet were selected and placed in a polystyrene box (60 x 40 x 30 cm) for 24 h and 48h simulated transport. Each box contained sufficient ice-gel bags covered by a moist foam

layer and a temperature data logger (Onset HOBO). Marron from each treatment group were placed in a ventilated container prior to placing in the polystyrene boxes. Placing the marron in the ventilated container not only avoided the marron from mixing with different treatment groups, but also protected the marron from the moist foam layer and ice-gel bags, and was based on the method used in a previous study (Sang *et al.* 2009). Subsequently, another moist foam layer and ice bag were placed over the marron ventilated containers, before the outer polystyrene box was sealed with a lid. The sealed boxes were placed on a trolley at room temperature and being moved intermittently to give simulated transport effects.

Twenty four and forty eight hours post simulated live transport, the animals were returned to the culture tanks once the parameters for intestinal bacteria population, total haemocyte count (THC), bacteraemia, morbidity, dehydration and mortality were measured and recorded.

6.2.4. Measurements of the parameters

- ***Intestinal Bacteria Population***

Bacterial population of the marron was measured before and during simulated transport at 24h and 48h. In brief, marron from each treatment group were sacrificed by placing them at -20^{o}C for 5 min before aseptic removal of the GIT. The marron dorsal shell was cut-off horizontally from tail to head until the intestines were exposed. The intestine from individual animals was collected aseptically and placed in a sterilised pestle, weighed and then homogenised. The homogenates of intestines were serially (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) diluted using sterile normal saline. Fifty microliters of each serial dilution was inoculated onto a blood agar (BA) plate and incubated overnight in a CO_2 incubator at 25^{o}C. A colony count was performed for each dilution to determine the total number of aerobic bacteria (Buller 2004).

- ***Total haemocyte count (THC)***

The total haemocyte count was measured following the established methods used in western rock lobsters *Panulirus cygnus* (Fotedar *et al.* 2001) and marron (Sang *et al.* 2009). In brief, 0.5 mL of haemolymph withdrawn from the second last ventral segment of marron was inserted into a haemocytometer (The Neubauer Enhanced Line, Munich, Germany) counting

chamber and immediately viewed under 100-fold magnification on a camera-equipped microscope and images were taken for THC counts. Cells were counted in both grids, and the mean was used as the haemocyte count. For each treatment group, the procedure was repeated using ten different animals. The total haemocyte count was calculated as THC = (cells counted x dilution factor x 1000)/volume of grid (0.1 mm^3).

- ***Haemolymph bacteria (Bacteraemia)***

Bacteraemia of marron was determined following the established method described by Sang et al. (2009), with a minor modification. Briefly, the haemolymph was withdrawn into a sterile syringe and placed onto a sterile glass slide to avoid bubbles before a 0.05 mL aliquot was lawn inoculated onto a BA plate. The plates were placed in a sterilised container and incubated overnight at 25^{o}C. The total colony forming units (CFU) for each plate and cfu/mL were calculated on the basis of a total volume of 0.05 mL/plate.

- ***Dehydration***

Dehydration of marron was measured using the established method (Jussila *et al.* 1999). Ten marron from each treatment group of equal size were weighed prior to the commencement of the simulated transport, then weighed at 24h and 48h post transport and the percentage of weight loss was recorded.

- ***Morbidity and survival rate (%)***

Morbidity (vigour index) of marron was measured following the established method proposed by Jussila et al., (1999). In this study, morbidity of marron was identified based on the response to stimuli at a time after simulated transport, and the time of recovery was recorded after being returned to the culture tanks.

Mortality of marron from each treatment group was measured at 24h and 48h post-simulated transport up to one day they were returned to the culture tanks. Determination of survival rate following the established equation;

SR = (Nt/No) x 100

where SR is the survival rate (%); Nt is the number of marron at time t and No is the number of marron at the commencement (o), respectively.

6.2.5. Data analysis

The data were analysed using SPSS statistical package version 22.0 for Windows and Microsoft Excel. The difference between means was determined using one way analysis of variance (ANOVA) and a t-test. All significant tests were performed at $P < 0.05$ level. All data were presented as mean ± SE, unless otherwise indicated.

6.3. Results

6.3.1 Intestinal Bacteria Population

Intestinal bacterial population of marron declined at 24 and 48 h simulated transport both for basal diet and probiotic fed marron. Reduction of the intestinal bacterial population occurred at 24h and 48h of transport; however a significant reduction of more than half the initial population levels were observed at 48h, both in basal diet and probiotic fed marron (Table 6.1). This result suggests that the longer the stress disturbance, the greater the reduction of intestinal bacterial population. Nevertheless, at 48h post-transport, the bacterial population (646 ± 16.4) of probiotic fed marron was comparable to the initial bacterial population (626.7 ±19.7) of basal diet fed marron.

Table 6.1. Intestinal bacteria population (million CFU ± SE) of marron fed different diets at 24h and 48 post transport.

Treatment	Intestinal bacteria population (million CFU/g of gut)		
	0h	24h	48h
Basal diet	626.7 ±19.7$^{a.1}$	531.1 ± 15.7^{a,1}	260.0 ±67.1^{b,1}
B. mycoides diet	1656 ± 167.7^{a,2}	1318 ± 131.5^{a,2}	646 ± 16.4^{b,2}

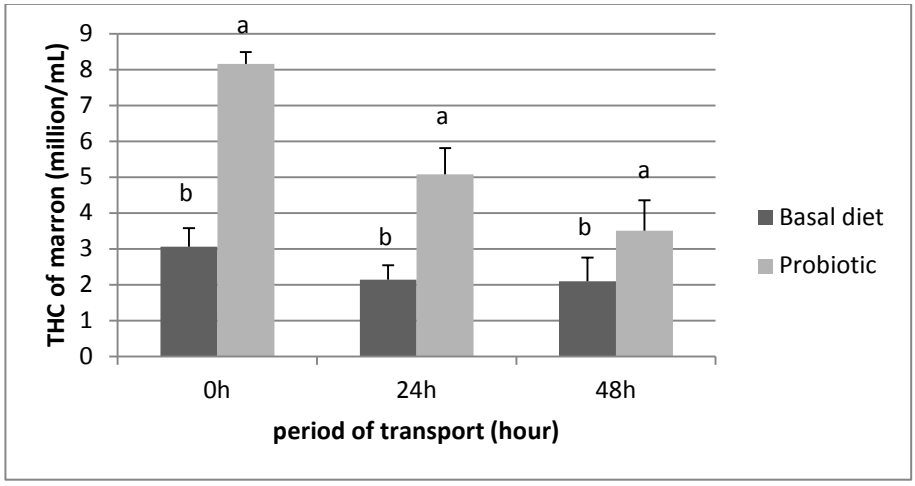
*Data in the same column with different superscript letters (1, 2) are significantly different at a level of 0.05. Data in the same row with different superscript (a,b) indicates significantly different at a level of 0.05.

6.3.2 Total haemocyte count (THC)

Prior to the simulated transport test, the THC of marron fed probiotic and basal diets for 10 weeks was measured. The THC of marron fed probiotic supplemented diet was significantly higher compared to THC of basal diet fed marron at the commencement of simulated

transport test (Fig.1). This result indicates that the health status of marron fed probiotic was higher at the initiation of the simulated transport test.

After 24h and 48h post transport, the THC in both treatment groups declined indicating that transport affects the THC in marron.



*Different superscript (a,b) over bars indicates significantly different at a level of 0.05.

Figure 6.1. THC of marron fed basal and probiotic diets at 24h and 48h of transport.

6.3.3 Haemolymph bacteria (Bacteraemia)

Haemolymph bacteria were observed in basal diet and probiotic fed marron after feeding with the test diets for ten weeks prior to the simulated transport test. Total bacterial count in the haemolymph of probiotic fed marron was significantly ($P < 0.05$) lower compared to basal fed marron, indicating the customized probiotic of host origin may induce protection from bacteria and other foreign particles in the haemolymph. This result was strongly related to THC of marron in each treatment group, as haemocytes play an important role in removal of bacteria and foreign particles from the haemolymph of crayfish (Chisholm & Smith 1995; Johansson *et al.* 2000).

6.3.4 Dehydration

Dehydration occurred in marron fed both the basal diet or the probiotic supplemented diet. During the first 24h, dehydration in the basal diet fed marron was 3.8 %, whereas in probiotic fed marron it ranged between 3.0 and 3.7 percent. After 48h of transport, the

dehydration still occurred in both treatment groups but in the basal diet it was 0.45 percent while in probiotic fed marron the extra dehydration was 0.55 percent. There was no significant different (P> 0.05) in the mean dehydration between basal diet and probiotic fed marron after 24h and 48h of transport.

6.3.5 Morbidity and survival rate (%)

Marron showed a very weak response to stimuli after 24h of transport and this condition was more noticeable after 48h of transport. Marron started to show response to stimuli after 30 minutes during the temperature acclimation in the boxes. As most marron in the boxes were actively crawling and swimming, they were returned to the tanks for mortality observation. No mortality was observed in marron fed probiotic supplemented diet; while in basal diet fed marron mortality (6.7 %) occurred at 48h of transport. No clinical signs were observed on dead marron shells.

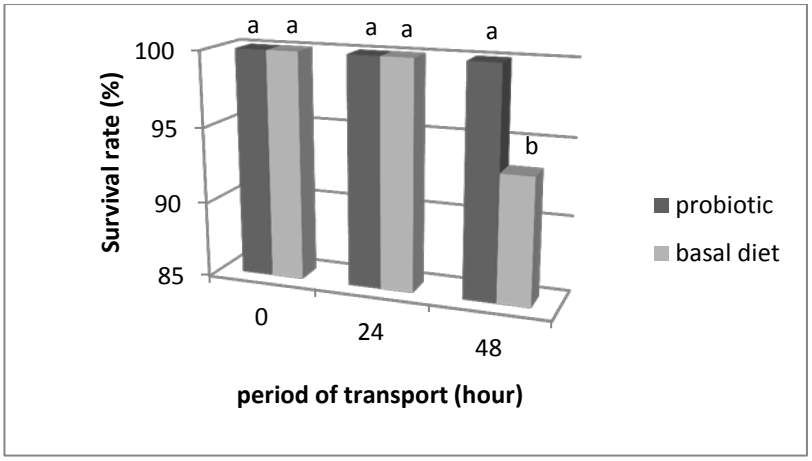


Figure 6.2. Survival rate (%) of marron fed basal and probiotics diets at 24h and 48 of transport.

6.4. Discussion

A number of immune and physiological parameters are involved in the stress response following handling and live transport of crustaceans and fish such as behaviour, morbidity and vigour, THC, blood glucose, dehydration , oxygen uptake, blood composition, pH, hormones and ion (Lorenzon *et al.* 2007; Fotedar & Evans 2011). In marron, the common selected parameters for testing following handling and simulated transport include dehydration (Morrissy *et al.* 1992) THC, haemolymph/plasma glucose, serum protein (Jussila *et al.* 1999), proportion of granule cells, clotting time and bacteraemia (Sang *et al.* 2009).

The circulating haemocytes of crustaceans are an essential part of the immune system, and perform functions such as phagocytosis, encapsulation, and lysis of foreign cells and much research in the defensive role of haemocyte in crustacean is being conducted (For review see; Soderhall & Cerenius 1992; Bachere 2000; Johansson *et al.* 2000). The results suggest total haemocyte count (THC) is a reliable indicator of stress in crustaceans (Lorenzon *et al.* 2001; 2008) including in marron (Jussila *et al.* 1999; Sang *et al.* 2009).

In the present study, THC was investigated as an indicator for stress tolerance. THC of marron fed probiotic was significantly higher compared to basal diet fed marron both at 24h and 48h post simulated transport, indicating that the customized probiotic *B mycoides* was able to improve marron immunity. Enhancement of THC in probiotic fed marron leads to increased stress tolerance and diseases resistance, which results in a significantly higher survival rate (100%) over 24h and 48h post live transport. Higher THC of probiotic fed marron also provides better protection to the gill from parasites and bacteria pathogens which may cover and reduce respiration efficiency. Tinh *et al.* (2008), suggested that probiotic bacteria can also be active on the gills and skin of the host. Inefficient respiration during handling and live transport may contribute to marron mortality in basal diet fed marron. Thus prior to transport, purging is generally essential in freshwater crayfish (Fotedar & Evans 2011) to evacuate the GIT content and clean the gills and skin.

Handling and live transport creates physiological stresses which reduce THC in many crustacean species such as American lobster *Homarus americanus* (Lorenzon *et al.* 2007), crab *Cancer pagurus* (Lorenzon *et al.* 2008), western rock lobster *Panulirus cygnus* (Fotedar *et al.* 2001; Jussila *et al.* 2001), marron (Jussila *et al.* 1999; Sang *et al.* 2009), and a mollusc abalone *Haliotis tuberculata* (Cardinaud *et al.* 2014). Therefore, increasing the THC by feeding probiotic supplemented diets may improve stress tolerance and protect the animals from pathogens. This has been demonstrated in crayfish, *Pacifastacus leniusculus*, where a higher THC corresponded to improved defence reactions of the animal when infected with the Oomycete Fungus *Aphanomyces astaci* (Persson *et al.* 1987), and in marron where a higher THC corresponded to reduced bacteraemia and improved immunity against the pathogen *Vibrio mimicus* (Sang *et al.* 2009; Ambas *et al.* 2013).

Bacterial population and diversity in the GIT is an important health component for aquatic animals (Gomez & Balcazar 2008; Gaggìa *et al.* 2010) significantly affected by acute stress (Olsen *et al.* 2005). Stress due to high stocking density could also affect the performance of probiotics (Nayak 2010). In the present study, the bacterial population level decreased both in probiotic and basal diet fed marron at 24h and a significant reduction was observed at 48h of transport indicating that prolonged stress significantly reduces intestinal bacteria of the animal. However, the higher bacterial population level of probiotic fed marron prior to transport resulted in the bacterial population remaining higher at 48h of transport compared to the population level of basal diet fed marron at the initiation of the transport test. The reduction of intestinal bacterial population due to handling and transport stress was also determined in other species. In Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss*, adherent bacterial population in the mid-gut and hindgut were significantly reduced following acute handling stress, whereas the level increased in faeces, which suggests that considerable amounts of mucus was lost following stress (Olsen *et al.* 2002; 2005). This significant reduction of the intestinal bacterial population implies decline in the health status of the animal. The microbiota within the GIT can be considered a metabolically active organ, is an essential health component, provides protection against infection and instructs mucosal immunity (Gaggìa *et al.* 2010). Beneficial bacteria such as lactobacilli and bifidobacteria decrease following a stress response (Gaggìa *et al.* 2010) and this may provide opportunistic pathogens to become established (Lorenzon *et al.* 2001; 2008; Ringø *et al.* 2010).

Haemolymph bacteria (bacteraemia) of marron fed probiotic diet was significantly lower compared to basal diet fed marron suggesting that greater THC plays an important role in reducing bacteria and foreign particles in marron haemolymph. Once pathogens or foreign particles enter the haemocoel, the haemocytes initiate phagocytosis (Li *et al.* 2010). In crayfish, hyaline cells are chiefly involved in phagocytosis, whereas semi-granular cells are active in encapsulation (Johansson *et al.* 2000). Crustacean haemocytes contain antibacterial activity (Haug *et al.* 2002; Van de Braak *et al.* 2002), which can reduce the viable count of bacteria within 4 hours, however the antibacterial potency (per unit protein) varies from species to species (Chisholm & Smith 1995).

Morbidity and mortality post live transport often occurs as a result of stress (Fotedar & Evans 2011). In the present study, morbidity and weakness indicated by no response to stimuli were observed both in basal diet and probiotic fed marron at 24h and 48h post simulated transport. However, after returning to the culture tanks the probiotic fed marron fully recovered and swam normally in less than 30 min, whereas basal diet fed marron took more time to recover and had several mortalities.

Other than mortality, marron also could be losing weight through dehydration from tissue and gill chambers while out of water during handling and live transport (Morrissy *et al.* 1992; Jussila *et al.* 1999; Fotedar & Evans 2011). The present study indicated that dehydration of marron was observed in both test diets at 24h and 48h post transportation, however the dehydration was not significantly different between the two treatments. The results revealed that the dehydration of marron was comparable to the previous marron handling and live transport studies indicating that dehydration of 4 to 5 % of the body weight over 24 to 72h transportation is a common phenomenon. Jussila et al., (1999) observed a minor dehydration of marron during the first 4 h that remained at 4.0 to 4.5 % up to 24h post handling and transportation, whereas Morrissy et al., (1992) observed wet dehydration of 3.9% during the first 24h, with a further additional loss of 0.4 % over the next 24h. Acute stress requires high energy which could reduce the hepatosomatic indices and contribute to the dehydration of the animal (Cruz *et al.* 2012). In marron, hepatopancreas significantly reduced after 24h of transport (Jussila *et al.* 1999). Therefore, dehydration should be considered when crayfish are going to be transported for a long distance (Jussila *et al.* 1999).

Probiotics have improved stress tolerance and immunity of various aquatic animal groups such as fish species including sea bream, *Sparus aurata* (Rollo *et al.* 2006), grouper *Epinephelus coioides* (Sun *et al.* 2010), nile tilapia *Oreochromis niloticus* (Pirarat *et al.* 2011); shrimp such as western king prawns, *Penaeus latisulcatus* (Hai *et al.* 2009b) Pacific white shrimp, *Litopenaeus vanname*i (Li *et al.* 2007; Tseng *et al.* 2009; Liu *et al.* 2010), and tiger shrimp, *Penaeus monodon* (Rengpipat *et al.* 2000). Among the bacterial genera evaluated for probiotic use, *Bacillus* species have been successful in improving the stress tolerance in these aquatic animal hosts and more recently in black swordtail, *Xiphophorus helleri* (Hoseinifar *et al.* 2015). Our previous work using *B. mycoides* also showed improved

marron immunity when experimentally challenged with the pathogen *Vibrio mimicus* (Ambas *et al.* 2013).

6.5 Conclusion

The present study demonstrated that supplementation with host origin customized probiotic *B. mycoides* significantly improved the health status of marron by increasing their tolerance to a live transport stress test, which resulted in no mortality up to 48h of simulated live transport.

CHAPTER 7. Synbiotic effect of *B. mycoides* and organic selenium (OS) on immunity and growth of marron, *Cherax cainii* (Austin, 2002).

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7.1. Introduction

Prebiotics and probiotics have been extensively used in aquaculture (Burr & Gatlin, 2005; Denev et al., 2009; Ganguly et al., 2010; Daniel & Zhou, 2011; Dimitroglou et al., 2011; Kristiansen et al., 2011). Prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon and thus improves host health (Gibson & Roberfroid, 1995; Ringø et al., 2010). Prebiotics have shown beneficial effects on numerous aquatic animals (Zhou et al., 2010), and reviews of their potential use in aquaculture have been documented (Merrifield et al., 2010; Ringø et al., 2010; Daniel & Zhou, 2011). The prebiotics commonly used and evaluated in aquatic animals to date includes inulin, fructooligosaccharides (FOS), mannanoligosaccharides (MOS), galactooligosaccharides (GOS), xylooligo-saccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltooligosaccharides (IMO) and GroBiotic-A (Ringø et al., 2010), chitosan oligosaccharides (COS) and organic selenium (OS). In marron, the prebiotics evaluated were MOS (Sang et al., 2009; Sang & Fotedar, 2010) and organic selenium (OS) (Nugroho & Fotedar, 2013b).

Probiotics, defined as live microorganisms when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002), have been recommended as alternatives for use as growth promoters, diseases control and for the safety of sustainable aquaculture (For reviews see; Ringø et al., 1995; Ringø & Gatesoupe, 1998; Gatesoupe, 1999; Verschuere et al., 2000; Irianto & Austin, 2002; Austin, 2006; Balcázar et al., 2006; Farzanfar, 2006; Das et al., 2008; Sahu et al., 2008; Denev et al., 2009; Ninawe & Selvin, 2009; Qi et al., 2009; Merrifield et al., 2010; Nayak, 2010; Prado et al., 2010; Cerezuela et al., 2011; Kolndadacha et al., 2011; Lara-Flores, 2011; Cruz et al., 2012; Ibrahem, 2013; Lakshmi et al., 2013; Pandiyan et al., 2013) and more recently (Lazado et al., 2014; Michael et al., 2014; Newaj-Fyzul et al., 2014; Ghanbari et al., 2015; Hai, 2015).

Probiotics and prebiotics generally have been studied separately (Li et al., 2009; Cerezuela et al., 2013). Synbiotics as a combination of probiotics and prebiotics have not been intensively explored (Li et al., 2009). To date, the synbiotic studies on aquatic animals are limited to gilthead sea bream *Sparus aurata* L. (Tapia-Paniagua et al., 2011; Cerezuela et al., 2012; Cerezuela et al., 2013), rainbow trout *Oncorhynchus mykiss* (Rodriguez-Estrada et al., 2009), Japanese flounder *Paralichthys olivaceus* (Ye et al., 2011), shrimp *Penaeus japonicus* (Zhang et al., 2011), shrimp *Litopenaeus vannamei* (Li et al., 2009; Thompson et al., 2010), European lobster *Homarus gammarus* (Daniels et al., 2010), sea cucumber, *Apostichopus japonicus* (Zhang et al., 2010), yellow croaker *Larimichthys crocea* (Ai et al., 2011), cobia *Rachycentron canadum* (Geng et al., 2011) and Zebrafish *Danio rerio* (Nekoubin et al., 2012)**.** A review of synbiotic use of probiotics and prebiotics for fish in aquaculture has also been documented (Cerezuela et al., 2011).

Sel-plex, an organic form of selenium derived from yeast produced by Alltech, showed beneficial effects in marron when administered at 0.2 g $kg^{-1}$of feed (Nugroho & Fotedar, 2013b). *Bacillus mycoides* was a strain isolated from the gastrointestinal tract of marron demonstrated favourable probiotic properties such as growth inhibition of *Vibrio mimicus* and *Vibrio cholerae* non-01 (Ambas, Buller, et al., 2015), and improvement in marron health and immunity (Ambas et al., 2013; 2015). The present study examined the synbiotic effect of the probiotic *B. mycoides* and the prebiotic OS (Sel-Plex) on growth, health condition and immunity as tested by THC, DHC, bacteraemia, clotting time and glutathione peroxidase enzyme activity of marron.

7.2. Materials and Methods

7.2.1 Animal and feed preparation

Marron, *Cherax cainii* (Austin, 2002) 10.83±0.28g were supplied by Marron Growers Association (MGA) of Western Australia located in Northcliffe and Manjimup. The marron were acclimated to experimental tanks and feed for 2 weeks before the experiment commenced. During the acclimation, marron were fed with a basal diet at 1.5 % of total biomass per tank once a day at 1700 hours.

Commercial feed was purchased from Glenn Forrest Pty. Ltd with a composition of 26% crude protein, 9% crude fat and 5% crude ash. The commercial marron feed was homogenised using a blender to obtain a desirable pellet size for marron and for inclusion of *B. mycoides*, OS (Sel-Plex) and synbiotic of *B. mycoides* and OS (Sel-Plex) before storing at 4^{o}C. *Bacillus mycoides* was added at 10^{8}CFUg^{-1}of feed following the previous study (Ambas et al., 2013), whereas OS (Sel-Plex) was supplemented at 0.2 gkg^{-1}of feed (Nugroho & Fotedar, 2013b).

7.2.2 Experimental Set up

The experimental tanks were 250 L cylindrical plastic tanks (80 cm diameter and 50 cm high) filled with freshwater and supplied with constant aeration. The tank was also equipped with a submersible thermostat set to 24^{o}C. Water in each tank ran continuously at a rate of approximately 3 L min^{-1} using recirculating biological filtration. Sufficient PVC pipes of appropriate diameters were placed into each tank as marron shelters. Following the acclimation, the marron were distributed equally into twelve experimental culture tanks at the density of 12 marron tank^{-1}, where each group was in triplicate.

7.2.3 Data Collection

- ***Total and Differential Haemocyte Count***

The total haemocyte count (THC) and differential haemocyte count DHC (preferably proportion of hyaline cell) of marron were measured on the final day of the feeding trial. Haemocyte sample preparation and calculation were done using an established method (Jussila et al., 1997; R. Fotedar, 1998). Briefly, 0.5 mL of haemolymph withdrawn from the second last ventral segment of marron was placed into a haemocytometer (Neaubauer, Munich, Germany) immediately viewed under a camera equipped microscope then images were taken for THC and DHC counts. For each treatment group, the procedure was repeated ten times using a different animal. The total haemocyte count was calculated as THC = (cells counted x dilution factor x 1000)/volume of grid (0.1 mm^{3}).

The proportion of hyaline cells (DHC) was also prepared using established methods (Bancroft & Stevens, 1977; Hai & Fotedar, 2009). One drop of the mixture of anticoagulant and haemolymph was smeared onto a glass microscope slide and air-dried before fixing in

70% methanol for 10 min. The slides were stained in May–Grunwald and Giemsa for 10 min each. Identification of the haemocyte cell groups followed the criteria described by Bauchau (1981) and Johansson et al., (2000). On each slide, a total of 200 cells were counted and the percentage of a haemocyte group was calculated using the equation:

DHC (%) = (Number of haemocytes cell type/total haemocytes cells counted) x 100

- ***Haemolymph bacteria (Bacteraemia)***

Assessment of bacteraemia was performed following the established procedure described by Fotedar *et al*., (2001) with a minor modification. The haemolymph was withdrawn into a sterile syringe and placed onto a sterile glass slide from which a 0.05 mL aliquot was smeared onto a blood agar (BA) plate. This technique was effective in removing bubbles of the haemolymph so that an accurate volume was smeared onto the plates. Subsequently, the plates were placed in a sterilised container before overnight incubation at 25^{o}C. The total colony forming units (CFU) for each plate and CFU mL^{-1} were calculated on the basis of a total volume of 0.05 mL^{-1} plate.

- ***Haemolymph clotting time***

Determination of haemolymph clotting time followed the established method (S. Fotedar et al., 2001; Jussila et al., 2001). Briefly, the haemolymph of marron was withdrawn using a sterile syringe and dispensed into an Eppendorf tube. A 30 µL aliquot was quickly transferred and drawn into a capillary tube then the tube was repeatedly inverted until the haemolymph stopped moving, which was noted as haemolymph clotting time (seconds).

- ***Glutathione peroxidase* (*GPx*)**

Sample preparation and determination of the GPx activity in marron tissue followed established method (Rotruck et al., 1973)**.** In brief, marron tissue was diluted with physiological saline at a ratio of 1:1 and stored at 4^{o}C until used. To calculate GPx activity, 0.2 mL tissue homogenates (homogenized in 0.4 M sodium phosphate buffer, pH 7.0), 0.1 mL 10 mM sodium azide, 0.2 mL 0.2 mM reduced glutathione, and 0.1 mL 0.2 mM hydrogen peroxide were mixed. Subsequently, the mixtures were incubated for 10 min at 37°C after which 0.4 mL of 10% trichloroacetic acid (TCA) was added to stop the reaction. The mixtures were centrifuged at 3200 rpm for 20 min. The supernatant was assayed for

glutathione content using Ellman's reagent (9.8 mg 5,5´-dithiobis-[2-nitrobenzoic acid] [DTNB] in 100 mL 0.1% sodium citrate). The GPx activity was expressed as micrograms of GSH consumed per minute per milligram of protein.

- ***Intestinal bacterial density***

Bacterial density in marron intestine was determined following the established protocol (Hai & Fotedar, 2009). In brief, five marron of equal size from each treatment group were selected and rinsed in distilled water. Subsequently, the shells were cleaned with 70% alcohol then rinsed again in distilled water to remove the external bacteria. Following dissection, the intestine of each marron was removed and weighed, then homogenised using a sterilise pestle and mortar. Subsequently, the homogenates were diluted serially with sterilise normal saline then lawn inoculated to nutrient agar (NA) plates prior to incubation for 24 h at 25 ^{o}C. The total colony forming units (CFU) for each plate and CFU mL^{-1} were calculated on the basis of a total volume of 0.05 mL plate^{-1} (Buller, 2004).

- ***Effect of OS on B. mycoides***

Sel-Plex (Alltech) does not dissolve in water, but does dissolve in NaOH. The recommended dose as a feed additive is 0.2g kg^{-1}. To determine whether OS (Sel-Plex) dispersed in water, or dissolved in 0.08 % sodium hydroxide would affect the growth of *B. mycoides*, an experiment was performed by adding the Sel-Plex and solvent to media as treatments in triplicate as follows; (1) *B. mycoides* in TSB only, (2) *B. mycoides* in TSB with 0.2 g kg^{-1} OS (Sel-Plex), (3) *B. mycoides* in sodium hydroxide added to TSB. To achieve the lowest concentration of sodium hydroxide which dissolved Sel-Plex 0.2 g kg^{-1} OS (Sel-Plex), a working solution of OS (Sel-Plex) in sodium hydroxide was prepared by a serial dilution to the desired concentration.

Prior to the experiment, pure *B. mycoides* was grown on BA plates and incubated overnight at 24^{o}C, then emulsified in sterile normal saline to be used as an inoculum. Subsequently, 100 uL of the inoculum was added to each media of the three treatments then incubated 24h at 25^{o}C. Determination of *B. mycoides* density in each treatment was obtained by total bacterial count on BA plates (Buller, 2004).

- ***Specific Growth Rate (SGR)***

The specific growth rate (SGR) of marron was determined by measuring the average weight of each marron from each treatment group at the beginning and end of the feeding trial. The specific growth rate was calculated as follows:

SGR (%) = 100 x (lnWt – lnWo) t^{-1},
where SGR is the specific growth rate in weight (% g day^{-1}), Wt is the weight of marron at measurement (t) and at the commencement (W0), where t is experimental period (day).

- ***Survival rate (%)***

At the end of the experimental period, the number of marron in each tank was counted and survival rate was calculated using the following formula;

SR = (Nt/No) x 100

where SR is the survival rate (%); Nt is the number of marron at time t and No is the number of marron at the commencement (0), respectively.

- ***Water Quality***

The water quality in each tank was kept at optimum conditions for marron by performing water exchange at a rate of 10-15% of the total water volume twice a week, after siphoning out the faeces and uneaten feed. Several water quality parameters were monitored weekly including nitrite which was measured using Calorimeter PR 1890, USA; temperature and pH using a digital pH/mV/C meter, Cyberscan pH300, Eutech instruments Singapore; and dissolved oxygen using a digital DO meter SM600, Milwaukee, Romania.

7.2.4 Data analysis

The data were analysed using SPSS statistical package version 22.0 for Windows and Microsoft Excel. Significant differences among treatment means were determined using one way analysis of variance (ANOVA). All significant tests were performed at $P < 0.05$ level. The results were presented as means ± SE (standard error).

7.3. Results

- ***Immune competence***

Immune competences selected in this study (THC, DHC, bacteraemia, clotting time and GPx activity) are presented in Table 7.1 and Figure 7.1. All immune parameters observed were significantly higher (P<0.05) in supplement-fed marron compared to basal diet-fed marron.

Table 7.1. Immune competence (mean ± SE) of marron fed *B. mycoides*, OS and synbiotic diets.

Parameters	Control	*B. mycoides*	OS	*B. mycoides*+OS
THC (10^6/mL)	2.858±1.1^{c}	9.471±0.8^{a}	4.162±1.0^{b}	8.129±1.4^{a}
Hyaline cell (%)	24.4±3.2 ^{c}	40.8±4.2 ^{a}	32.5±3.1 ^{b}	38.3±2.9 ^{a}
Bacteraemia (CFU/mL)	4020±1.686 ^{c}	668±433 ^{a}	2584±832 ^{b}	2576±470 ^{b}
Clotting time (sec)	71.7±6.9 ^{c}	36.4±6.8 ^{a}	56.9±5.6 ^{b}	53.7±12.8 ^{b}

B. mycoides was added at 10^8CFUg-^{1}of feed, whereas OS was supplemented at 0.2 ppm kg-1of feed. Data in the same row having different superscripts indicates significantly different at 0.05 .

Synbiotic use of *B. mycoides* and OS synergistically improve immunity particularly the GPx enzyme activity of marron and to some extent THC and DHC (hyaline cell). In general, the synbiotic feed improved marron immunity which significantly higher compared to OS and basal diet fed marron but comparable to the single use of *B. mycoides.*

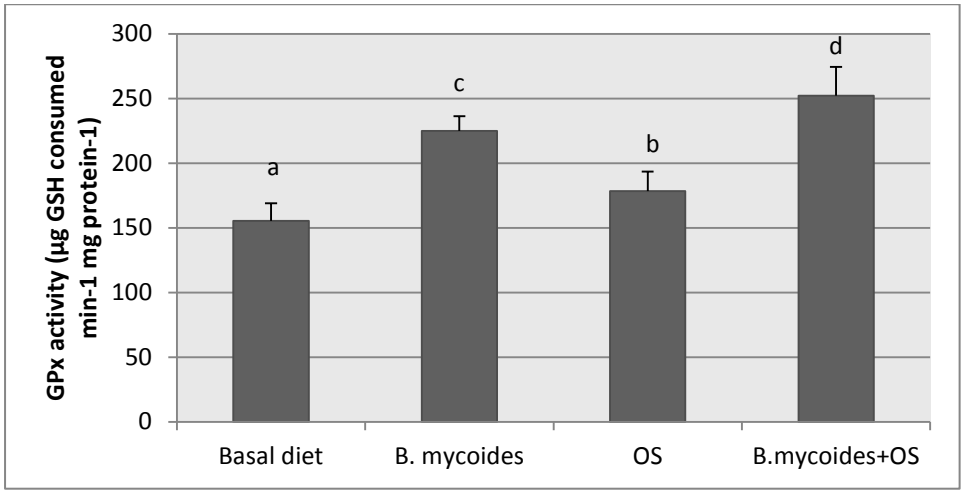

Figure 7.1. GPx activity (µg of GSH consumed per minute per milligram of protein) of marron muscle fed basal, *B. mycoides*, OS and synbiotic diets. Different letters over bars indicates significantly different at 0.05.

- ***Intestinal bacteria density***

At the termination of the feeding trial, the intestinal bacterial density (million $g^{-1}$of gut) was significantly different ($P<0.05$). Marron fed *B. mycoides* showed the highest intestinal bacterial density (3.399±825 CFU$g^{-1}$of gut) compared to basal diet-fed marron (519±176 CFU $g^{-1}$of gut) (Fig. 7.2).

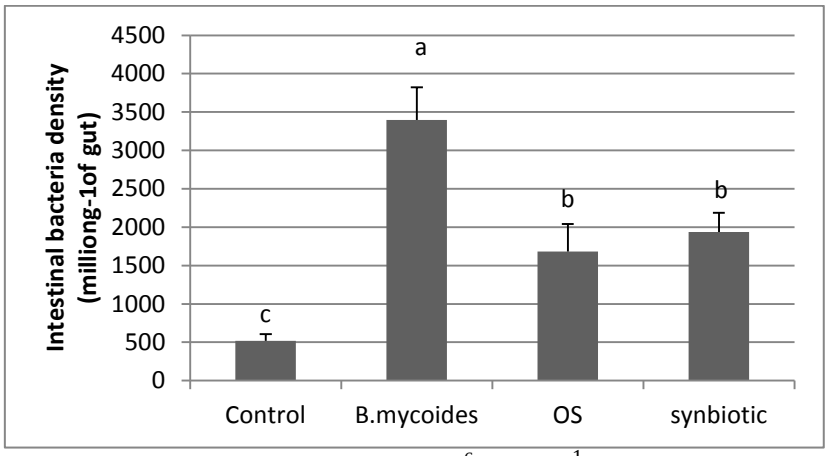

Figure 7.2. Intestinal bacterial population (10^6 CFU $g^{-1}$of gut) of marron fed various diets. Different letters over bars indicates significantly different at 0.05.

- ***Effect of OS on B. mycoides***

The growth (number of colonies) of *B. mycoides* was measured by total bacterial count after 24h incubation and indicated that dissolved OS (Sel-Plex) in TSB media did not improve the growth of *B. mycoides*. In fact, the number of *B. mycoides* colonies was significantly lower ($186.6x10^6$ CFU mL^{-1}) in TSB media containing OS compared to a count of $382.6x10^6$ CFU mL^{-1} in TSB media without OS. In addition, 0.08 % of sodium hydroxide used as an OS solvent did not inhibit *B. mycoides* growth, as the total colony count in sodium hydroxide prepared with TSB media was similar ($366.6x10^6$ CFU mL^{-1}) to the total colony of *B. mycoides* in TSB only.

- ***Specific Growth Rate (SGR)***

Growth of marron fed various supplemented diets at the end of the feeding trial is presented in Table 7.2. The highest specific growth rate was detected in marron fed with *B. mycoides* supplemented diet. Although the lowest growth rate was observed in marron fed with the basal diet, the growth rate was not significantly different to OS or synbiotic-fed marron.

Table 7.2. Specific growth rate (mean ± SE) of marron fed *B. mycoides*, OS and synbiotic diets.

Parameters	Basal diet	*B. mycoides*	OS	*B. mycoides*+OS
Initial weight (g)	10.83±0.28	10.83±0.28	10.83±0.28	10.83±0.28
Final weight (g)	13.06+1.52	14.29±1.55	13.21±1,57	13.71±1.63
SGR (% day^{-1})	0.16±0.05^{a}	0.21±0.07^{b}	0.17±0.06^{a}	0.18±0.04^{a}

B. mycoides was added at 10^{8}CFUg-1of feed, whereas OS was supplemented at 0.2 ppmkg-1of feed. Data in the same row having different superscripts indicates significantly different at 0.05.

- ***Survival rate (%)***

The survival rate of marron fed various supplemented diets at the termination of the feeding trial is presented in Fig.7.3. No mortality was observed in tanks of marron fed *B. mycoides*, OS (Sel-Plex) or a combination of both, but deaths occurred in basal diet-fed marron.

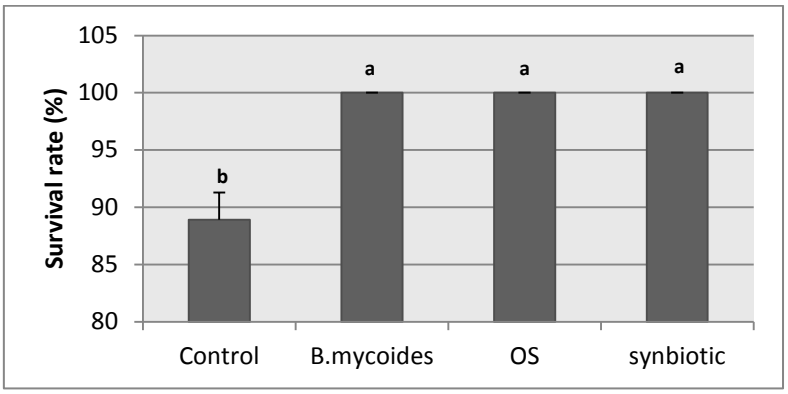

Figure 7.3. Survival rate (%) of marron fed different diets at the termination of the feeding trial. Different letters over bars indicates significantly different at 0.05.

- ***Water quality***

The water quality in the present study was maintained within the optimum range for marron growth, as each tank was equipped with a bio-filter recirculation system and regular water exchange was conducted. No significant difference was observed for the water quality in tanks among the treatments (Table 3).

Table 7.3. Water quality parameters in experimental tanks during trial

Parameters	Control	*B. mycoides*	OS	*B. mycoides*+OS
Nitrite (ppm)	0.045±0.01^{a}	0.034±0.01^{a}	0.035±0.01^{a}	0.033±0.01^{a}
pH	7.60±0.05^{a}	7.62±0.07^{a}	7.58±0.05^{a}	7.65±0.03^{a}
Temperature (^{o}C)	23.27±0.81^{a}	23.47±0.62^{a}	23.65±0.84^{a}	23.53±0.43^{a}
Dissolved oxygen (ppm)	6.21±.020^{a}	6.25±0.18^{a}	6.24±0.42^{a}	6.21±0.23^{a}

*Data in the same row having different superscripts indicates significantly different at 0.05.

7.4. Discussion

Probiotics and prebiotics have been proven to improve immunity and physiological aspects in various aquaculture species; hence studies on feed supplements are now focused on synbiotics to explore further synergistic effects in aquatic animals.

Total Haemocyte Count, proportion of hyaline cell, bacteraemia and haemolymph clotting time (Jussila et al., 1997; Sang et al., 2009), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) (Nugroho & Fotedar, 2013a) have been successfully used as tools to evaluate immunity and health status in marron studies. The present study demonstrated that supplementation with either *B. mycoides*, OS (Sel-Plex), or their combination as a synbiotic significantly improved marron immune parameters, particularly GPx enzyme activity and THC . The significant increase in the GPx and THC in the synbiotic-fed marron compared to use of OS (Sel-Plex) alone or the basal diet-fed marron indicated a synergistic effect of the synbiotic on marron immunity. Previous studies using OS (Sel-Plex) also demonstrated improved marron THC (Sang et al., 2009; Nugroho & Fotedar, 2013c, 2013a; Sang et al., 2011b), and the use of *B. mycoides* (Ambas et al., 2013). Increased THC and improved immunity of aquatic animals fed a synbiotic diet has also been observed in shrimps *Penaeus japonicus* (Zhang et al., 2011) and *Litopenaeus vannamei* (Li et al., 2009), rainbow trout *Oncorhynchus mykis*s (Rodriguez-Estrada et al., 2009; Mehrabi et al., 2012), Japanese flounder *Paralichthys olivaceus* (Ye et al., 2011), sea cucumber *Apostichopus japonicas (Zhang et al., 2010)* and koi *Cyprinus carpio koi* (Lin et al., 2012). However, synbiotic use of β-glucan and *Bacillus subtilis* or *Pediococcus acidilactici* did not improve THC of shrimp, *Litopenaeus vannamei* (Wongsasak et al., 2015).

The haemocyte is responsible for destroying invasive microorganisms (bacteraemia) and foreign particles, thus greater THC contributes to better cellular immunity (Sang et al., 2011a). In Australia, bacteraemia has been associated with mortalities in crayfish (Eaves & Ketterer, 1994; Edgerton & Owens, 1999), as a result of exposure to environmental stressors (Jussila et al., 1997; Evans & Edgerton, 2002; Sang et al., 2009). Once pathogens or foreign particles enter the haemocoel, the haemocyte initiates phagocytosis (Li et al., 2010), where in crayfish the phagocytosis is chiefly executed by hyaline cells (Johansson et al., 2000). In the present study, bacteraemia of probiotic, OS and synbiotic fed marron was significantly lower

compared to basal diet-fed marron indicating that probiotic, OS and their synbiotic effectively reduces foreign particles in the haemolymph as a result of higher THC and proportion of hyaline cells.

Chronic bacteraemia results in immunosuppression, reduced growth rates in *C. quadricarinatus* (Edgerton & Owens, 1999) and mortality, thus clearance of the bacterial pathogens in the circulating haemolymph is essential to maintain animal health. Sang et al., (2009) and Nugroho & Fotedar (2013c) demonstrated that greater THC and a higher proportion of hyaline cells reduce bacteraemia in marron. Our previous study also confirmed a significant bacteraemia reduction in marron fed *B. mycoides* at 48h and 96h post injection challenge test with the pathogen *V. mimicus* (Ambas et al., 2013). Chisholm & Smith (1995) evaluated antibacterial activity of four species of crustaceans and found that their haemocytes contained factors able to reduce the viable count of injected bacteria within 4 hours.

The antioxidant enzymes, including glutathione peroxidase (GPx), which is a very potent antioxidant, protect the body from damage from oxidation by free radicals (Chiu et al., 2010), which can cause cellular damage and oxidative stress (Parrilla-Taylor & Zenteno-Savín, 2011). In this study, GPx was significantly higher in muscle of synbiotic-fed marron (Fig.1) compared to others test diets. Supplementation with OS (Sel-Plex) also significantly improved the antioxidant enzymes glutathione-S-transferase (GST) and GPx in marron haemolymph (Nugroho & Fotedar, 2013a). In contrast, synbiotic use of β-glucan and *B. subtilis* in feed resulted in significantly lower superoxide dismutase (SOD) activity in shrimp *Litopenaeus vannamei,* compared to β-glucan alone (Wongsasak et al., 2015).

The specific growth rate (SGR) of marron improved significantly when fed *B. mycoides*, OS OS (Sel-Plex) and their synbiotic in supplemented feeds compared to basal diet-fed marron. However, marron fed synbiotic feed was not significantly different to singular supplementation of either *B. mycoides* or OS (Sel-Plex). Improved growth using synbiotic feed has been demonstrated in rainbow trout *Oncorhynchus mykiss* fingerlings (Rodriguez-Estrada et al., 2009; Mehrabi et al., 2012), European lobster *Homarus gammarus* (Daniels et al., 2010), Japanese flounder *Paralichthys (Ye et al., 2011),* and Nile tilapia *Oreochromis niloticus* (Hassaan et al., 2014).

The main purpose of using both prebiotics and probiotics in aquatic animals is to improve health, immunity and growth by stimulating commensal intestinal bacteria of the host. The feed supplementation of *B. mycoides* or OS (Sel-Plex) alone significantly improved intestinal bacterial density compared to the basal diet-fed marron; however, the synbiotic use did not further improve the total bacterial population compared to single supplementation. The highest intestinal bacterial load of *B. mycoides*-fed marron compared to basal diet fed marron and other probiotic candidates fed marron was also demonstrated in the previous study using various probiotic sources (Ambas et al., 2013). Zhang et al., (2011) observed a significant increase in total bacterial counts of shrimp *P. japonicus* fed a synbiotic diet. Microbiota of GIT can be considered as a metabolically active organ (Gaggìa et al., 2010), which provides an initial barrier to pathogen entry (Sugita et al., 1988; Verschuere et al., 2000; Ramirez & Dixon, 2003; Ringø et al., 2003), thus its stability (density and diversity) is essential for the health of an organism (Rollo et al., 2006; Denev et al., 2009).

The effect of the Sel-Plex, yeast-derived OS, on *B, mycoides* evaluated in this study demonstrated that density of *B. mycoides* was higher in OS-free TSB media compared to *B. mycoides* in TSB+OS media indicating that OS (Sel-Plex) did not improve the growth of *B. mycoides*. Cerezuela *et al*.(2011) reviewed synbiotic studies and concluded that dietary synbiotic effect is most likely dependent on the fish species, feeding dose and duration, and the type of prebiotics and probiotics synbiotic combinations. In shrimp, *L. vannamei*, a combination of β-glucan and *Pediococcus acidilactici* was better than β-glucan and *B. subtilis* in terms of SOD activity of the animal (Wongsasak et al., 2015).

Synergistic effect of *B. mycoides* and OS was observed on marron immunity (GPx and THC), but not on the growth rate. A similar result was observed in koi, *Cyprinus carpio koi*, using dietary chitosan oligosaccharides and *B. coagulansi*, which synergistically improved innate immunity and resistance but did not improve the growth (Lin et al., 2012). It was due to the fact that the probiotic *B.mycoides* did not grow well on OS added substrate and resulted in a lower intestinal bacterial population (Fig.2), as each strain has substrate preference (Rastall & Maitin, 2002). No synbiotic studies have examined *in vitro* the effect of pure prebiotics solely on the selected probiotics to determine whether the prebiotics improve or inhibit the growth of the probiotics. This test is essential to avoid any adverse effects of the synbiotic feeds on

the hosts as was found in sea bream, *Sparus aurata*, where the combined administration of inulin and *B. subtilis* increased susceptibility to infection (Cerezuela et al., 2012).

Members of the *Bacillus* genus represent the majority of bacteria used as probiotics due to their physical and biological characteristics (Wang et al., 2008). The most widely used in synbiotic studies include *B. coagulans* (Lin et al., 2012), *B. subtilis* (Zhang et al., 2010; Geng et al., 2011; Zhang et al., 2011; Cerezuela et al., 2012; Cerezuela et al., 2013; Zhang et al., 2014; Wongsasak et al., 2015), *B. licheniformi*s (Zhang et al., 2011; Hassaan et al., 2014), *B. megeterium* (Li et al., 2009), and *B. clausii* (Ye et al., 2011). In the present study, *B. mycoides* was selected as it was of host origin and possessed a number of probiotic properties (Ambas, Buller, et al., 2015) such as improved marron immunity against *Vibrio mimicus* (Ambas et al., 2013) and improved GIT health status (Ambas, Fotedar, et al., 2015).

7.5. Conclusion

The present study suggested that *B. mycoides* and OS (Sel-Plex) contribute a synergistic effect on marron immunity particularly on oxidative enzyme activity (GPx) and the THC, but to a lesser extent on growth rate. In addition, an *in vitro* test revealed that dissolved OS (Sel-Plex) in TSB media did not improve *B. mycoides* growth.

CHAPTER 8. Effect of supplementation period of probiotic *Bacillus mycoides* on marron *Cherax cainii* (Austin, 2002) health

(Abstract accepted at Indonesia Aquaculture Conference, 2015)

8.1. Introduction

Supplementation with probiotics is becoming an integral part of aquaculture practices to obtain high production and has shown enough evidence to play an important role in aquaculture (Nayak 2010; Newaj-Fyzul *et al.* 2014), however probiotics often exert host specific and strain specific effects (Hai *et al.* 2010; 2015). Many factors significantly affect various desired outcomes including probiotic strains, form of the supplements, vector of administration, dosage and duration of the probiotic application (Nayak 2010; Mohapatra *et al.* 2013).

The use of probiotic bacteria is a strategy to overcome microbial problems (Skjermo *et al.* 2015), whereas modulation of the immune system is one of the most commonly purported benefits of the probiotic (Nayak 2010), thus the majority of probiotic application is for disease prevention (Kesarcodi-Watson *et al.* 2012). However, other than immunity modulation, numerous health benefits could be obtained with probiotic supplementation (Nayak 2010) such as improved growth (Gildberg *et al.* 1997 ; Rengpipat *et al.* 1998; Hai *et al.* 2009a; Merrifield *et al.* 2010a; Rahiman *et al.* 2010; Sun *et al.* 2010; Giannenas *et al.* 2015), improved enzyme activity (Ziaei-Nejad *et al.* 2006; Wang Y-B 2007; Gómez & Shen 2008; Zhou *et al.* 2009; Ye *et al.* 2011; Ray *et al.* 2012) and improved stress tolerance (Rollo *et al.* 2006; Liu *et al.* 2010; Hoseinifar *et al.* 2015), whereas modulation and restoration of intestinal bacteria population (Balca´zar *et al.* 2007; Denev *et al.* 2009; Gaggìa *et al.* 2010; Dimitroglou *et al.* 2011; Tapia-Paniagua *et al.* 2011; Ige 2013) as a contribution to the overall health status of the host has been underestimated (Gómez *et al.* 2007; Ghanbari *et al.* 2015).

Modulation and persistency of the probiotic in the gastrointestinal tract (GIT) in the host is commonly used as primary indicator of probiotic action. Gatesoupe (1999), surmised persistency of probiotics supplemented either daily or continuously in various fish species and shellfish is greatly varied. To improve persistency of probiotics, and for safety reasons, it is widely accepted that host origin (autochthonous) strains, especially mucus inhabitants (Jöborn *et al.* 1997; Vine *et al.* 2004; Kim *et al.* 2007; Merrifield *et al.* 2010c) with probiotic

properties, offer improved safety, ability to colonise and multiply, and can remain predominant and persist in the same host (Gatesoupe 1999; Verschuere *et al.* 2000; Rollo *et al.* 2006; Balca´zar *et al.* 2007; Merrifield *et al.* 2010b) after changing to basal diets (Nayak 2010) compared to commercial probiotic supplemented diets (Ridha & Azad 2015).

Duration of supplementation significantly affects the establishment, persistence and subsequent induction of immune responses and desirable outcomes (Sharifuzzaman & Austin 2009; Nayak 2010), but the effect of feeding duration on probiotic efficacy remains scarcely investigated (Merrifield *et al.* 2010b). Probiotic studies have assessed potential applications for a periods as short as 24h (Skjermo *et al.* 2015) or 3 days (Sakai *et al.* 1995; Gram *et al.* 1999), up to 5 months (Aubin *et al.* 2005); but the effect of long-term efficacy is not available (Merrifield *et al.* 2010b) and the basis for choosing the probiotics supplementation period is often unclear (Sharifuzzaman & Austin 2009).

The present study evaluated the effect of feeding duration using a marron *Cherax caiini* (Austin, 2002) host origin strain, *Bacillus mycoides* (Ambas *et al.* 2015a) with previously demonstrated health benefits (Ambas *et al.* 2013; Ambas *et al.* 2015b) by measuring intestinal bacterial population, hepatosomatic index (Hiw), total haemocyte count (THC), haemolymph bacteria (bacteraemia), glutathione peroxide (GPx) enzyme activity and survival rate.

8.2. Materials and Methods

8.2.1. Animals and Experimental system

Marron, *Cherax cainii* (Austin, 2002) were supplied by Aquatic Resource Management Pty Ltd., Manjimup, Western Australia. Prior to commencement of the feeding trial, the juveniles were acclimated to the experimental conditions and basal diet for two weeks in the culture tanks. During the acclimation period, marron were fed the basal diet at a rate of 1.5% of the total biomass per tank once per day at 1700 hours.

Twelve cylindrical culture tanks (80 cm diameter and 50 cm high and 250 L in capacity) were used in this study. The tanks were placed on three steel standing racks in a purpose-built laboratory designed for Curtin Aquatic Research Laboratory (CARL) Unit, Curtin University Australia. Each tank was filled with freshwater and supplied with constant aeration and

provided with a submersible thermostat set to 24^{o}C and a re-circulating biological filtration system (Fluval 205, Askoll, Italy) to maintain good water quality. Continuous running water in each tank was provided at a rate of approximately 3 L/min. The tanks contained sufficient marron shelters using PVC pipes of appropriate diameters.

After acclimation, marron of similar size were selected and distributed randomly into twelve culture tanks at12 marron/tank. The marron were divided into four treatment groups as follows and each consisted of three replicates;
Marron fed solely on basal diet until termination of the trial (BD)
Marron fed with probiotic diet for one week then changed to basal diet (Pro-1w)
Marron fed with probiotic diet for two weeks then changed to basal diet (pro-2w)
Marron fed with probiotic diet for four weeks then changed to basal diet (pro-4w).
This basal diet was fed to marron up to one week after probiotics feeding ceased.

8.2.2. **Feed and test diets**

The commercial marron diet supplied by specialty feeds, Glen Forrest Western Australia was used as control or basal diet. The proximate composition of the basal diet was: 26% crude protein, 9% crude fat and 5% crude ash.

The experimental diets (basal and probiotic supplemented diets) were prepared prior to the feeding trial. The probiotic supplemented diet using *Bacillus mycoides*, a host-origin strain isolated from mature healthy marron with probiotic properties (Ambas *et al.* 2015a) was prepared on a weekly basis. Identification of the probiotic isolate was performed by the Bacteriology Laboratory, Animal Health Laboratories, Department of Agriculture and Food, Western Australia, using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Bioscience Corporation), Vitek Compact II (Biomerieux) and conventional biochemical methods according to standard procedures and identification methods (Buller 2004). The strain was suspended into 1 mL aliquots of GLL (Glycerol Lab Lemco broth) and stored at - 80°C.

The probiotic diet was prepared according to Hai and Fotedar (2009). In brief, a pure culture of *B. mycoides* was grown on blood agar (BA) plates for 24h at 25°C. Colonies in logarithmic phase of growth were emulsified in sterilised distilled water and serially diluted. The optical

reading of each serial dilution was recorded, and a viable count performed to obtain a standard curve for inoculum density. From the standard curve, the amount of the diluted probiotic was calculated to achieve the desirable supplementation density of 10^8 colony forming unit (CFU) per gram of feed; a density used in most *Bacillus* species studies. The pellets were air dried, packed and stored at 4°C until used.

8.2.3. Data Collection

The immune parameters including total haemocyte count, total bacterial count in haemolymph (bacteraemia), Hiw and survival rate were performed at the termination of each probiotic feeding period, except the intestinal bacteria population which was measured one week after changing to the basal diet.

- ***Intestinal bacteria population***

Intestinal bacterial density as indicator of marron heath was determined following the established protocol (Hai *et al.* 2009a). Five equal size marron from each treatment group were selected and rinsed in distilled water prior to intestinal bacterial count. The shells were cleaned with 70% alcohol then rinsed in distilled water to remove the external bacteria. Following dissection, the intestine from individual marron was removed and weighed, then homogenised using a sterilised pestle and mortar. Serial dilutions of the homogenates in sterilised normal saline were performed and lawn inoculated to blood agar (BA) plates prior to incubation for 24 h at 25^{o}C. The total colony forming units (CFU) for each plate and CFU/mL were calculated on the basis of a total volume of 0.05 mL/plate from each serial dilution (Buller 2004). Along with intestinal bacterial density, persistency of supplemented probiotic, *B. mycoides* was also observed up to two weeks post probiotic supplementation or basal diets.

- ***Total haemocyte count (THC)***

Measurement of THC followed the established methods described by Jussila *et al.* (1997). In brief, 0.5 mL of haemolymph withdrawn from the second last ventral segment of marron placed into a haemocytometer (The Neaubauer Enhanced Line, Munich, Germany) counting chamber and immediately viewed under 100-fold magnification of camera-equipped microscope and images were taken for later THC counts. Cells were counted in both grids,

and the mean was used as the haemocyte count. For each treatment group, the procedure was repeated using five different animals. The total haemocyte count was calculated as THC = (cells counted x dilution factor x 1000)/volume of grid (0.1 mm^3).

- ***Glutathione peroxidase (GPx) enzyme activity***

The preparation and determination of the GPx activity in marron tissue followed established methods (Rotruck *et al.* 1973). Marron tissue was diluted with physiological saline at a ratio of 1:1 and stored at 4^oC until used. To calculate GPx activity, 0.2 mL tissue homogenates (homogenized in 0.4 M sodium phosphate buffer, pH 7.0), 0.1 mL 10 mM sodium azide, 0.2 mL 0.2 mM reduced glutathione, and 0.1 mL 0.2 mM hydrogen peroxide were mixed., The mixtures were incubated for 10 min at 37°C after which 0.4 mL of 10% trichloroacetic acid (TCA) was added. The mixtures were then centrifuged at 3200 rpm for 20 min. The supernatant was assayed for glutathione content using Ellman's reagent (9.8 mg 5,5′-dithiobis-[2-nitrobenzoic acid] [DTNB] in 100 mL 0.1% sodium citrate). The GPx activity was expressed as micrograms of GSH consumed per minute per milligram of protein.

- ***Hepatosomatic indices*** **(**Hiw**)**

The wet hepatosomatic indices (Hi_w) of marron fed probiotic supplemented diets at various periods were calculated as per established equations (Jussila 1997; Fotedar 1998). In brief, hepatopancreas of marron from each treatment group were removed, placed in foil and weighed. The wet hepatosomatic indices (Hiw), was calculated as follows;

$Hi_w = W_{wh} \times 100\ W_t^{-1}$

Where;
Hi_w = Wet hepatosomatic indices (%)
W_{wh} = Weight of hepatopancreas (wet)
W_t = Total weight of marron (g)

- ***Survival rate (%)***

Survival rate of marron was calculated at the termination of the experimental period by counting the number of marron in each tank and calculated using the following formula;

SR (%) = (Nt/No) x 100

where SR is the survival rate (%); Nt is the number of marron at time t and No is the number of marron at the commencement (o), respectively.

- ***Water Quality***

To maintain optimum water quality conditions for marron, water exchange at a rate of 10-15% of the total water volume was performed once a week, after siphoning out the faeces and uneaten feeds. Several water quality parameters were monitored weekly including total ammonia (ppm) measured using Calorimeter PR 1890, USA; temperature (^{o}C) and pH using a digital pH/mV/C meter, Cyberscan pH300, Eutech instruments Singapore; and dissolved oxygen (ppm) using a digital DO meter SM600, Milwaukee, Romania.

8.2.4. Data analysis

The data were analysed using SPSS statistical package version 23.0 for Windows and Microsoft Excel 2010. Significant differences among treatment means were determined using one way analysis of variance (ANOVA). All significant tests were performed at $P < 0.05$ level and results were presented as means ± SE (standard error).

8.3. Results

8.3.1. Intestinal bacterial population

Overall, the intestinal bacterial population increased in marron fed both the basal diet and the probiotic over all feeding times. However, intestinal bacterial population of probiotic fed marron was significantly higher ($P<0.05$) than the basal diet fed marron. A significant increase of bacterial population was observed in probiotic fed marron from week-1 to week-2, but increased at slow rate in basal diet fed marron. Intestinal bacterial population among the probiotic fed marron was observed significantly higher ($P<0.05$) in marron fed probiotic for four weeks than the other time periods and the basal diet fed marron.

Table 8.1. Intestinal bacterial population (million CFU/g of gut) of marron fed probiotic supplemented diets for different duration.

Diets	0 week	1 week	2 weeks	4 weeks
Basal diet	$421\pm35.5^{1,a}$	$530.2\pm24.6^{1,b}$	$585\pm20.5^{1,bc}$	$615 \pm 35.5^{1,c}$
Probiotics	$421\pm35.5^{1,a}$	$637.5 \pm 26.7^{2,b}$	$980\pm 54.5^{2,c}$	$1085\pm51.5^{2,c}$

Data in the same row having the same superscript letter (a;b) indicate a similar mean which is not significantly different at α level of 0.05.
Data in the same column within an index having different subscript letters (1, 2) are significantly different at α level of 0.05.

8.3.2 Total haemocyte count (THC)

During the feeding trials the highest THC was observed in marron fed probiotic supplemented diet for four weeks compared to other diets. In general, the THC of probiotic fed marron (one to four weeks) was higher than THC of basal diet fed marron.

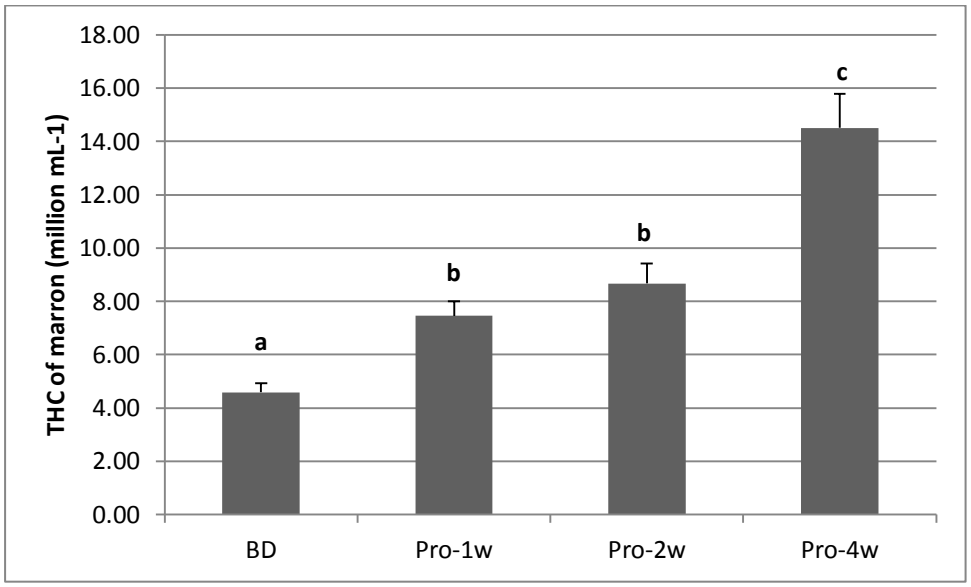

Figure 8.1. THC (million/mL) of marron fed basal and probiotic diets at various period (week). Different letters over bars indicates significantly different at 0.05.

8.3.3. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) enzyme activity of marron fed a basal diet or a probiotic supplemented diet for one to four weeks is presented in Figure 8.2. The results show that the highest GPx enzyme activity (83.27 ug GSH consumed min^{-1} mg protein $^{-1}$) was observed in

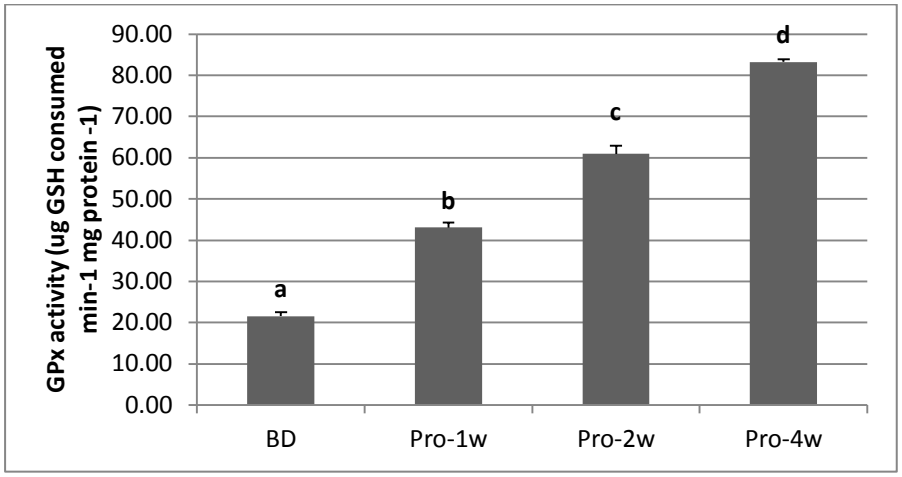

Figure 8.2. GPx enzyme activity of marron tissue muscle fed basal and probiotic diets at various duration (week). Different letters over bars indicates significantly different at 0.05.

probiotic application and the basal diet fed marron. One week probiotic application significantly (P <0.05) improved the GPx enzyme activity of marron compared to GPx of the basal diet fed marron, however the longer probiotic feeding up to four weeks the higher GPx enzyme activity of the marron.

8.3.4 Hepatosomatic indices (H_{iw})

Hepatopancreas has been used as indicator of health and nutritional status of crustacean including marron (Jussila & Mannonen 1997). The wet hepatosomatic indices (Hiw) was significantly higher (P < 0.05) in marron fed probiotic diets for four weeks compared to other feeding periods. The Hiw of two weeks probiotic fed marron is not significantly different from 1 week probiotic fed marron, whereas the Hiw of basal diet fed marron was not significantly (P>0.05) different with the Hiw of 1 week probiotic fed marron.

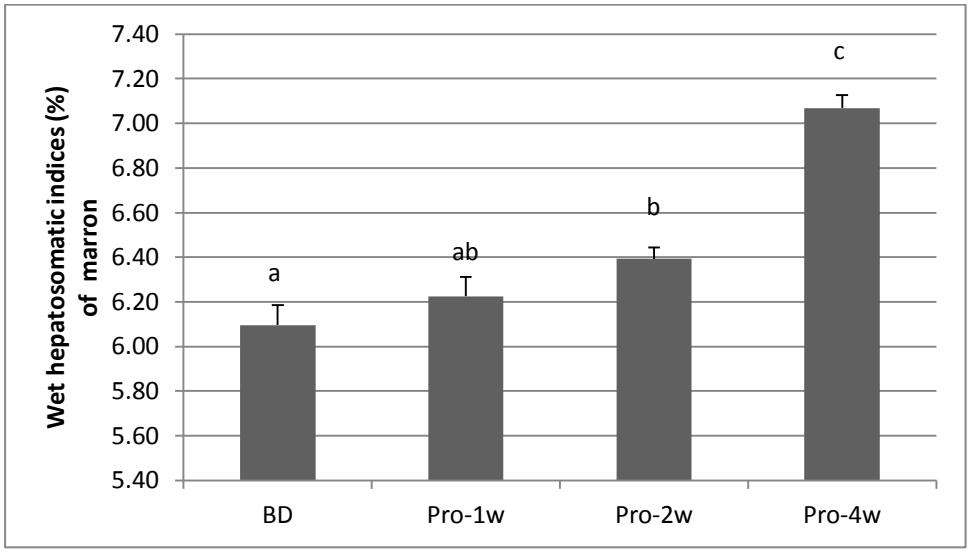

Figure 8.3. Hepatosomatic indices (Hi_w) of marron fed basal and probiotic diets at various duration (week). Different letters over bars indicates significantly different at 0.05.

8.3.5. Survival rate (%).

No mortality was observed in any treatment group during the 6 weeks of feeding. Both probiotic and basal diet feeds nutritionally met the lowest nutrient requirement for marron to survive.

8.3.6. Water quality

The selected parameters namely dissolved oxygen (ppm), temperature (^{o}C), pH and ammonia (ppm) are presented in the Table 8.2. The results revealed that the water quality was within the range for optimum growth of marron (Jussila 1999) as each tank was equipped with bio-filtration and regular water exchanges were performed. There was no significant difference in any of the water quality parameters among the treatments.

Table 8.2. Water quality parameters in experimental tanks during feeding trial

Parameters	Basal diet	1 week	2 weeks	4 weeks
Ammonia (ppm)	0.041±0.02[a]	0.035±0.03[a]	0.038±0.02[a]	0.045±0.05[a]
pH	7.55±0.08[a]	7.65±0.05[a]	7.78±0.05[a]	7.65±0.03[a]
Temperature (^{o}C)	24.27±0.55[a]	24.47±0.65[a]	23.95±0.35[a]	24.35±0.33[a]
Dissolved oxygen (ppm)	6.25±.025[a]	6.55±0.15[a]	6.35±0.45[a]	6.28±0.25[a]

Data in the same row having the same superscript letter (a;b) indicate a similar mean which is not significantly different at α level of 0.05.

8.4. Discussion

The study of the effect of feeding duration on probiotic efficacy remains scarcely investigated (Sharifuzzaman & Austin 2009; Merrifield *et al.* 2010b). Short term supplementation with probiotics has proved beneficial (Merrifield *et al.* 2010b), whereas long-term administration may reduce the efficacy and induce immunosuppression in fish; however the side effects of a long-term administration, especially with probiotics have not been well-studied (Sakai 1999).

The intestinal bacterial population of marron increased significantly one week after probiotic feeding commenced for up to four weeks, indicating that *B. mycoides* was able to stimulate the gut microbial population of marron. Our previous work also demonstrated that *B. mycoides* significantly improved the intestinal bacterial population of marron compared to the commercial probiotic strains (Ambas *et al.* 2013). Modulation of GIT bacterial population and restoration of a normal microbiota constitutes the key components to maintain good health throughout the development stages of the animals (Gomez & Balcazar 2008) and this a stable commensal microbiota is part of the natural resistance to infections (Denev *et al.* 2009).

Supplementation of probiotics, particularly with host origin strains, restores and improves microbial population (diversity and density), as the added probiotic provides a favourable environment for the indigenous intestinal bacteria, whereas potential pathogens are suppressed (Purivirojkul *et al.* 2005; Dimitroglou *et al.* 2011; Tapia-Paniagua *et al.* 2011; Yang *et al.* 2011; Hoseinifar *et al.* 2015). Reduction of either population or diversity will reduce protection provided by the indigenous microbiota (Ige 2013) and diminish other beneficial contributions including enzymes, amino acids, and vitamins (Ringø & Gatesoupe 1998; Bairagi *et al.* 2002; Wang *et al.* 2008; Ray *et al.* 2012).

Persistency within GIT is often used as preliminary indicator for the induction of probiotic efficacy (Gatesoupe 1999; Vine *et al.* 2004), as the probiotic benefits are presumably lost after the probiotic disappear from the host (Merrifield *et al.* 2010b). This argument is supported by several findings where beneficial effects rely on viable cells compared to non-viable cells (Panigrahi *et al.* 2005; Thompson *et al.* 2010), as viability is an important property for adherence and colonization of the intestinal tract of the host (Nayak 2010). In the present study, persistency of *B. mycoides* in marron GIT was observed for up two weeks after changing to a basal diet indicated that this host origin strain may persist in the GIT from the animal they were isolated.

The reduction or disappearance from the GIT after changing to a basal diet was observed in several studies using host origin probiotics. Balcazar et al., (2007) evaluated fish origin lactic acid bacteria (LAB) groups *Lactococcus lactis*, *Lactobacillus sake*i and *Leuconostoc mesenteroides* fed at $x10^6$ cfu/g of feed to brown trout *Salmo trutta* for 2 weeks and detected high numbers in the intestines during the probiotic feeding period but then decreased gradually, although the bacteria could be detected at $1x10^2$ cfu/g two weeks after shifting to a basal diet. In abalone *Haliotis gigantea*, host-derived *Pediococcus* sp. was able to colonized the gut for 12 days after shifting to a commercial basal diet (Iehata *et al.* 2010). Higher gut colonization and longer retention time of host origin probiotic was observed in Nile tilapia *Oreochromis niloticus*, whereas the commercial probiotic disappeared rapidly from the gut (Ridha & Azad 2015). In trout *Oncorhynchus mykiss*, a two weeks probiotic *Lactobacillus rhamnosus* feeding trial resulted in a high number during the probiotic feeding but disappeared from the intestine, skin mucus and water within one week after changing to a

probiotic-free diet (Nikoskelainen *et al.* 2003), whereas a 24h probiotic treatment period in Atlantic cod *Gadus morhua* decreased to initial levels within 4 days (Skjermo *et al.* 2015).

In Atlantic cod *Gadus morhua* L, (Skjermo *et al.* 2015) proposed a strategy for introducing probiotic bacteria to the fish larvae either as a continuous supply or as repeated additions every 3–4 days. This short-term-cyclic feeding could provide direct benefits of short-term application during probiotic and probiotic-free feeding periods, but this could also help to avoid over-stimulating the immune response whilst maintaining a level of protection (Merrifield *et al.* 2010b).

On the other hand, a long dietary probiotic application is advantageous to the host in many aspects (Nayak 2010), as the continual application may lead to high levels of colonisation and modulate GIT microbial populations (Merrifield *et al.* 2010b; Tapia-Paniagua *et al.* 2014). Our previous work also confirmed that marron fed probiotic up to 70 days resulted in an improved THC at day 35^{th} and at day 70^{th} with no mortalitis after pathogen (*Vibrio mimicus*) challenge test (Ambas *et al.* 2013). In crayfish, haemocytes are involved in many immune functions such as phagocytosis, encapsulation, storage and release of the proPO system**,** and cytotoxicity (Johansson *et al.* 2000), whereas marron haemocytes also contain oxidative enzymes such as glutathione-S-transferase (GST) and glutathione peroxidase (GPx) (Nugroho & Fotedar 2013). However, the THC of shrimp *P. vannamei* fed *Vibrio* spp. as probiotic candidates for 4 and 8 weeks showed no differences (Thompson *et al.* 2010). Many studies demonstrated that 2 weeks probiotics feeding results in higher immunity of the animals than the other feeding periods (Nayak 2010), but the longevity of the health effect of probiotics is often uncertain (Newaj-Fyzul *et al.* 2014).

Hepatopancreas is the main energy reserve in crustaceans and a source of various enzymes which used as an indicator of nutritional status in marron (Jussila 1997; Fotedar 1998). In this study, the highest Hiw was observed in 4 week probiotic fed marron, which indicates a better nutritional status compared to other treatments. Our previous work also suggested that *B. mycoides* improved the Hiw of marron especially at day 35 (Ambas *et al.* 2013). The hepatopancreas is also as a source of enzymes (Hammer *et al.* 2000), thus different size of Hiw as a result of probiotic feeding periods also indicates different enzyme activities. In Atlantic cod *G. morhua* L, improvement in the intestinal enzyme activity was observed after

40 days probiotic feeding compared to 20 days of feeding (Lazado *et al.* 2012). Meanwhile, Gómez and Shen (2008) proposed that a prolonged (45 days) probiotic feeding period using the optimum percentage of *Bacillus* spp. could enhance the digestive enzyme activity, whereas short feeding periods (ontogenetic stages) using *B. coagulans* SC8168 on shrimp *P. vannamei* larvae significantly increased some digestive enzyme activities (Zhou *et al.* 2009).

8.5. Conclusion

The present study suggested that supplementation of host origin probiotic *B. mycoides* is required longer to maintain a high population and predominatant levels and for improved health benefits to the host. Further study is required to determine the period till the beneficial effects of dietary supplementation lasts in GIT after the probiotic supplementation is terminated. It is also imperative to know whether the reduction in initial levels of supplemented probiotic strain is sufficient to provide the desired beneficial outcomes and the level of protection to the host.

CHAPTER 9. Performance of customized probiotic *Bacillus mycoides* on marron *Cherax cainii* (Austin, 2002) in earthen commercial marron ponds.

9.1. Introduction

Today, it has been widely accepted that probiotics play a significant role in aquaculture (Nayak, 2010, Merrifield et al., 2010, Newaj-Fyzul et al., 2014, Hai, 2015) as an ecofriendly method for disease control for sustainable aquaculture (Sihag and Sharma, 2012), however there has been only a few *in vivo* studies on the use of probiotics in a controlled environment (Tinh et al., 2008). In screening a probiotic candidate, an *in vivo* test is essential (Verschuere et al., 2000, Balcázar et al., 2006, Kesarcodi-Watson et al., 2008, Vine et al., 2004) as *in vivo* physiology is more complex and different from *in vitro* monoculture (Tinh et al., 2008). No study has ever compared probiotic beneficial effects *in vitro* and *in vivo* (Sahu et al., 2008).

The complexities encountered by an added probiotic under outdoor conditions include (i) the uncertainty for the probiotic to remain viable in an aquatic environment (Newaj-Fyzul et al., 2014), (ii) interaction with other strains in the host environment (Wong and Rawls, 2012, Mickeniene, 1999), (iii) selection process by the host GIT (Ringø et al., 2007a), (iv) competition with the indigenous GIT inhabitants (Vine et al., 2004), (v) viability during storage (Burr and Gatlin, 2005). To complicate the issue, not all of the authors examined the viability of the probiotics during the feed preparations after the microbial cells have been added to the feed (Newaj-Fyzul et al., 2014). For these reasons, the host origin (host GIT and its environment) with favourable probiotic properties is preferable by most of the authors for an ideal candidate (Gatesoupe, 1999, Verschuere et al., 2000, Merrifield et al., 2010, Rollo et al., 2006, Hai et al., 2009b, Nayak, 2010) as its efficacy is likely to be highest in the host and particularly in its natural environment (Verschuere et al., 2000, O'Sullivan, 2001).

Bacillus mycoides is a predominated bacterium isolated from a number of healthy adult farmed marron GIT that exhibit favourable probiotic properties such as non-pathogenicity to marron, antagonism ability towards common crayfish pathogens (*Vibrio mimicus* and *V. cholerae* non-01), exhibition of a diverse enzyme profiles and non-susceptible to the majority of antibiotics tested (Ambas et al., 2015a), improved immunity (Ambas et al., 2013) and the gastrointestinal health status of marron (Ambas et al., 2015b).

To date, evaluating probiotic performance *in vivo* studies is limited to tiger shrimp *Penaeus monodon* (Rengpipat et al., 2000), shrimp *Litopenaeus vannamei* (Thompson et al., 2010) and beluga *Huso huso* (Salma et al., 2011). When evaluating probiotics *in vivo* for its nutritional benefit outcomes in aquatic animals, the probiotic candidates should be added to the diet and its effect evaluated on the growth and/or physiological status of the animals (Verschuere et al., 2000). The present study examined in vivo performance of customized probiotic *B mycoides* in commercial marron ponds by counting the total haemocyte (THC), hepatosomatic indices (Hiw), intestinal bacteria population, gluthathionine peroxide (GPx) enzyme activity, survival rate and marron pond productivity.

9.2. Materials and methods

9.2.1. Experimental marron farm site

The present study was conducted at an existing commercial marron farm located at 432 Boorara Road, Nortchcliffe Western Australia 6262 (Latitude -34.66001 N; Longitude 116° 9' 49.644 W). Six of the 900 m^2 existing commercial marron ponds with an average depth of between 1.6m to 1.7 m were used for the dietary supplemented probiotic feeding trial. As the marron production operation in these commercial ponds was already underway before the commencement of the trial, the commencement of this feeding trial was reflected by shifting the existing marron diets to the test diets (probiotic *B. mycoides* supplemented diet and the basal diet).

9.2.2. Preparation of the diet and feeding

The commercial marron diet supplied by specialty feeds, Glen Forrest Western Australia was used as a basal diet, which was used during previous laboratory scale studies. The proximate composition of the basal diet was: 26% crude protein, 9% crude fat and 5% crude ash.

Supplementation of the probiotic to the basal diet followed the established method (Hai and Fotedar, 2009). A pure colony of the isolate was grown on blood agar (BA) plates and incubated overnight at 25^{o}C. The overnight growth inoculum was diluted into 20 mL of sterilized normal saline before being sprayed onto the basal diet at a concentration of 10^{8} CFU/g of feed and then immediately covered with aluminium foil and stored in a refrigerator at 4^{o}C to avoid bacterial growth. Preparation of the probiotic supplemented diet was

performed on a weekly basis to maintain freshness. In addition, the probiotic diet was wrapped in aluminium foil as a1 kg/wrap for ease of feeding rate calculations.

The concentration (CFU/mL) of the probiotic bacterium sprayed onto the feed was determined using an established method (Hai et al., 2007) where optical density (Spectrophotometer, BOECO S-20, Hamburg, Germany) correlates to the bacterial concentration (CFU/mL) and confirmed by performing a total bacterial count using BA plates (Buller, 2004).

Feeding was performed once per day in the late afternoon and adjusted weekly after weight sub-sampling of the marron from each pond, which referred to demand feeding rates obtained from The Second Pemberton Grow Out data set 1990-1993, Department of Fisheries Western Australia.

9.2.3. Data collection

Most of the parameters measured in the present study used a comparable size of the two treatment groups such as hepatosomatic indices (Hiw), intestinal bacteria population, total haemocyte counts (THC), gluthathionine peroxide (GPx) enzyme activity, except for the survival rates and marron productivity in marron pond.

Data collection was performed at day 90th, day 160th and at the harvest (day 310th) for determination of the survival rate, pond production and GPx enzyme activity.

- ***Total haemocyte counts (THC)***

The total haemocyte count was measured following the established methods used for western rock lobsters *Panulirus cygnus* (Fotedar et al., 2001). The haemocyte samples preparation was performed on the farm site and mixed with an anticoagulant at a ratio of 1:1, injected into 2 mL cuvette tubes then kept in an iced cool box before taking to the laboratory for THC determination.

In brief, 0.5 mL of haemolymph and anticoagulant mixture was inserted into a haemocytometer (The Neubauer Enhanced Line, Munich, Germany) counting chamber and immediately viewed under 100-fold magnification on a camera-equipped microscope and

images were taken for THC. Subsequently, the cells were counted in both grids, and the mean was used as the haemocyte count. The total haemocyte count was calculated as THC = (cells counted x dilution factor x 1000)/volume of grid (0.1 mm^3).

- ***Hepatosomatic indices (Hiw)***

The hepatosomatic index (Hiw) of marron fed basal and *B. mycoides* supplemented diets were calculated as per established equations (Jussila, 1997, Fotedar, 1998). In brief, the hepatopancreas of marron from each treatment group were removed, placed in foil and weighed. Determination of the hepatosomatic indices (H_{iw}) used the following equation;

$$Hiw = Wwh \times 100\ W_t^{-1}$$

Where;
Hiw = Wet hepatosomatic indices (%)
Wwh = Weight of wet hepatopancreas (g)
W_t = Total weight of marron (g)

- ***Intestinal bacteria population(million CFU g^{-1} of gut)***

The intestinal bacterial population of marron from the different feeding groups was determined following our previous work (Ambas et al., 2015b). Before aseptic removal of the GIT, the marron was anaesthetized by placing the animal at -20^oC for 5 minutes. Subsequently, the dorsal shell was cut-off from tail to head until the intestines were exposed, then the intestine was collected and placed in a sterilised pestle, weighed and homogenised. The homogenates of intestines were diluted serially (from 10^{-1} to 10^{-6}) using a sterile normal saline. Fifty microliters of each serial dilution was inoculated onto a blood agar (BA) plate and incubated overnight in a CO_2 incubator at 25^oC. A colony count was performed for each dilution to determine the total number of aerobic bacteria (Buller, 2004).

- ***Gluthathionine peroxide enzyme activity***

The gluthathionine peroxide enzyme activity was determined followed the established method (Rotruck et al., 1973)**.** In brief, the marron muscle tissue was diluted with a physiological saline at a ratio of 1:1 and stored at 4^oC until used. To calculate GPx activity, 0.2 mL muscle tissue homogenates (homogenized in 0.4 M sodium phosphate buffer, pH 7.0), 0.1 mL 10 mM sodium azide, 0.2 mL 0.2 mM reduced glutathione, and 0.1 mL 0.2 mM

hydrogen peroxide were mixed, then incubated for 10 minutes at 37°C after which 0.4 mL of 10% trichloroacetic acid (TCA) was added to stop the reaction. Subsequently, the mixtures were centrifuged at 3200 rpm for 20 minutes. The supernatant was assayed for glutathione content using Ellman's reagent (9.8 mg 5,5 ´ -dithiobis-[2-nitrobenzoic acid] [DTNB] in 100 mL 0.1% sodium citrate). The GPx enzymes activity of the samples was measured at the Biochemistry Laboratory, Department of Agriculture and Food, Western Australia. The GPx activity was expressed as micrograms of GSH consumed per minute per milligram of protein.

- ***Survival rate (%) and pond production***

The survival rate of marron from each pond and treatment group was measured using the established equation as follows;

SR (%) = Nt/No x 100

Where ;
SR = survival rate (%)
Nt = No of marron at measurement (ind)
No = No of marron at initial stocking

In addition, the pond production from each pond was determined by counting and weighing total marron at harvest using the following equation;

Pond production (kg/m2) = Total weight (kg)/pond size (m2)

Whereas the average marron weight at harvest was calculated as follow;

Mean weight (g) = Tw/Tn

Where Tw = total weight of marron each pond (g)
Tn = Total number of marron (ind)

- ***Temperature (^{o}C)***

The temperature fluctuation was recorded by placing a temperature data logger (Onset HOBO) in each pond. In addition, each pond was equipped with two paddle wheels to ensure sufficient dissolved oxygen especially during critical periods.

9.2.4. Data analysis

The data were analysed using T-test Microsoft Excel for windows version 2010. The difference of means between the two treatment groups was determined at 0.05 level of significance.

9.3. Results

- ***Total haemocyte counts (THC)***

TheTHC of marron fed basal and probiotic, *B. mycoides* supplemented diets was not significantly different (P>0.05) at day 90th of marron rearing, however on day 160th the THC of probiotic diet fed marron was significantly higher (P<0.05) than the THC of basal diet fed marron (Fig.9.1).

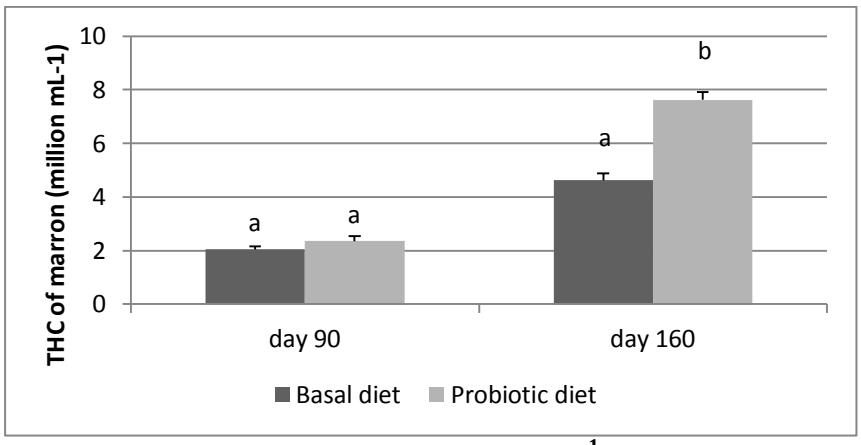

Figure 9.1. THC of marron (million cells mL^{-1}) fed basal and probiotic diets
*Different letters over bars indicates significantly different at 0.05.

- ***Wet hepatosomatic indices (%)***

In the present study, the wet hepatosomatic indices (Hiw) of probiotic diet fed marron was significantly higher (P<0.05) both at day 90th and day 160th of measurements (Fig. 9.2) than the Hiw of basal diet fed marron. The Hiw of basal diet fed marron was lower at day 90th than the Hiw at day 160th .

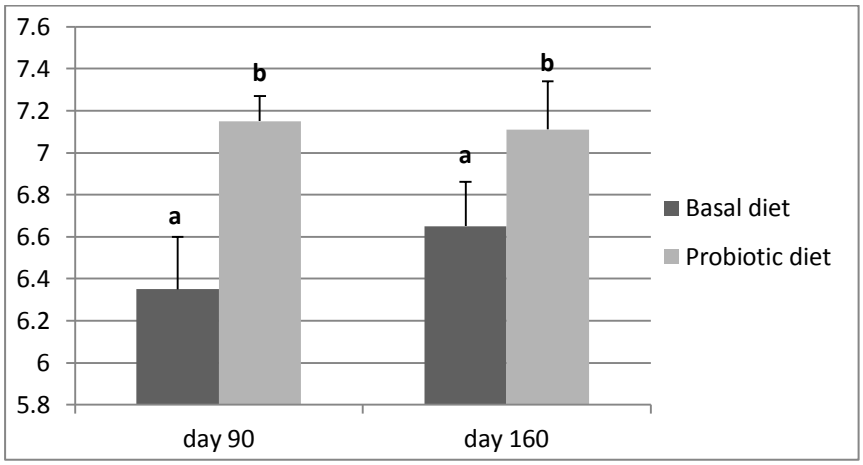

Figure 9.2. Hepatosomatic indices (Hiw) of marron fed basal and probiotic diets.
*Different letters over bars indicates significantly different at 0.05.

- ***Intestinal bacteria population (million CFU/g of gut)***

Supplementation of a marron-origin probiotic, *B. mycoides* to the diet significantly ($P<0.05$) improved the intestinal bacteria population of marron compared to intestinal bacteria population of the basal diet fed marron, both on day 90^{th} and day 160^{th}. In addition, there is an increase of intestinal bacteria population in both treatment groups on day 160^{th} compared to day 90^{th} (Fig 9.3)**.**

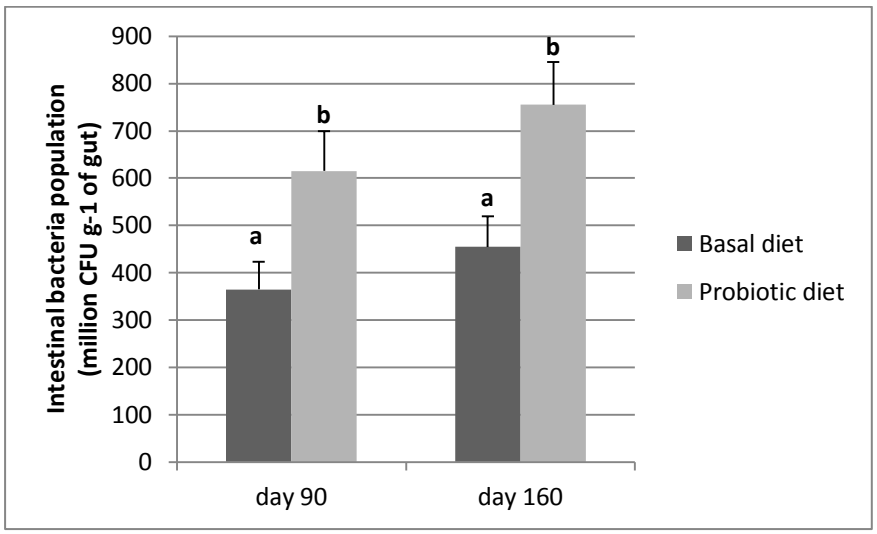

Figure 9.3. Intestinal bacteria population of basal and probiotic diets fed marron.
*Different letters over bars indicates significantly different at 0.05.

- ***Gluthathionine peroxide (GPx) enzyme activity of tissue muscle***

The GPx enzyme activity was significantly higher (P<0.05) in the marron tail muscle tissue fed with the probiotic *B. mycoides* supplemented diet compared to the GPx acitivity of basal diet fed marron both on day 90th and termination of the feeding trial (Fig 9.4).

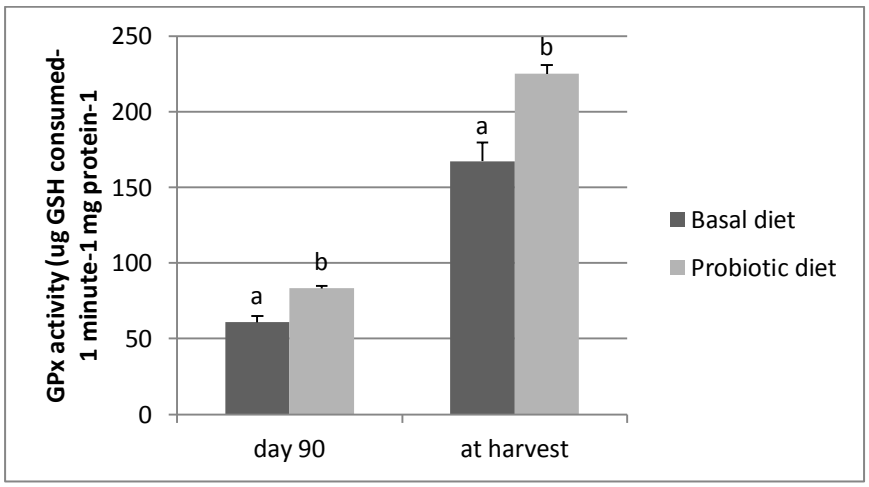

Figure 9.4. GPx enzyme activity in tissue muscle of basal and probiotic diets fed marron
*Different letters over bars indicates significantly different at 0.05.

- ***Survival rate (%) and pond production (kg/m2)***

The present study demonstrated that the survival rate (%) of probiotic fed marron ranged between 74.80±2.52 (%), which was significantly higher (P<0.05) than the survival rate of marron from the ponds fed with basal diets (66.15±6.33%).

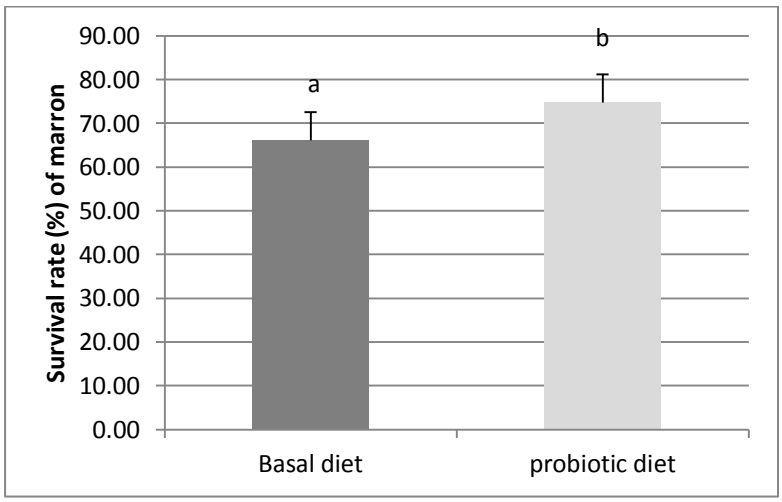

Figure 9.5. Survival rate (%) of marron fed basal and probiotic supplemented diets.
*Different letters over bars indicates significantly different at 0.05.

Meanwhile, the average pond production of the basal diet was 258.3±32.6 g/m^2 whereas the average pond production of the probiotic diet fed marron was 215±26.1 g/m^2 (Table 9.1).

Table. 9.1. Survival (%) and production (g/m^2) of marron fed basal and probiotic diets

	Basal diet			Probiotic diet		
	1	2	3	1	2	3
Initial stocking (n)	3200	3000	3200	3100	3200	3200
Total harvest (n)	1662	2287	2249	2139	2472	2474
Survival (%)	51.94	76.23	70.28	69.00	77.25	78.16
Weight harvest (kg)	137.64	195.8	363.8	192.65	148	241.3
Average weight (kg)	0.083	0.086	0.162	0.090	0.060	0.098
Production (kg/m^2)	0.153	0.218	0.404	0.214	0.164	0.268

- ***Temperature***

The water temperature in each pond fluctuated daily, but greater water temperature (^{o}C) fluctuation occurred in November (Figure 9.6). The highest water temperature in marron ponds in average was observed in January and February with an average of 25.61^{o}C to 25.84^{o}C, whereas the lowest (13.05±1.2^{o}C) was observed in July. In addition, a very extreme temperature fluctuation was detected on 21st November where the temperature at night (2400 hours) ranged between 12.7^{o}C to 13.9^{o}C but during the day time (1200 hours) jumped to 36.3^{o}C to 52.7^{o}C which caused high mortality especially in the basal diet fed marron ponds.

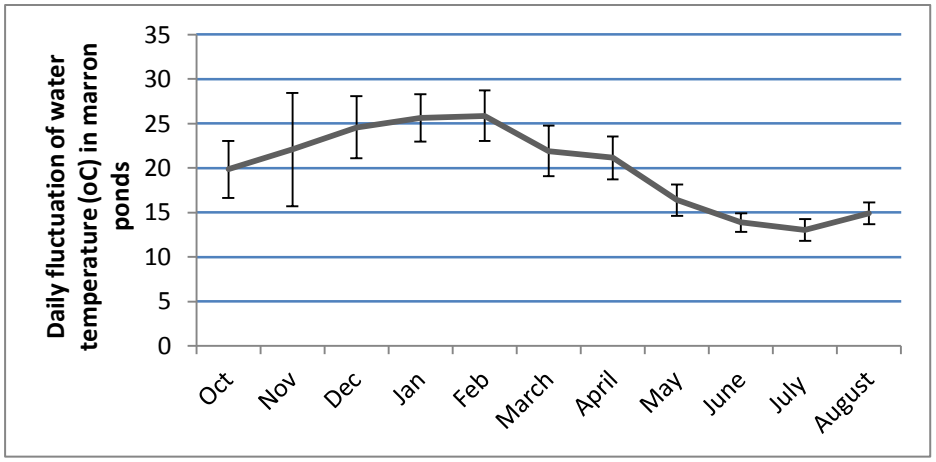

Figure 9.6. The average water temperature in marron ponds during feeding trial.

9.4. Discussion

In aquaculture, the higher immune status of an animal is crucial as the animal is exposed to a series of stress conditions including the natural rhythms of the environment (Rollo et al., 2006, Tapia-Paniagua et al., 2014) and the link between stress and higher susceptibility to diseases is conclusive (Tapia-Paniagua et al., 2014). Therefore, the improved immunity of the aquatic animal (Nayak, 2010, Hai, 2015, Ridha and Azad, 2015) is particularly important to reduce mortalities which lead to significant economic losses (Dagar et al., 2010) and to ensure a profitable aquaculture operation (Bachere, 2000). The present *in vivo* study suggested that customized probiotic *B. mycoides* significantly improved marron immunity and health status (THC, Hiw, intestinal bacteria population and GPx enzyme activity), which in turn improved the survival rate of marron.

The THC of marron fed *B. mycoides* supplemented diets improved significantly showing that the probiotic *B. mycoides* remained effective during the entire feeding trial and was able to play a crucial role in marron immunity. It has been proposed by most authors that the host origin probiotic candidate is preferred as its efficacy is likely to be highest in the host and environment from where it has been isolated (Verschuere et al., 2000, O'Sullivan, 2001). Improved THC by feeding probiotic diets has also been observed in many crustaceans such as tiger shrimp *Penaeus monodon* (Rengpipat et al., 2000), western king prawns *P. latisulcatus* (Hai et al., 2010), shrimps *P. japonicus* (Zhang et al., 2011) and *Litopenaeus vannamei* (Li et al., 2009). Moreover, the haemocytes have been successfully used as an immune indicators in various crustacean such as shrimps (Lorenzon et al., 2001, Van de Braak et al., 2002), lobsters (Lorenzon et al., 2007, Fotedar et al., 2001, Jussila et al., 1997), crabs (Lorenzon et al., 2008) and crayfishes (Persson et al., 1987, Soderhall et al., 1984) including marron (Jussila et al., 1999, Sang et al., 2009) as the haemocytes are involved in various defence mechanisms including recognition, phagocytosis, encapsulation, storage and release of the proPO system and cytotoxicity (Soderhall and Cerenius, 1992, Johansson et al., 2000, Sritunyalucksana and Soderhall, 2000).

It has been documented that the hepatopancreas of crustacean is not just an organ responsible for metabolism but it is an integrated part of immunity (Rőszer, 2014). The hepatopancreas is an important organ for absorption and storage of large amounts of energy particularly lipids and can synthesize digestive enzymes for food digestion (Wang et al., 2014). It has also been

used as an indicator of the marrons' condition (Jussila, 1997, Fotedar, 1998). As the hepatopancreas also serves as source of various enzymes, the larger hepatopancreas of crayfish could be an indicator of greater digestive enzyme activities (Hammer et al., 2000). In the present study, *B. mycoides* significantly improved Hiw of marron compared to Hiw of basal diet fed marron both on day 90^{th} and day 160^{th} which suggested that a supplemented probiotic in the marron diet was able to improve the metabolism and energy availability for the animals. These results are in line with Tapia-Paniagua et al. (2014) who suggested that probiotics increases energy availability of animals and thus improve stress tolerance.

In addition to hepatopancreas, the intestinal bacteria population also plays a significant role in metabolism and immunity. The beneficial bacteria not only protect the animal from the pathogen invasion, but also reflect the nutritional status of the animal (Ringø et al., 2007b, Denev et al., 2009, Gaggìa et al., 2010). The present study demonstrated that *B. mycoides* significantly improved the intestinal bacteria population of the marron and also that there was an increase in bacteria population both in basal and probiotic diets fed marron as the marron size increased on day 160^{th}. Ringø et al. (2003) suggested that there is a progressive increase of intestinal bacteria population of small intestines to larger intestines of aquatic animals. Modulation of the intestinal bacteria population have also been demonstrated in many groups of aquatic animals such as Atlantic cod (Lazado et al., 2014), Mediterranean teleosts (Dimitroglou et al., 2011) and Salmonids (Merrifield et al., 2010).

GPx is another immune parameter of marron, which was improved by feeding with a probiotic supplemented diet. The GPx of marron fed with a probiotic diet was significantly higher than the GPx of basal diet fed marron. Our previous study also revealed a progressive increase of GPx tail muscle tissue of marron with time from one to four weeks feeding. Improved antioxidant enzyme activity by feeding with probiotics have been detected in shrimp *Litopenaeus stylirostris* (Castex et al., 2010). GPx enzyme activity plays a crucial role in maintaining cellular homeostasis of crayfish (Borković et al., 2008) protects the body from oxidation by free radicals (Chiu et al., 2010), which can cause cellular damage and oxidative stress (Parrilla-Taylor and Zenteno-Savín, 2011). The GPx activity has also been detected higher in haemocyte of marron (Nugroho and Fotedar, 2013). In addition, probiotics especially lactic acid bacteria exhibit various antioxidant activity which is capable of limiting

excessive amounts of reactive radicals in vivo and thus potentially contributes in preventing and controlling several diseases associated with oxidative stress (Amaretti et al., 2013).

Survival rate and growth are critically important for a profitable aquaculture practices. In the present study, the average survival rate (74.8%) of *B. mycoides* diet fed marron was significantly higher than the survival rate (66.2 %) of basal diet fed marron as the probiotic was able to improve immunity and stress tolerance of marron when the culture condition extreme. An extreme fluctuation of water temperature on the 21st of November ranged between 12.78^{o}C to 13.94 ^{o}C at night (2400 hours) and between 36.29 ^{o}C to 52.72 ^{o}C during the day time (1200 hours) in the marron ponds triggered high marron mortality on that day and the following days. However, higher immune status particularly the Hiw of probiotic fed marron suggested the animals were more adaptable to this chronic environmental stress situation which resulted in a higher survival rate compared to the basal diet fed marron. Jussila et al. (1999) observed a decreased Hiw of marron during a post simulated transport stress test, which suggested that high energy utilization (hepatopancreas) induced by this stress conditions (Cruz et al., 2012).

Though immunity and the health status of marron given a probiotic fed diet were significantly higher than basal diet fed marron as described above, the average pond production was still relatively low. This could be partly attributed to a broad spectrum of juvenile size and/quality and their sources used at the initial stocking of the experimental ponds. The basal diet fed ponds were initially stocked with the juveniles produced from the same experimental ponds, whereas most of the juveniles for the probiotic fed marron ponds were obtained from the non-experimental ponds and out sourced and were relatively smaller in size than the juvenile sizes of basal diet fed marron. Lack of juvenile's sources at initial stocking time contributed to the relatively larger variations in sizes and quality. Therefore, further study is required by using only one source of equal sized juveniles as an initial stocking to evaluate the performance of probiotic diets on marron in commercial marron farms. A comparable size of animal from treatments groups is recommended by some authors when measuring a particular parameters as several parameters vary greatly according to animal size or organs such as bacteria density and microvilli of similar size GIT (Ringø et al., 2003, Cerezuela et al., 2012).

9.5. Conclusion

In summary, the customized marron origin probiotic *B. mycoides* worked effectively in vivo (commercial marron farm) as indicated by a significant improvement of marron immunity and health status (THC, GPx enzyme activity, intestinal bacteria population and Hiw) which in turn enhanced survival rates when compared to basal diet fed marron.

CHAPTER 10. General discussion

The common steps used when screening a probiotic candidate for a particular aquatic species are: (1) the screening process following the outlined criteria and steps (2) evaluating the performance of the selected probiotic candidates in vitro or in laboratory conditions (3), and evaluating the performance of the selected candidates *in vivo* to validate the *in vitro* or laboratory based findings, however the last step is scarcely investigated.

In this study, a series of laboratory and a commercial scale trial were conducted to screen and evaluate an appropriate probiotic candidate for marron culture. The study was initiated with a selection of probiotic candidates, using established screening techniques and steps (Verschuere et al., 2000, Vine et al., 2004, Balcázar et al., 2006, Kesarcodi-Watson et al., 2008, Merrifield et al., 2010c, Ringø E, 2008, Nayak and Mukherjee, 2011, Leyva-Madrigal et al., 2011, Kesarcodi-Watson, 2009, Hamid et al., 2012, Lim et al., 2011, Hai et al., 2007, Haeri et al., 2012, Gullian et al., 2004, Gomez-Gil et al., 2000), followed by several laboratory based studies to evaluate its potential use as a probiotic candidate by measuring its effect on marron's immunity and physiology, then finally *in vivo* validation in an existing commercial marron farm.

10.1. Screening of the probiotic candidates for marron aquaculture

The selection of probiotics is a critical step as inappropriate microorganisms may lead to undesirable effects to the host (Nayak, 2010). The screening of probiotic candidates to be used in aquaculture have been demonstrated in various aquatic species including fish (Hamid et al., 2012), shrimps (Hai et al., 2007, Leyva-Madrigal et al., 2011, Gullian et al., 2004) and bivalves (Kesarcodi-Watson, 2009). Many criteria were proposed when screening a probiotic candidate (Merrifield et al., 2010c). However the absence of inhibition is not sufficient to exclude a strains as a candidate (Newaj-Fyzul et al., 2014) as until recently most of the probiotic screening studies merely focussed on the production of antibacterial compounds or antagonism ability towards pathogens (Kesarcodi-Watson et al., 2012), while other criteria were often overlooked (Nayak and Mukherjee, 2011).

The list of criteria proposed by most authors when screening probiotic candidates are:

- non-pathogenic to the host or other aquatic animals and to humans host origin (gastro intestinal tract or environment of the host)
- non- resistance to antibiotic or free of plasmid-encoded genes
- resistant to bile salts and low pH
- antagonistic towards common virulent pathogens of the host
- ability to compete and grow well in intestinal mucus
- ability to colonise the intestinal epithelial surface
- exhibit fast growing in the host environment
- exhibit various enzyme profiles and contribute digestive enzymes to the host
- ability to remain viable during feed processing, storage conditions or before being ingested by the host

The more of these characteristics that are fulfilled, the more likely is its effectiveness on the host (Merrifield et al., 2010c). In the current study, six strains of bacteria from two sources (marron GIT and commercial probiotic products) including *Bacillus mycoides* (A10) and *Shewanella* sp (A12) isolated from a number of healthy adult marron intestines, *Bacillus* sp. (PM1), *Bacillus subtilis* (PM3), *Bacillus* sp. (PM4) and *Bacillus* sp. (AQ) from commercial probiotic products were investigated for their potential to use in marron culture applying various tests and criteria used by some authors. These criteria are (i) pathogenic tests (ii) antibiotic susceptibility tests (iii) antagonism ability towards pathogens (*Vibrio mimicus* and *V. cholerae* non-01) (iv) enzyme production and profiles.

Based on the criteria and the results of the various tests performed (Chapter 3), *Bacillus mycoides* was selected as a probiotic candidate for marron culture. Host-origin strains with favourable probiotic properties, especially in a strain with an antagonism ability against the known or probable pathogen(s) of the host, is the most recommended approach for the selection as it is the most beneficial to the host (Nayak, 2010, Gildberg et al., 1997 , Rollo et al., 2006, Balca´zar et al., 2007, Farzanfar, 2006, Kesarcodi-Watson et al., 2008). As *Vibrio mimicus* and *V. cholerae* non-01 are two opportunistic bacteria that cause septicaemia in crayfish and tail blister in marron (Eaves and Ketterer, 1994, Wong et al., 1995, Evans and

Edgerton, 2002, Buller, 2004), antagonistic ability towards these pathogens is an important selection criteria when screening a probiotic candidate for the marron.

Host-origin isolates, especially a predominant GIT mucus inhabitant, indicates that the isolated strain has fulfilled the criteria of (i) non-pathogenicity to the host, (ii) resistance to bile salts and low pH of the host GIT, (iii) ability to compete and grow well in the ambience of intestinal mucus, (iv) ability to colonise the intestinal epithelial surface, and (v) ability to remain viable in the host environment. Thus, host-origin strains require less screening tests and process than those of the non-host origin candidates. The isolate of *B. mycoides* also fulfilled other primary criteria of non- resistance to the majority of antibiotics; exhibited a wide range of enzyme profiles and could remain viable during feed processing and storage as *Bacillus* spp.

In brief, the use of host-origin candidates predominant healthy strains with probiotic properties especially antagonism ability towards a common, multi- strained virulent pathogens, and thus is proposed as a first primary criterion. The host origin strains may also remain viable in the host environment and the host GIT, stay predominant in the host, multiply and finally exert numerous health benefits to the host. Application of *B. mycoides* at early larvae stages may improve its ability to stay on the intestinal mucus and then become predominant in the marron GIT at later development stages (Ringø and Gatesoupe, 1998, Gatesoupe, 1999).

10. 2. Performance of *B. mycoides* on health status of marron

Probiotics have been shown conclusively to improve the health status of various animals such as shrimps (Farzanfar, 2006, Lakshmi et al., 2013, Hai et al., 2009b), fishes (Dimitroglou et al., 2011, Merrifield et al., 2010c, Lazado et al., 2014) and bivalves (Prado et al., 2010, Kesarcodi-Watson et al., 2012). This section describes several selected marron health parameters (intestinal bacteria population, hepatosomatic indices, GIT health and growth) are discussed which have been derived from the previous published and unpublished chapters.

In crustacea, animal health can ultimately be assessed by growth and acceptable survival, and/or other physiological or growth related indicators. In most crayfish studies including marron, the health status of the animals were assessed by measuring growth, hepatosomatic

indices, tail muscle indices and the health status of GIT (Morrissy, 1979, Sommer et al., 1991, Kartamulia and Rouse, 1992, Jussila and Mannonen, 1997, Fotedar, 1998, Jussila, 1999, Fotedar, 2004, Sang and Fotedar, 2010).

The microflora of the aquatic animal digestive tract plays a crucial role in digestion and metabolism (Ganguly and Prasad, 2012) and can be considered as a metabolically active organ (Gaggìa et al., 2010); however its contribution to the digestive process is not well investigated (Lazado et al., 2012) and even its effect on the overall health status of the animal has been underestimated (Gómez et al., 2007) so far.

The modulation of both gut microbiota and enhanced gut morphology, may improve the nutritional status and growth of the host (Merrifield et al., 2010c). Therefore, manipulation of the gut microbiota through probiotic supplementation is a novel approach from a nutritional point of view (Nayak, 2010, Dimitroglou et al., 2011, Pirarat et al., 2011, Tapia-Paniagua et al., 2011) and is relevant to aquaculture practices and its sustainability (Ghanbari et al., 2015). The modulation of gut microbiota through probiotic supplementation could improve nutritional status and effective use of diets in several ways. This includes improved appetite (Newaj-Fyzul et al., 2014); direct contribution of beneficial enzymes and co-enzymes as nutrients (Ramirez and Dixon, 2003, Lazado et al., 2014), or by stimulating the host digestive enzymes and the endogenous enzyme-producing microbiota (Lazado et al., 2014). Whereas, indirect contribution of probiotic supplementation includes improve GIT health reflected in size and density of the microvilli, and the increased hepatosomatic indices (Ambas et al., 2015b), all mediated through the improved nutrient digestions, better feed conversion and enhanced growth (Newaj-Fyzul et al., 2014).

The present study suggested that *B. mycoides* significantly improved the intestinal bacteria population in marron GIT throughout the experimental series (Fig 10.1). Numerous improvements were observed in relation to various size of marron in different studies. A progressive increase in the number of GIT bacteria population from approximately 10^5 cfu/g of gut in small intestine to 10^7 cfu/g of gut in large intestine in adult fish species has been noted (Ringø et al., 2003), whereas Pérez et al. (2010) suggested that generally the fish GIT contains bacteria with an estimated $10^7 – 10^8$ cfu/g gut. Modulation of intestinal bacterial population as one of the health benefits of using probiotics have also been demonstrated in

Atlantic Cod (Lazado et al., 2014), Mediterranean teleosts (Dimitroglou et al., 2011) and salmonids (Merrifield et al., 2010c).

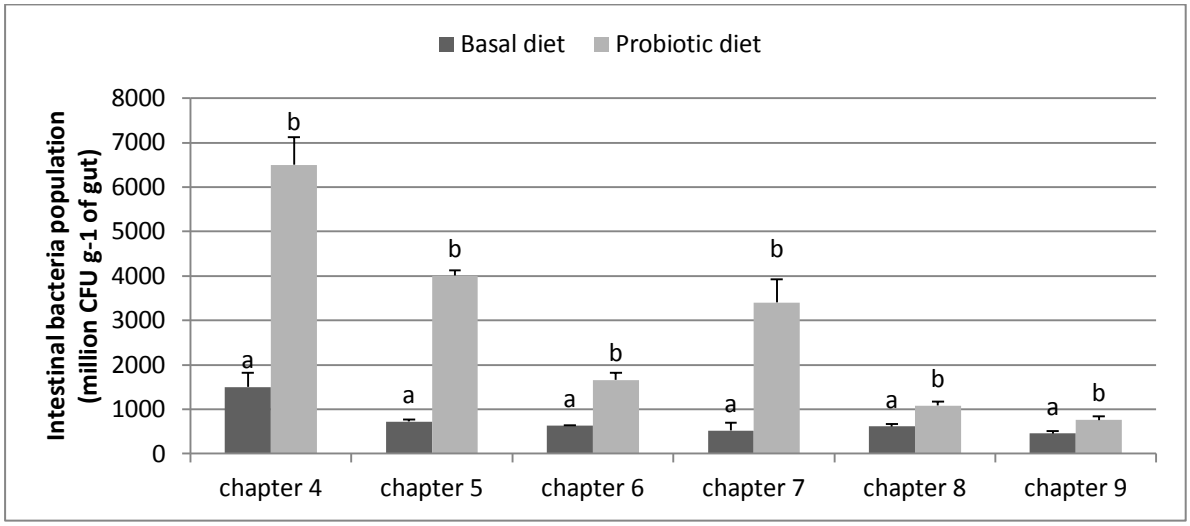

Fig 10.1. Intestinal bacteria population (CFU/g of gut) of marron fed basal and probiotic diets
*Different letters over bars indicates significantly different at 0.05.

Another crucial digestive organ which plays a significant role in nutrient digestion is the hepatopancreas (Thompson et al., 2010) as a source digestive enzymes (Hammer et al., 2000), which are important for nutrient digestion and absorption and acts as a storage organ for a large amount of energy in the form of lipids (Wang et al., 2014). The hepatopancreas has been used as an indicator of nutritional status in various crustaceans such as crab *Portunus trituberculatus* (Wang et al., 2014), freshwater prawn *Macrobrachium rosenbergii* (Sureshkumar and Kurup, 1999), red swamp crayfish *Procambarus clarkii*, (Shi et al., 2010) and marron *Cherax cainii* (Austin, 2002) (Jussila, 1997, Fotedar, 1998, Sang et al., 2011).

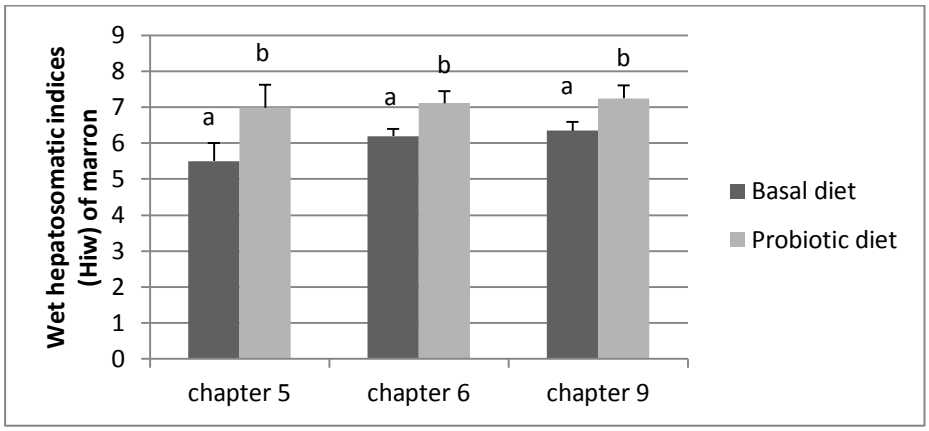

Figure 10.2. Comparison of wet hepatosomatic indices (Hiw) of marron fed basal and *B. mycoides* supplemented diet. Different letters over bars indicates significantly different at 0.05.

In the present study, the Hiw of *B. mycoides* diet fed marron was significantly higher compared to the Hiw of basal diet fed marron (Fig 10.2). This shows that the marron fed *B. mycoides* are able to improve nutrient digestion efficiency, which then deposited in the hepatopancreas or muscles, which reflected in the improved growth of the marron. The larger size of the hepatopancreas (Hiw) and higher carbohydrate content are indications of faster growth (Sureshkumar and Kurup, 1999), higher digestive enzyme activities (Hammer et al., 2000), and as an indication of higher nutritional status in marron (Fotedar, 1998, Jussila and Evans, 1998).

GIT health is the main determinant in the nutrient metabolism of aquatic animals (Merrifield et al., 2010b, Tuohy et al., 2003) including marron (Sang and Fotedar, 2010, Nugroho and Fotedar, 2013b, Ambas et al., 2015b), whereas the health status of GIT is most likely determined by the microbial balance of indigenous microbiota (Ramirez and Dixon, 2003, Denev et al., 2009) with the density and diversity of bacteria in the intestine having the most impact on intestinal health (Ringø et al., 2007). Many studies demonstrated that probiotics improve the GIT health status of aquatic animals as demonstrated in rainbow trout, *Oncorhynchus mykiss* (Dimitroglou et al., 2009, Merrifield et al., 2010d), Atlantic salmon *Salmo salar* (Kristiansen et al., 2011), gilthead seabream *Sparus aurata* (Cerezuela et al., 2012) and Senegalese sole *Solea senegalensis* (Batista et al., 2015). In the present study, *B. mycoides* significantly improved the health status of marron GIT indicated by a higher intestinal bacteria population, larger hepatopancreas (Hiw), more dense and higher microvilli, and thicker intestinal epithelium, which in turn improved the growth of marron (chapter 5). Longer and dense microvilli provide greater nutrient absorption ability due to their increased surface area (Cerezuela et al., 2012, Caspary, 1992, Pirarat et al., 2011, Merrifield et al., 2009) and provide a larger surface area for bacterial colonisation as in Arctic charr, *Salvelinus alpinus* (Ringø et al., 2001), whereas larger hepatopancreas in crayfish is an indication of higher digestive enzyme activities (Hammer et al., 2000, Coccia et al., 2011). All these features of a healthy GIT suggested that the marron benefited from the probiotic, *B. mycoides* supplementation.

Improved growth and feed digestion efficiency is a crucial strategy in aquatic feed formulation as feed accounts for 40–60% of the production costs in marron aquaculture and aquaculture of other similar species (Fotedar, 2004, Burr and Gatlin, 2005). Improved growth

and feed digestion efficiency are mediating factors when animals are fed probiotics as the probiotic improves appetite (Newaj-Fyzul et al., 2014), modulate intestinal bacteria population (Merrifield et al., 2009, Dimitroglou et al., 2011) and improve GIT health status of aquatic animals (Dimitroglou et al., 2009, Merrifield et al., 2010d). Improved growth and feed digestion efficiency by feeding probiotic diets have been observed in various groups of aquaculture species including shrimps (Rengpipat et al., 2003, Hai et al., 2009a), lobster (Daniels et al., 2010), fishes (Merrifield et al., 2010a, Dimitroglou et al., 2011) and molluscs (Macey and Coyne, 2005, Prado et al., 2010, Kesarcodi-Watson et al., 2012).

In brief, *B. mycoides* significantly improve the health status of marron indicated by improved intestinal population (chapters 5, 6, 7, 8 and 9), improved hepatosomatic indices (chapters 5, 6 and 9), improved GIT health status (chapter 6) and improved growth of marron.

10. 3. Performance of *B. mycoides* on marron immunity

In aquaculture, the higher immune status of animals is vital as aquaculture practices especially in an intensive culture expose the animal to a series of stress conditions (Rollo et al., 2006, Tapia-Paniagua et al., 2014) including: handling and transport (Lorenzon et al., 2007, 2008, Fotedar and Evans, 2011) to the farm environment; high stocking density (Nayak, 2010, Tapia-Paniagua et al., 2014); periodic manipulation (Cruz et al., 2012) and the natural rhythms of the environment (Le Moullac and Haffner, 2000, Hoseinifar et al., 2015). Furthermore, their combination effects cause even more chronic stress conditions and weakened immunity of the animals (Li et al., 2010) including in marron (Jussila et al., 1999, Sang et al., 2009). Mortalities due to stress routinely occur in aquaculture which lead to significant economic losses (Dagar et al., 2010). A link between stress and higher susceptibility to diseases has been established (Tapia-Paniagua et al., 2014), therefore improving the immunity of aquatic animals is extremely important to ensure a successful aquaculture (Bachere, 2000). This section describes the performance of *B. mycoides* in marron immunity as measured by THC, GPx enzyme activity and tools such as the Hiw and intestinal bacteria population as part of the immune defence system in aquatic animals (Shi et al., 2010, Rőszer, 2014, Gaggìa et al., 2010).

In crayfish, the haemocytes and hepatopancreas are playing a crucial role in immunity (Shi et al., 2010). Each cell type of haemocytes is active in defence reactions (Johansson et al., 2000, Vazquez et al., 2009) that are responsible for destroying invasive microorganisms (bacteraemia), foreign particles (Li et al., 2010) and fungus (Vazquez et al., 2009, Persson et al., 1987, Ding et al., 2012, Soderhall et al., 1984). Meanwhile, the hepatopancreas especially its epithelial cells are major sources of immune molecules (for review see:Rőszer, 2014) and as an immune defence organ in crayfish as demonstrated in red swamp crayfish, *Procambarus clarkii* (Shi et al., 2010).

The haemocyte defence mechanisms involve various continuous or discreet steps such as recognition, phagocytosis, encapsulation, storage and release of the proPO system and cytotoxicity (Johansson et al., 2000, Soderhall and Cerenius, 1992, Sritunyalucksana and Soderhall, 2000, Vazquez et al., 2009), antimicrobial proteins, lectins and clottable protein (Vazquez et al., 2009). The haemocytes also contain antioxidant enzymes as in spiny cheek crayfish *Orconectes limosus* (Borković et al., 2008) including glutathione-S-transferase (GST), glutathione peroxidase enzymes (Nugroho and Fotedar, 2013a), immune-related genes (Shi et al., 2010) and antibacterial activity, which is very effective against both gram-positive and gram-negative organisms (Chisholm and Smith, 1995, Le Moullac and Haffner, 2000).

The present study demonstrated that *B. mycoides* significantly improved marron THC (Fig 10.3). A higher THC effectively reduces bacteraemia in marron (Sang et al., 2009, Nugroho and Fotedar, 2013b). A significant bacteraemia reduction was also observed in marron fed *B. mycoides* diet at 48h and 96h post injection with the pathogen *Vibrio mimicus* (Ambas et al., 2013). Thus, a low circulating haemocyte number in crustaceans is strongly correlated with a greater sensitivity to pathogens (Soderhall et al., 2003, Le Moullac and Haffner, 2000). In general, the haemocytes of crustaceans effectively reduce the viable count of injected bacteria within 4 hours (Chisholm and Smith, 1995).

Improved THC by feeding probiotic diets have also been demonstrated in tiger shrimp *Penaeus monodon* (Rengpipat et al., 2000), western king prawns *P. latisulcatus* (Hai et al., 2010), shrimp *P. japonicus* (Zhang et al., 2011) and *Litopenaeus vannamei* (Li et al., 2009).

However the underlying mechanism in the processes involved in the improvement of THC when fed probiotic diets, is not well understood.

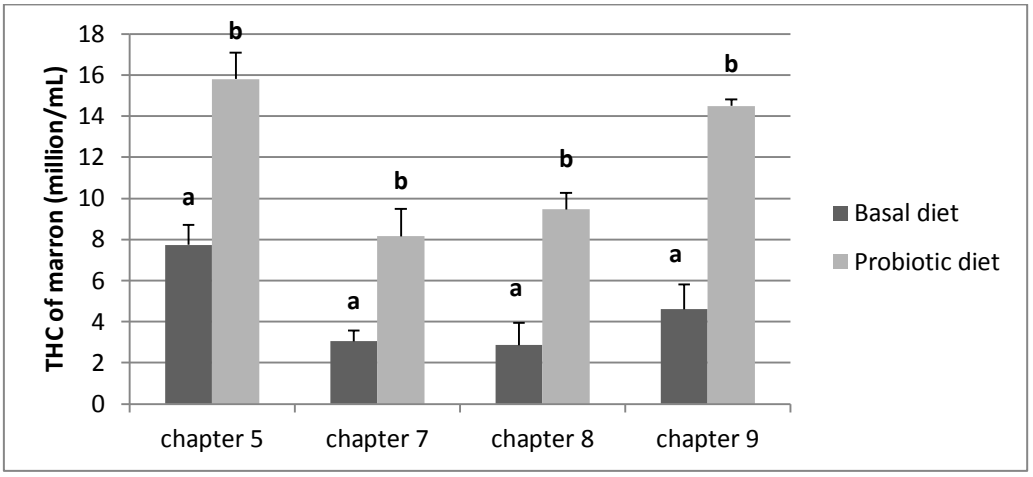

Figure 10.3. THC of marron fed basal and *B. mycoides* added diets at various studies. Different letters over bars indicates significantly different at 0.05.

It is widely accepted that digestion and immunity are complicated physiological processes that have co-evolved (Cerezuela et al., 2013, Liu et al., 2013). The contribution of intestinal bacteria population to the nutritional health status of aquatic animals has been described in the previous section (chapter 10.2), however the microbial community population dynamics also determine vulnerability of the host to diseases (Ghanbari et al., 2015), as GIT is one of the three main entries of pathogen invasions in aquatic animals (Ringø et al., 2003).

The commensal bacteria in the GIT is crucial to immunity, thus a reduction in either diversity or quantity of the GIT commensal microbiota is likely to reduce the effective barrier mechanism (Denev et al., 2009, Ige, 2013) and increase the permeability and alterations of the immune defences (Tapia-Paniagua et al., 2014), which provide opportunistic pathogens to become established (Lorenzon et al., 2001, Ringø et al., 2010). Probiotics secrete a variety of antimicrobial compounds against pathogens (Sugita et al., 1996, Chythanya et al., 2002, Vine et al., 2004, Seehanat, 2005, Hai et al., 2007, Ariole and Oha, 2013, Muñoz-Atienza et al., 2013, Verschuere et al., 2000, Gómez and Shen, 2008) including antiviral activity (Lakshmi et al., 2013). In the present study, *B. mycoides* demonstrated antibacterial activities against pathogens *Vibrio mimicus* and *V. cholerae* non-01 (Ambas et al., 2015a), restored and modulated the commensal bacteria in marron GIT (section 10.2). In grouper *Epinephelus*

coioides, some potentially harmful strains such as *Staphylococcus saprophyticus* were suppressed after feeding probiotic *Psychrobacter* sp. (Yang et al., 2011).

It has been stated earlier that the hepatopancreas is an integrated organ of immunity and metabolism (Rőszer, 2014). There is a crosslink between hepatopancreas gland cells and phagocytes which aids the initiation of the immune response and the clearance of pathogens. Moreover, the epithelial cells of the hepatopancreas are the major sources of immune molecules such as lectins, hemocyanin, ferritin, antibacterial and antiviral proteins, proteolytic enzymes and nitric oxide (Rőszer, 2014, Shi et al., 2010, Wang et al., 2014). Thus, larger hepatopancreas (Hiw) of probiotic fed marron (Ambas et al., 2013, Ambas et al., 2015b) implies more immune molecules of the hepatopancreas epithelial cells and more energy deposited. Acute stress requires high energy which could reduce the hepatosomatic indices (Cruz et al., 2012) as observed in marron handling and transport tests (Jussila et al., 1999), thus aquaculture species should be fed probiotics as probiotics increase energy availability (Tapia-Paniagua et al., 2014). Marron fed *B. mycoides* also showed improved energy availability (Hiw) which suggested that they were more adaptable to stress conditions than basal diet fed marron which resulted in a significant low mortality during water temperature stress condition as observed in commercial marron ponds (Chapter 9).

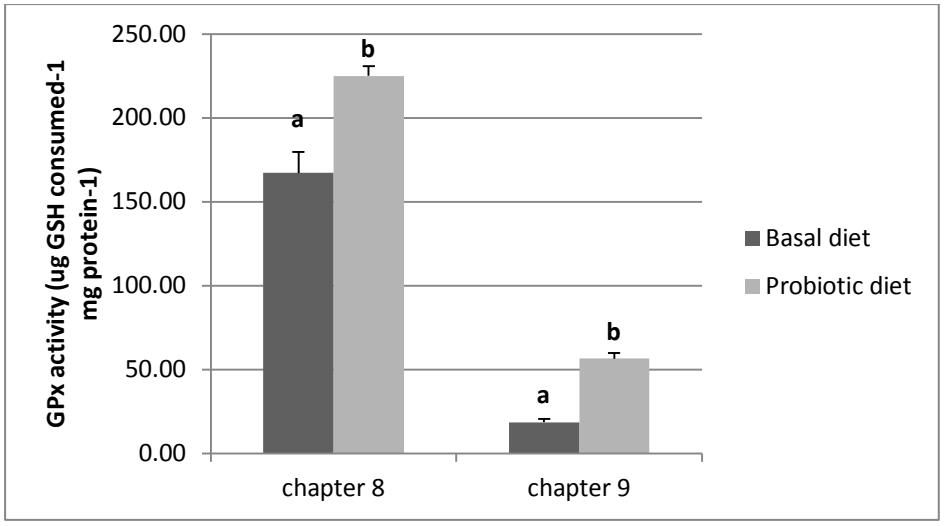

Figure 10.4. Glutathione peroxidase enzyme activity of basal and probiotic diet fed marron. Different letters over bars indicates significantly different at 0.05.

In addition, antioxidant defence (AD) enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play a crucial role in all animals including crayfish

in maintaining cellular homeostasis (Borković et al., 2008). These various antioxidant enzymes have been detected in hepatopancreas, gills and muscle in spiny cheek crayfish *Orconectes limosus* (Borković et al., 2008) and in the haemocyte of marron (Nugroho and Fotedar, 2013a). Improved antioxidant enzyme activity by feeding probiotic have been detected in shrimp *Litopenaeus stylirostris* (Castex et al., 2010). Probiotics especially lactic acid bacteria exhibit various antioxidant activity that are capable of limiting excessive amounts of reactive radicals *in vivo* and thus potentially contribute to prevent and control several diseases associated with oxidative stress (Amaretti et al., 2013). In the present study, the GPx enzyme activity of probiotic fed marron was significantly higher than basal diet fed marron (Fig. 10.4) suggesting that *B. mycoides* is able to enhance the GPx enzyme activity of marron, however the potential antioxidant enzyme activity of *B. mycoides* was not examined.

It is evident from this research that *B. mycoides*, a predominant marron originated strain with favourable probiotic properties is significantly contributes towards various health benefits in marron. First, it is a potential anti-pathogen as it produces antibacterial (pathogens) compounds (chapter 3), then provides a good environment and modulates commensal bacteria diversity in the GIT (chapters 3 to 9) as a key component of GIT health status (chapter 5). Along with healthy GIT, the high density and diverse commensal GIT bacteria and larger Hiw contribute various digestive enzymes, all are important for improving nutrient digestion efficiency and improved nutritional status; this in turn improves growth and the survival of the marron by enhancing the immune status and tolerance towards various stressors (chapters 6 and 9).

10.4. Conclusion and recommendation

From this research, the following conclusions are drawn:

1. *B. mycoides* and *Shewanella sp* are the two predominant strains that can be isolated from a number of healthy farmed adult marron's gastro intestinal tract (GIT) (objective 1).
2. *B. mycoides* exhibited favourable probiotic properties such as antagonism ability towards crayfish's common pathogens (*Vibrio mimicus* and *V. cholerae* non-01), non-pathogenic to the host, diverse enzyme profiles and non-resistant to the major classes of antibiotics (objective 1).

3. Host origin (host GIT and its environment) strains with probiotic properties especially with antagonism ability towards the virulent common pathogens of the host are an ideal source of probiotic candidates, as their efficacy are more likely obvious in the host and its environment from where they are isolated (objectives 1- 6).
4. Supplementation of probiotic candidates (host and non-host origin) improved health and the physiological condition of marron in terms of significantly higher tail muscle indices, THC, haemolymph bacteria (bacteraemia) and intestinal bacteria population (Objective 2).
5. Application of *B. mycoides* in marron feed before the pathogen *Vibrio mimicus* injection improved the marron survival and resulted in no mortality (objectives 2 and 4).
6. Supplementation of host origin *B. mycoides* at 10^{8} CFU/g of feed in the marron diet improved the health status of the gastrointestinal tract (GIT) of marron as indicated by an increase in bacterial density, increased and longer microvilli, thicker intestinal epithelium and higher hepatosomatic indices (objective 3).
7. Synbiotic use of *B. mycoide*s at 10^{8} colony forming unit (CFU) g^{-1} of feed and organic selenium (OS) as Sel-Plex at 0,2 g/kg of feed synergistically improved the immune status (GPX and THC) of marron but to a lesser extent on growth rate (objective 4). In vitro tests revealed that OS (Sel-Plex) dispersant in TSB media did not improve *B. mycoides* growth (objective 5).
8. Application of customizedcustomized probiotic *B. mycoides* for two weeks significantly improved marron immunity in terms of THC and GPx enzyme activity which was comparable to four weeks application. However, a continued or periodic application could maintain a higher level of the intestinal bacteria population in the GIT for the better health benefits to the host (objective 6).
9. The customizedcustomized marron origin probiotic *B. mycoides* worked *effectively in vivo* (commercial marron farm) as indicated by the significant improvement in marron immunity and health status which in turn enhanced the survival (objective 6).
10. The customizedcustomized probiotic *B. mycoides* improved the physiological and immunological status of marron (objectives 2-7).

Based on the research outcomes, several recommendations and further research are warranted:

1. Prior to stocking in commercial grow out ponds, the marron juveniles should be fed probiotic *B. mycoides* supplementary diets to improve the growth, health and immune status of marron which in turn can improve the survival and pond production for profitable sustainable marron culture.

2. Predominant healthy host origin (GIT mucosal inhabitants and the host environment) strains with favourable probiotic properties particularly the antagonism ability towards the most common virulent bacteria pathogens of the host is recommended as a favourable probiotic candidate.

3. In probiotic studies, identification of intestinal bacteria should use an advanced identification technique/method to get a comprehensive picture of the intestinal bacteria population and diversity (both culturable and non-culturable) and persistency of the added probiotic.

4. Further study is required to evaluate the extent to which the supplemented probiotic remain viable in the feed after feed preparation.

5. A comparable intestinal size and similar sections of animals should be used when examining intestinal health status (microvilli and histological section) of probiotic and probiotic-free diet fed animals.

6. Performing the *in vivo* test is essential to evaluate the effectiveness of a probiotic candidate on the target animals in its natural environment which is different from controlled laboratory conditions.

7. Prior to evaluating any synbiotic diets (prebiotic and probiotic) for synergistic beneficial effects, the growth of probiotics should be tested using media containing the prebiotic dispersant to determine whether the prebiotic improves or inhibits the growth of the probiotic.

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