Aquaculture and Environment Centre
Department of Environment and Agriculture

Role of immunostimulants in the culture of decapod crustacean

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PREAMBLE

The purpose of this research is to improve the productivity of the commercially important decapods crustacean farming by enhancing their growth, survival, health status and immune competence through the application of immunostimulants, beta glucan (BG) and mannan oligosaccharide (MOS). The effectiveness of these immunostimulants on performances of marron (*Cherax tenuimanus*), tropical rock lobster (*Panulirus ornatus*) and yabbies (*Cherax destructor*) were evaluated by measuring their survival, growth, physiological parameters, health of digestive tract, immunological parameters and resistant ability to bacterial infection and to other abiotic stressors when they were fed with immunostimulants supplemented diets.

This thesis consists of eleven chapters. Chapter 1 is an introduction which briefly highlights current issues of the aquaculture industry, an overview about the use of immunostimulants in marron aquaculture, tropical rock lobster and yabbies. This chapter also justifies and underlines the need, aim and objectives of the current research.

Chapter 2 briefly reviews the research into aquaculture aspects of marron, yabbies and tropical rock lobster. Biology of those crustaceans is also presented in the chapter. The relevant information on the immune system of crustacean is described in this chapter. The use of immunostimulants in aquaculture is also main part of the chapter. Some of relevant criteria to evaluate the performance of crustacean used in this research are also mentioned.

Chapter 3 to chapter 10 reports the main research of this thesis and attempts to evaluate the effectiveness of dietary MOS and BG on the performances of marron, tropical rock lobster and yabbies. All these chapters form an essential component of this research and can be viewed as independent experiments bound by a common theme. These chapters were published in separate peer-reviewed journals or presented at international conference. Because of that, the readers may find some repetitions in 'Introduction', 'Materials and Methods' and "Discussion" sections.

Chapter 3 presents the physiological, immunological and morphological characteristics of digestive track of different size classes of marron as baseline data. Chapter 4 and 5 describe an experiment which aims to find the optimum levels of MOS supplemented for the marron. The effectiveness of different MOS supplemented diets is evaluated by comparing growth, survival, physiological condition and immunological indicators of marron (Chapter 4). Health status of digestive tract of marron including intestinal bacterial community, gut morphology and histology of hepatopancreas and gut of different MOS treated marron groups are detailed in the Chapter 5.

Chapter 6 consists of three trials aiming to investigate the ability of MOS-fed marron to resist bacterial infection and stressors including air and NH_3 exposure. The optimum MOS inclusion levels determined in the chapter 4 and 5 are used in these trials. Survival and immunological parameters including total haemocyte count (THC), different haemocyte counts (DHC), haemolymph clotting time, bacteraemia and lysosomal membrane stability of haemocyte are compared between MOS-fed and non-MOS-fed marron.

Chapter 7 details the experiment with the aim to find out the approximate inclusion level of BG in the diets of marron. Growth, survival, physiological and immunological parameters of marron fed different BG supplemented diets are described and discussed in this chapter.

In chapter 8, the application of MOS is further tested in tropical rock lobster on survival, growth, health of digestive track and resistant capacity to bacterial infection. Chapter 9 describes the effects of dietary MOS on performance of yabbies. Results in terms of growth, survival, immunological parameters and digestive enzyme activities are presented and discussed.

Chapter 10 summarizes the effectiveness of dietary MOS and BG on crustacean. The data collected from this research are also dicussed to obtain a more complete picture in the use of MOS and BG. An assessment of the role of the MOS and BG in crustacean culture is also discussed. In chapter 11, the main conclusions are highlighted which are then followed by the recommendations for future research.

ABSTRACT

Increased concerns on antibiotics used in aquaculture have promoted research toward alternative products. Immunostimulants have been approved to be good alternatives for antibiotics used in the culture of many species. A series of experiments were conducted under laboratory conditions to investigate the roles of immunostimulants, beta glucan (BG) and mannan oligosaccharides (MOS) in the culture of marron (*Cherax tenuimanus*), tropical rock lobster (*Panulirus ornatus*) and yabbies (*Cherax destructor*).

BG showed survival, physiological and immunological improvements in marron through dietary supplementary levels of 0.1 to 0.2%. Dietary MOS at 0.2 to 0.4% inclusion level improved survival, health status and immune system of marron. Similar improvements of dietary MOS were observed in yabbies and tropical rock lobster. In addition, the growth of yabbies and tropical rock lobster increased with MOS supplemented diets. Dietary MOS also benefited marron, tropical rock lobster and yabbies by enhancing the biological functions of their digestive system. Marron and lobster fed MOS diets revealed a healthier gut condition with improvement in morphological structure and microbiota structure. MOS also changed digestive enzyme activities of yabbies. Further, dietary MOS lowered mortality of marron and tropical rock lobster when they were infected with bacteria. The resistance ability of marron to environmental stressors such as NH_3 and air exposure during live transportation was also enhanced in marron fed MOS. In addition, there were no adverse effects of BG and MOS on the performances of above crustaceans.

The current research implies that MOS and BG could be the used as alternatives to antibiotics in crustacean cultured under laboratory environment. In order to attain the optimum benefits of MOS and BG dietary supplementation in the commercial production of crustaceans, however, further research is suggested.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BI	Biomass increment
Cal/g	Calorie per gram
CARL	Curtin Aquatic Research Laboratory
CFU/mL	Colony forming unit per milliliter
DHC	Differential haemocyte count
FCR	Feed conversion ratio
g	Gram
GC	Granulocyte(s), granular cell(s)
HC	Hyalinocyte(s), hyaline cell(s)
HM%	Moisture content of hepatopancreas
HSIwet	Wet hepatosomatic index
HSIdry	Dry hepatosomatic index
L	Litre
LSD	Least Significant Difference
mg/L	Milligram per litre
Min	Minute
mL	Millilitre
MOS	Mannan oligosaccharide
NRR	Neutral red retention
OC	Osmoregularoty capacity
PO	Phenoloxidase
ppt	Parts per thousand, ‰
ProPO	Prophenoloxidase
S	Second
SE	Standard error
SGC	Semi-granulocyte(s), 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

TM%	Moisture content of tail muscle
TMIwet	Wet tail muscle index
TMIdry	Dry tail muscle index
WA	Western Australia

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

1. Articles

1. Sang, H. M., Ky, T. L., & Fotedar, R. (2009). Dietary supplementation of mannan oligosaccharide improves the immune responses and survival of marron, *Cherax tenuimanus* (Smith, 1912) when challenged with different stressors. *Fish & Shellfish Immunology, 27*, 341 - 348.

2. Sang, H. M., & Fotedar, R. (2010). Effects of mannan oligosaccharide dietary supplementation on performances of the tropical spiny lobsters juvenile (*Panulirus ornatus*, Fabricius 1798). *Fish & Shellfish Immunology, 28*, 483 - 489.

3. Sang, H. M., & Fotedar, R. (2010). Effects of dietary be 1, 3 Beta glucan on the growth, survival, physiological and immune response of marron, *Cherax tenuimanus (*smith, 1912). *Fish & Shellfish Immunology, 28,* 957 - 960.

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6. Sang, H. M., Fotedar, R., & Filer, K. (2010). Effects of dietary mannan oligosaccharide on the survival, growth, immunity and digestive enzyme activity of freshwater crayfish, *Cherax destructor* Clark (1936). *Aquaculture Nutrition,* In press.

2. Book chapter

1. Sang, H. M. & Fotedar, R. The applications of mannan oligosaccharide in aquaculture. NOVA publisher (in press).

3. Conference abstracts

1. Sang, H. M. & Fotedar, R. Size dependent physiological, immunological and gut - morphological characteristics of marron, *Cherax tenuimanus* (smith, 1912) – A base line data. In Australasian Aquaculture 2008 International Conference and Trade Show. Brisbane - Australia, 2008.

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND INFORMATION

Aquaculture is the fastest growing animal food-producing sector which outpaces the world population growth, with per capita supply from aquaculture increasing from 0.7 kg in 1970 to 7.8 kg in 2006, an average annual growth rate of 6.9 percent (FAO 2008). The industry is considered to be the main source of food fish for humans in the future (Watanabe 2002). From a production of less than 1 million tones per year in the early 1950s, production in 2006 was reported to be 51.7 million tones with a value of US$ 78.8 billion, representing an annual growth rate of nearly 7 percent. It is estimated that with the current population growth, aquaculture production must keep increasing and reach at least 100 million tones per annum by 2030 to maintain the current per capita consumption (FAO 2006).

Inherently, aquaculture carries the risk of financial losses due to disease either through mortality or reduced meat quality, resulting in reduced profit margins. The economic cost of this to the aquaculture industry can be considerable (Smith *et al.* 2003b). Aquaculture practices themselves may further exacerbate the problem because animals are often cultured under stressful conditions of overcrowding, high food levels, sub-optimal water temperature and poor water quality (Lee and Wickins 1992). In these stressful environments, diseases associated with opportunistic bacteria, such as *Vibrio* spp. or *Pseudomonas* spp., can become prevalent. This can compound the problem associated with more pathogenic organisms and is often worsened by the repeated restocking of cages and ponds, leading to the accumulation of pathogens and opportunistic bacteria in the water and sediment in the surrounding environment. The potential for a disease outbreak poses a continual threat to the existence of any crustacean farm or hatchery and once an infection occurs it can prove devastating to the entire stock (Smith *et al.* 2003b).

The application of antibiotics and other chemicals to culture ponds is expensive and undesirable as it risks contamination of both the environment and the final product (Brown and Higuera-Ciapara 1991; Capone *et al.* 1996; Collier and Pinn 1998), as well as causing mortality or impaired growth in juvenile stock (Stuck *et al.* 1992). In

addition, the repeated application of antibiotics, in the long term, is also encouraging the spread of drug resistant pathogens (Brown 1989; Aoki 1990; Karunasagar *et al.* 1994). Moreover, chemical disinfection may be incompatible with geographical location of the crustacean farm or with the physical requirements of the stock. Thus, there is a very great need to maximise the immunocompetence of the stock whilst minimising the use of therapeutic chemicals (Bache`re *et al.* 1995).

The word "immunostimulant" refers to any substance that is used in aquaculture with intent to boost immune reactivity and improve resistance to, or survival following infection by harmful micro-organisms (Anderson 1992; Chang *et al.* 2003; Smith *et al.* 2003b; Bagni *et al.* 2005; Soltanian *et al.* 2007). Those substances have been receiving the most attention and claims for success in promoting survival of crustaceans against experimental exposure to infectious micro-organisms. There are five mains types of substances considered to be immunostimulants for crustacean aquaculture and research. These are: (i) live bacteria; (ii) killed bacteria (bacterins or bacterial antigen); (iii) glucans; (iv) peptidoglycans; and (v) lipopolysaccharides (LPS). Recently, mannan oligosaccharide (MOS) derived from the cell wall of a specific strain of yeast (*Saccharomyces cerevisiae* 1026) has been receiving heightened attention for it's prebiotic and immunostimulant function in aquaculture species. Research and commercial trials have demonstrated its efficacy as a feed additive in a range of fishes and shrimp species (D'Abramo and Robinson 1989; Dimitroglou *et al.* 2006; Dimitroglou *et al.* 2007; Dimitroglou *et al.* 2008; Dimitroglou *et al.* 2009; Dimitroglou *et al.* 2010).

The freshwater crayfishes, marron (*Cherax tenuimanus*) and yabby (*Cherax destructor*) are considered to be important potential aquaculture species in Australia. The distribution of marron in Western Australia has been extended as far east as Esperance and as far north as Geraldton and global interest in marron farming has led to the species being introduced into South Africa, Zimbabwe, Japan, USA, China and the Caribbean as well as several Australian states (Morrissy *et al.* 1990). On the other hand, yabbies are widely distributed in temperate and sub-tropical south-eastern and central Australia. They have also been introduced into farm dams in southwestern Australia for recreational fishing and aquaculture (Austin 1985). Like all freshwater crayfishes, marron can have several small epibionts attached to their exoskeleton and

gills. Two such epibionts are *Epistylis* and *Temnocephala*. These are symptomatic of poor water quality and result in poor growth rates, particularly in un-aerated ponds containing excessive organic matter. This infection reduced appeal of marron to consumer (Morrissy *et al.* 1990). Although there is no report on the losses in marron aquaculture caused by bacterial infection, the threat to consumers of organism infected marron needs to be considered. Similar to marron aquaculture, some commercial yabby farms are encountering sporadic disease problems (Wong *et al.* 1995).

Among the rock or spiny lobster species, the tropical rock lobster (*Panulirus ornatus*) is probably the most suitable candidate for aquaculture due to its shortest oceanic larval development phase (4–6 months) and the fastest growth rate (1 kg within 2 years after hatching) (Phillips et al. 1992; Pitcher, Skewes & Dennis 1995). The industry is totally based on the wild seed and trash fishes as food source. Food conversion ratios for lobsters in Vietnam on trash fish have been reported to be as high as 28:1 (Tuan *et al.* 2000). This leads to serious implications on the availability of the trash fish and environmental impacts. Further, the introduction of disease through trash fish and the translocation of the trash fish remain greatest impediments in lobster aquaculture. Together with degradation of the culture environmental quality, diseases have occurred and become the main constraint of tropical rock lobster industry. Several serious diseases have been reported such as black gill, shell necrosis and red body (Tuan and Mao 2004).

However, limited published information is available on the health management of marron, yabby and tropical rock lobster culture. Further, there is no published information on the effects of supplementing diet with any immunostimulant on the performance of those decapod crustaceans under laboratory and commercial condition. This research, therefore, focuses on the roles of immunostimulants on the culture of marron, yabby and tropical rock lobster.

1.2 AIM

To improve the growth, productivity, survival, immunity and health status of the crustacean farming by dietary supplementation of the selected immunostimulants.

1.3 OBJECTIVES

The above aim of the research can be achieved by fulfilling the following specific objectives:

1. To obtain the base line data on physiology and immunology of marron.
2. To evaluate the effects of supplementing different levels of mannan oligosaccharide and beta glucan in the formulated diet on the growth, survival, health status and health of digestive tract of the marron.
3. To investigate the effects of mannan oligosaccharide on growth, survival, and digestive enzyme activities of yabbies.
4. To evaluate the effectiveness of mannan oligosaccharide on growth, survival, health status and digestive tract condition of tropical rock lobster.
5. To investigate the effectiveness of mannan oligosaccharide and beta glucan on immune system of marron.
6. To investigate the effects of mannan oligosaccharide on immune system of tropical rock lobster and yabbies.
7. To investigate the efficacy of mannan oligosaccharide on the resistant capacity of marron and tropical rock lobster to bacteria infection and different environmental stressors including air and NH_3 exposures.
8. To provide the selected recommendations for further research based on the outcomes of the current research which the aim to understand the roles of immunostimulants in crayfish aquaculture.

1.4 SIGNIFICANCES

The research aims to make significant contributions in improving the crustacean aquaculture industry by contributing to understanding of the effective use of immunostimulants for marron, tropical rock lobster and yabby aquaculture. The specific significances of the current research are as follows:

1. The study will assist in understanding the mechanisms of the role in which immunostimulants play in aquaculture.
2. The present study will contribute to the knowledge of the immune response of crustacean to environmental and biological stressors.
3. The research will assist in creating a better understanding of the use of immunostimulants through their administration in the diets used by crustacean aquaculture industry.

4. The research will reinforce the suggestion that an alternative to chemicals and antibiotics in the crustacean aquaculture industry is warranted.

5. The research will contribute to an increase in the production of crustacean via improvement in the survival and growth.

6. The research will contribute to sustain the crustacean aquaculture industry via development of a new environmentally friendly aquaculture industry by elimination the use of chemicals and antibiotics.

7. The findings of this study may be used as references for the other related studies in other commercial decapod crustaceans.

CHAPTER 2: LITERATURE REVIEW

2.1 OVERVIEW OF RESEARCH ON FRESHWATER CRAYFISH AND TROPICAL ROCK LOBSTER AQUACULTURE

2.1.1 Marron

Marron (*Cherax tenuimanus* Smith) is the world's third largest freshwater crayfish found in Australia and capable of reaching 2 kg in weight (Morrissy 2000). Native to Western Australia, it has been recognized as a potential aquaculture species during the past few years due to its attractive attributes (Rouse and Kartamulia 1992). These attributes include rapid growth (40-l20 g/year), delicate flavor, a non-aggressive and non-burrowing behavior, a simple life cycle where juveniles are released from the female, so complicated hatcheries for larval rearing is not required, and ability to transport alive in international markets (Morrissy 1976; O'Sullivan 1988; Lawrence 2005). Marron farming in Australia was pioneered by Dr. Noel Morrissy in the mid 1970s (Lawrence 1998) and has significantly increased since the 1980s (O'Sullivan 1988). There are around 280 marron farmers licensed by Fisheries Department, Western Australia (Morrissy 2000). Recently, marron was also introduced into South Africa, Zimbabwe, Japan, USA, China and the Caribbean as a commercial aquaculture species. As a result of the national and international interests in marron farming, its distribution has been widely extended (Bryant and Papas 2007).

Research related to marron cultured has attracted many researchers especially on nutrition (Tsvetnenko *et al.* 1995; Fotedar *et al.* 1997; Fotedar 1998b; Fotedar *et al.* 2000; Fotedar 2004), culture technique (Morrissy 1979; Jussila and Evans 1996a, 1996b; Whisson 1997; Fotedar 1998a; Fotedar *et al.* 1998; Fotedar 1999; Henryon *et al.* 1999; Henryon and Purvis 2000), culture environment and disease (O'Sullivan 1988; Langdon 1991b, 1991a; Lawrence 1995, 2005). Dietary protein has received the most attention. The reduced protein levels in diets showed no effects on the growth performance of marron, but higher wet tail muscles-to-body weight ratio was obtained in marron fed animal protein diets than those fed plant protein diets (Fotedar 2004). Low protein content (i.e. 23%) in diets was not a limiting factor for growth and survival of marron when compared to diets containing 43.6% protein, but

water stable diets produced significantly higher specific growth rate than unstable diets (Jussila and Evans 1998).

Temperature, stocking density and size grading are other interesting subjects studied for marron aquaculture (Morrissy 1990; O'Brien 1995; Whisson 1995). The optimum temperature for growth of marron is 24 ºC (Morrissy 1990). The stocking density of 6 marron/m^2 is recommended for commercially acceptable growth and maximum harvest rates in a recirculating culture system (Fotedar *et al.* 1998). Interestingly, size grading during grow-out period did not enhance growth, survival and production of marron (Qin *et al.* 2001). Research on marron aquaculture has also focused on other areas contributing to value adding marron including effects of salinity on molting and survival (Rouse and Kartamulia 1992), intensive culture in tanks (Jussila and Evans 1996b; Jussila 1997b), selectice breeding program (Henryon *et al.* 1999), live transportation (Jussila *et al.* 1999), hatchery production (Henryon and Purvis 2000) and polyculture with finfish (Whisson 1997; Storer 2005).

However, there is a scarcity of information on diseases of marron. *Epistylis* spp. and *Vorticella* spp. were found on the cephalothoraxes of marron cultured in southern Queensland (Herbert 1987). The presence of protozoan *Epistylis* spp. on the exoskeleton of marron in a commercial earthen pond was reported by Villarreal and Hutchings (1986) and epibionts, *Temnocephala* infected on marron was described in detail by Edgerton *et al.* (2002). A parasite *Vavraia parastacida* causing disease named microsporidiosis on marron was also found (Langdon 1991b, 1991a; Langdon and Thorne 1992). Blisters on the edges of the telson and uropod of marron were observed by Herbert (1987) presuming that the blisters may be caused by physical damage to the cuticle and subsequent irritation of the underlying tissue. Although no mortalities are directly caused by epibionts (Villarreal and Hutchings 1986; Herbert 1987; Edgerton *et al.* 2002) or by microsporidiosis (Langdon 1991b), or the presence of the blisters (Herbert 1987), their occurrence may affect the growth and health status of marron (Lawrence 1998), and may also reduce marketing appearance of marron to consumers (Morrissy *et al.* 1990).

2.1.2 Yabbies

Yabbies, *Cherax destructor* Clark (1936), are widely distributed in temperate and sub-tropical south-eastern and central Australia. They have been introduced into farm dams in southwestern Australia for recreational fishing and aquaculture (Austin 1985). Commercial culture of the yabbies emerged in Australia in the early 1970s. However, despite this industry being one of the oldest aquaculture industries in Australia, production levels have never reached their predicted potential and have stagnated around 300–350 tones per year (Piper 2000). The yabbies have many biological, economic and marketing attributes considered to make a good candidate species for aquaculture. Some of these attributes include rapid growth, good feed conversion efficiency and their direct life cycle. Yabby farms are also relatively inexpensive to construct compared to other forms of farming with some farmers utilizing existing farm dams to produce yabbies. As yabby farming is practiced on privately-owned land, it is an industry that is readily accessible to the majority of South Australian primary producers (Austin *et al.* 1997).

Many studies have focused on several aspects related to aquaculture of yabbies such as growth, nutrient requirement, and culture technique. Growth and survival of yabbies were found to differ with respect to diet in which zooplankton meal resulted in higher growth and greater survival comparing to the pellets containing 15 and 30% protein diets. Diet had a pronounced effect on the degree of variability in yabby size within tanks with zooplankton fed animals showing substantially lower levels of variability compared with pellet fed animals of equivalent size (Austin *et al.* 1997). Protein digestibility by yabbies was not influenced by the principal protein source such as fish, meat, soybean, snail, yabby and zooplankton meals. The high digestibility coefficients obtained for a wide variety of diet-types suggests that yabbies have a versatile digestive system which may reflect its natural polytrophic omnivorous feeding behavior (Jones and De Silva 1997). Mean weight, percentage weight gain, and specific growth rate were reported substantially higher for yabbies fed 30% protein diet than yabbies fed 15% protein diet. The higher protein diet resulted in an increase in carcass protein and ash and a decrease in carcass lipid and energy relative to the low-protein diet. Carcass moisture and calcium were not affected by feed types. The time spent in the intermolt phase of growth was highly dependent on the premolt weight and varied according to diet. The elevated dietary

protein caused a reduction in the intermolt period of yabbies. Dietary induced morphological changes were also recorded. Animals of a standard carapace length had significantly larger abdomens when they was fed the higher protein diet (Jones *et al.* 1996).

Geddes and Smallridge (1993) reported the growth, survival and yield of yabbies (3.9 g) cultured at densities of 3 and 9 individuals/m^2 in four 0.2-ha commercial ponds with forage crop planted. After 10 months of culture, the average weights of yabbies were 24.9, 33.7, 36.6 and 40.0 g with final mean weight lower in the high density ponds. There was wide variation in individual growth, with weight at harvest varying from 10 to 80 g and only 10.7% of yabbies harvested were premium market size (>50 g). Reproduction in the ponds provided a second generation of juveniles and large numbers of berried females at harvest. Only 22 to 44% of yabbies stocked were collected at harvest but refilling and further harvesting showed that perhaps 30% of the population had avoided harvest by burrowing. Yabbies below 50 g were restocked to grow-on ponds and more than 50% achieved market size in the period of 4 months. The biomass harvested after 10 months varied from 406 to 689 kg/ha. After grow-on and carry-over harvests, total yield from the original cohort was 666 kg of which 405 kg comprised individuals above 50 g. This yield was from an area approximately equivalent to 1 ha of ponds. Thus, extensive aquaculture using forage crops can produce modest yields of premium size yabbies (Geddes and Smallridge 1993). The provision of shelter to aquaria did not significantly enhance growth, survival or yield, however, increasing density reduced the average weight of yabbies and increased yield, but did not affect survival (Verhoef and Austin 1999a). By contrast, the growth of yabbies in the intensive culture system could be improved by providing the synthetic substrate (Jones *et al.* 2002). Survival of yabbies was reported to inversely relate to stocking densities (Geddes *et al.* 1993). On the contrary, Verhoef and Austin (1999b) reported that survival of yabbies was not affected by densities, but declined with increasing temperature. Temperature was reported to affect the molt increment of yabbies (Verhoef *et al.* 1998). In the temperature range of 22 to 28°C intermolt period and molt increment decreased with increasing temperature. The mean intermolt periods were 6.7, 5.5, and 5.0 days and mean molt increments were 102, 98, and 93% at 22, 25, and 28°C, respectively. Regression models derived by combining molt increment and intermolt period data

indicated that overall growth increased with increasing temperature (Verhoef *et al.* 1998).

Research on genetic improvement were also conducted to improve the productivity of yabby industry. Within-family selection strategy is very effective in increasing growth rate in yabbies (Jerry *et al.* 2005). A selection program was established based on founder stock from two populations previously identified as having good traits for aquaculture and involved a within-family selection protocol, coupled with a circular mating strategy, to select for faster growth rate (i.e. weight at age). A control line was also maintained to estimate the rate of genetic response. After two generations of selection, weight of males and females from the selected families were 29.5% and 32.7% higher than controls, respectively. This represented an average genetic gain per generation of approximately 15.5% (Jerry *et al.* 2005).

The information on disease and health management of yabby culture is relatively limited. Some commercial yabby farms are encountering sporadic disease problems. Wong *et al*. (1995) sampled 42 individuals of yabbies from Western Australia and isolated bacteria from all of them. They found that 45% of the sample had more than 100 CFU/mL of bacteria in haemolymph. In addition, the discoveries of an introduced microsporidian *Thelohania* sp. and a previously un-described virus associated with mortalities in yabby farms were potential threat to the sustainability of yabbies. Other parasites and ectocommensals have also been recorded from yabbies in Western Australia including temnocephalans, cilliates, *Psorospermium* spp. and the nematode *Gammarinema* spp. (Jones and Lawrence 2001). In addition, Edgerton (1996) reported two viruses in yabbies. They are the intranuclear inclusions due to bacculovirus in yabbies from South Australia and the virus found in Western Australia. The bacculovirus has not been seen in yabby hepatopancreas from Western Australia. The virus found in yabbies from Western Australia is associated with significant pathology and associated mortalities, but the conditions under which epizootics occur are not understood. The survey indicates that the virus is widespread at low prevalence throughout southwestern Western Australia. The low survival rates that occurred in the experimental ponds at Avondale in 1997 and which led to the discovery of the virus have not recurred at that site (Jones and Lawrence 2001).

2.1.3 Tropical rock lobster

The tropical rock lobster (*Panulirus ornatus* Fabricius 1798) is considered to be the good candidatefor aquaculture as they have a shortest oceanic larval development phase of 4–8 months (Dennis *et al.* 1997; Dennis *et al.* 2001) and a fastest post-larval growth rate, attaining a market size of 100–105 mm carapace length (approximately 1 kg) within 18 months after settlement (Phillips *et al.* 1992; Dennis *et al.* 1997). In Vietnam, a tropical rock lobster aquaculture industry has developed for nearly two decades. Cultured lobsters are fed exclusively on fresh whole or chopped fish and shellfish (Tuan *et al.* 2000; Tuan and Mao 2004). Finfish comprise about 70% of the diet, with lizard fish (*Saurida* sp.), red big-eye (*Priacanthus* sp.) and pony fish (*Leiognathus* sp.) being the predominant species. Oysters (*Crassostrea* spp., *Pinctada* spp.), cockles (*Anadara* spp.), paddy snail (*Pomacea* spp*., Pila* spp.) and swimming crab (*Portunus pelagicus*) are the predominant types of shellfish, making up the other 30% of the diet (Tuan and Mao 2004). The use of trash fish as a food source for lobster aquaculture is seasonal dependent and has negative impacts on the environment and resource. Thus, there has been considerable interest related to sustainable aquaculture of this species for the past decades. Several attempts have been made to achieve the target including research on nutrient requirements, disease, biology and culture technique of the tropical rock lobster.

Development of the pelleted formulated feed is one of the main priorities toward the sustainable aquaculture of the tropical rock lobster. Evaluation of nutrient requirements is the first step in formulation of the pelleted feed, which was reviewed by Williams (2007a). The best growth occurred at dietary crude protein asymptotes of 47 and 53% (dry matter basis) for the 6 and 10% lipid feeds, respectively. At the higher protein levels, lobster growth was better for the high lipid level than low lipid feeds (Smith *et al.* 2003a). The dietary protein requirement of tropical rock lobster was more recently examined by Smith *et al.* (2005). The lobster's acceptance of the pellet feed was improved by including freeze-dried krill hydrolysate and freeze-dried krill meal in the basal formulation and increased feeding frequency from twice to four times daily. Lobsters fed - pelleted feeds had high survival (79%) and responded to increasing dietary crude protein content with progressively higher growth rates, with the daily growth coefficient increasing from 0.72 to 1.38% as the dietary crude protein content increased from 33 to 61% (Smith *et al.* 2005).

Micro-nutrient requirements of tropical rock lobster have also been researched. Although dietary astaxanthin did not affect growth rate or survival, there was a dose–response increase in tissue carotenoid content and darkening of the exoskeleton pigmentation, which may have important implications for immunocompetency and marketing of the lobster (Barclay *et al.* 2006).

Beside nutrient requirement, culture technique is one of the main focuses for sustainable aquaculture of the lobster. Survival and growth of the lobster was improved when they were fed by live green mussel (*Perna viridis*) either as the sole food or as a supplement to fish by-catch compared to lobster fed only fish by-catch (Du *et al.* 2005). In addition, the survival and growth of the lobster was also improved by co-cultured with green mussel (Hoang and Sang 2007). A series of experiments were conducted to evaluate of partial replacement of live and fresh feeds with a formulated diet and the influence of weaning tropical rock lobster phyllosomata onto a formulated diet during early ontogeny. Results demonstrated that hatcheries can potentially replace 75% of live on-grown artemia with a formulated diet 7 days after hatch (Johnston *et al.* 2008).

The research on health management, especially, replacement of antibiotic usage in tropical rock lobster aquaculture is very scarce. There is some literature describing the common diseases of lobster culture in Vietnam and proposing the preventive and treatment methods. However, most of the current methods to control diseases rely on chemicals and antibiotics (Nha 2004). A few recent reports described the use of probiotics for the cage rearing lobster (Thuy *et al.* 2009; Thuy *et al.* 2010).

2.2 MARRON, YABBY AND TROPICAL ROCK LOBSTER BIOLOGY

2.2.1 Taxonomy

Marron can be readily distinguished from other *Cherax* species by the presence of five keels on the dorsal surface of their head and two small spines on the telson. Yabby (*Cherax destructor/albidus*), the other Cherax species within Victoria, in contrast have four keels along the head with only two easily discernible, no spines on

the telson and have elongate and large chelipeds (Bryant and Papas 2007). The systematic classifications of marron and yabbies are as follows:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Suborder: Pleocyemata

Infraorder: Astacidea

Family: Parastacidae

Genus: *Cherax*

Species: *C. tenuimanus* (Smith 1912) and *C. destructor* Clark (1936)

There are two discrete forms of marron, a smooth form and hairy form. They were considered to be subspecies (Austin and Knott 1996) until 2002 when allozyme evidence indicated these forms were distinct species (Austin and Ryan 2002). The hairy form, restricted to the Margaret River system in WA, maintained the name *Cherax tenuimanus* (Smith), while the smooth and widely translocated form was given the name *Cherax cainii* (Austin) (Bryant and Papas 2007).

Panulirus is a genus of spiny lobster in the family Palinuridae of the suborder Pleocyemata. The genus includes 20 species which have long flagella on their first antennae. The systematic classification of tropical rock lobster is as follows:

Kingdom Animalia

Phylum Arthropoda

Class Malacostraca

Order Decapoda

Suborder Pleocyemata

Family Palinuridae

Genus Panulirus

Species *Panulirus ornatus* (Fabricius, 1798)

2.2.2 Biology

In natural environment, marron live in the clear, deep water (Mosig 1998; Wingfield 1998). They prefer sandy reaches with structure (e.g. snags and rocks) for shelter (Molony *et al.* 2004). Marron are not considered to be burrowers (Clunie *et al.* 2002), however, Mosig (1998) reported that burrowing may occur in the banks of dams where refuge habitat is limiting. Marron farming has proven to be extremely successful in clay bottomed farm dams and aquaculture ponds (Morrissy 1976, 1990; Lawrence 1998, 2005). Habitat in the form of tyres, rope fibre, piping or other materials is often added to the dams to provide shelter for marron (Bryant and Papas 2007).

Marron are the third largest freshwater crayfish species in the world and largest *Cherax* species, reaching a length of 380 mm and weighing up to 2.7 kg (Molony *et al.* 2004). Maturity under favorable conditions can be reached in two years, although is commonly three years (Merrick and Lambert 1991). Breeding occurs annually in spring, triggered by water temperature and day length. Females have been reported to carry between 95 and 900 berries but generally average 150 (Merrick and Lambert 1991; Bryant and Papas 2007).

Biology of the yabbies was well compiled by Withnall (2000). They are semi-aquatic freshwater crayfish commonly found in a wide range of habitats throughout most of Victoria and New South Wales, including low-lying swamp ground, streams, rivers and dams. They may also be found in southern Queensland, South Australia, and parts of the Northern Territory. Yabbies are generally found in areas where oxygen levels are high and where there is plenty of vegetation. They are wide-range adapted temperature species which is able to survive in water temperatures between 1°C and 35°C. However, when water temperatures drop below 16°C the yabbies falls into a state of partial hybernation where metabolism, feeding and growth virtually cease. Temperatures higher than 35°C will result in cessation of growth and eventually mortality. The ideal temperature range for optimum growth is between 20°C and 25°C (Withnall 2000).

Yabbies are able to tolerate a wide range of elevated salinities, up to 8 ppt. However, high salinity levels will result in increased stress on the animal. Growth will often stop at salinities over 8 ppt and mortalities will occur as salinity levels increase. As dissolved oxygen decreases, feeding and therefore growth, also decreases. Yabbies are commonly found on muddy or silted bottoms and are rarely found in clear water habitats, preferring water with moderate levels of turbidity. Muddy waters afford some protection from predators such as fish and birds giving the yabbies a better chance at survival (Withnall 2000).

Reproduction of yabbies is primarily related to water temperature and day length. Research has shown that mating begins in the spring and early summer when water temperatures reach above 15°C and day length has increased. Spawning reaches its peak between October and January. Females often spawn two or more times in the one season. The number of fertilized eggs carried by the female ranges from 100 to 1000 related to the female size. The fertilized eggs undergo a series of larval stages, taking approximately 19 to 40 days to hatch. The length of time taken for the eggs to hatch is entirely dependent on temperature. In water temperatures of 20°C, the eggs hatch within 40 days. As temperature increases, the length of time taken to hatch decrease until water temperatures reach 30°C. Temperatures above 30°C adversely affect both the adult and the juvenile (Withnall 2000).

Tropical rock lobster inhabits shallow, sometimes slightly turbid coastal waters, from 1 to 8 m depth, with a few records from depths as great as 50 m on sandy and muddy substrates, sometimes on rocky bottom, often near the mouths of rivers, but also on coral reefs. The species has been reported as solitary or as living in pairs, but has also been found in larger concentrations (Holthuis 1991).

The maximum size of the male tropical rock lobster was reported bigger than the female. Length at first sexual maturity is approximately 110 mm of carapace length for males and approximately 98 mm of carapace length for females lobster. Spawning seasons of tropical rock lobster in the central coastal area of Vietnam is from January to May and from August to October. A female releases from 1.7 to 2.3 millions eggs. Fertilized eggs pass through different embryonic development stages

including morula - blastula - gastula - nauplius before hatching to first stage of phyllosoma (Thuy 2004b).

2.3 CRUSTACEAN IMMUNE SYSTEM

2.3.1 Haemocyte types and their roles

The different haemocyte types in crustaceans can be distinguished based on the morphological and biological functions (Bauchau 1981). Cornick and Stewart (1978) reported four different haemocyte types in American lobster (*Homarus americanus*) including prohyalocytes, hyalinocytes, eosinophilic granulocytes and chromophobic granulocytes, whereas Hose *et al*. (1990) recommended two types of haemocytes in decopod crustacean, hyalinocytes and granulocytes. Most research suggests that decopod crustacean haemocytes can be classified into three main types, hyalinocytes, semigranocytes and granulocytes (Hearing and Vernick 1967; Söderhäll and Cerenius 1992; Jussila *et al.* 1997; Fotedar *et al.* 2001; Smith *et al.* 2003b). In crayfish, haemocyte types has been also identified as hyalinocytes, semigranocytes and granulocytes (Cárdenas *et al.* 2000; Johansson *et al.* 2000). Differentiating between those cells type are based on their shape, nuclear, endoplasmic reticulum, free rebosomes, golgi, granules, lysosomes and mitchondria (Bauchau 1981) (Table 2.1).

Different haemocyte types are involved in different biological functions (Söderhäll and Cerenius 1992) (Table 2.2). The hyaline cells can execute phagocytosis (Johansson and Söderhäll 1989), initiate or contribute to haemolymph clotting in crustaceans (Hose *et al.* 1990) and contribute to formation and hardening of cuticle at molt (Vacca and Fingerman 1983). The semi-granular cells are the cells acting in encapsulation (Johansson and Söderhäll 1989) and having some phagocytic capacity (Smith and Soderhall 1983). These cells are also involved in prophenoloxidase (proPO) activating system (Johansson and Söderhäll 1989). The granular cells are reported to take part in haemolymph clotting system (Aono *et al.* 1993) and involved in cuticle hardening, tanning, basement membrane formation and wound healing (Vacca and Fingerman 1983). They are the primary phagocytic haemocytes (Hryniewiecka-Szyfter and Babula 1996) which are able to encapsulate foreign particles and phagocytose bacteria (Hose *et al.* 1990). More importantly, the main

function of the granular cells is a repository for the prophenoloxidase activating system (Johansson and Söderhäll 1985, 1989; Söderhäll and Cerenius 1992).

Table 2.1: Three different haemocyte types in crustacean

Criteria	Hyaline cell	Semi-granular cell	Granular cell
Shape	round or oval	oval or spindle	oval
Nucleus	central, round, large	central or eccentric, oval, lobed	eccentric, kidney shape
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

Table 2.2: Haemocyte types and known biological functions

Haemocyte type	Phagocytosis	Encapsulation	Cytotoxicity	ProPO activating system
Hyaline	Yes	No	Unknown	No
Semi-granular	Limited	Yes	Yes	Yes
Granular	No	Very limited	Yes	Yes

2.3.2 Non-specific immune system

Unlike fishes which have specific immune system, the defence mechanism in decopod crustaceans relies on a non-specific or innate immune response to recognise and fight foreign micro-organisms and pathogens (Söderhäll 1997). The crustacean defence system consists of cellular and humoral components, of which haemocytes play extremely important roles in the immune system (Bachere 2003). The haemocytes perform several biological functions such as phagocytosis, encapsulation, nodule formation, and mediation of cytotoxicity (Söderhäll and Cerenius 1992). They can execute the release of microbicidal proteins (Smith *et al.* 2003b) and are responsible in direct sequestration and killing of infectious agents

(Söderhäll and Cerenius 1992). The simplified flow diagram of the cellular defence system in crustacean is shown in Figure 2.1 (Smith *et al.* 2003b).

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β-1,3-glucan binding protein

β-1,3-glucans
Peptidoglycan
Live bacteria
Bacterial antigen

Semigranular
Haemocytes

Granular
Haemocyte

Hyalinocytes

Degranulation

Phagocytosis

Antibacterial
peptides

Inactive serine
proteinase

Prophenoloxidase
(ProPO)

Active serine
proteinase

Phenoloxidase

Cell adhesion
Release of reactive O_2^-
Opsonization

Phenolic compounds ⟶ Quinones ⟶ Melanin ⟶ Encapsulation

Figure 2.1: Simplified flow diagram of the crustacean defence system.

2.3.3 Defence mechanisms in crustacean

In decopod crustaceans, the physical barriers are the first obstacle to detain pathogenic micro-organisms (Söderhäll 1982). When there is damage and the micro-organisms invade the tissue, proteolytic pathways take place instantly, to eliminate

the microbes invading the organism (Ratcliffe *et al.* 1985; Vazquez *et al.* 2009). The effecter mechanisms for crustacean immune responses include the coagulation cascade, which avoids the loss of haemolymph and stimulates oxidative metabolites and production of melanin by activating the prophenoloxidase (proPO) system (Sritunyalucksana *et al.* 1999; Vargas-Albores and Yepiz-Plascencia 2000; Vazquez *et al.* 2009). Prophenoloxidase activation stimulates other important biological reactions in the immune response, such as phagocytosis, encapsulation and nodule formation (Table 2.3). Activation of such reactions is mediated through the specific recognition of glycosylated pathogen-associated molecular patterns (PAMPs) by crustacean proteins (Vazquez *et al.* 2009).

Table 2.3: Defence mechanisms in crustacean

Defense mechanisms	Haemocytes involved	Target
ProPO	Semi-granular cell, granular cell	Bacteria and fungi
Antimicrobial proteins	Granular cell	Bacteria and fungi
Phagocytosis	Hyaline cell, Semi-granular cell	Bacteria and micro-organisms < 10 μm
Encapsulation	Semi-granular cell, Granular cell	Fungal spore and yeast, organisms > 10 μm
Lectins	Hyaline, granular and semi-granular cells	Distinguish between the self and non-self particles, inducing agglutination and phagocytosis
Clottable protein	Clottable proteins from haemocytes	Bacteria and fungi

2.3.4 Clotting reaction

The haemolymph clotting is an efficient defence reaction in crustaceans which serves to seal wounds, preventing blood loss and pathogenic micro-organisms from penetrating the wound (Smith and Chisholm 1992). Haemolymph clotting prevents diffusion of bacteria in the circulation of crustaceans (Aono *et al.* 1993). Like other arthropods, crustacean haemolymph clotting involves aggregation of the circulating cells and the gelation of the plasma by factors released from the haemocytes (Durliat 1985) especially from granular cells and hyaline cell (Aono *et al.* 1993). Clotting can

be induced by microbial polysaccharides, such as β-1,3-glucan (Söderhäll 1981) and bacteria (Aono *et al.* 1993).

2.3.5 Prophenoloxidase activating system

The prophenoloxidase system functioning as an immunological recognition system (Johansson and Söderhäll 1989) is believed to be a dominant part of the crustacean defence system (Söderhäll 1997). When the cellular defence reactions are initiated, melanisation will occur (Ratcliffe *et al.* 1985). In the synthesis of the pigment melanin, the key enzyme is phenoloxidase. It is present in haemolymph as an inactive pro-enzyme called prophenoloxidase (proPO) (Johansson and Söderhäll 1985). ProPO is activated to form phenoloxidase when it reacts with microbial cell wall components such as β-1,3-glucan, lipopolysaccharides and peptidoglycan (Johansson and Söderhäll 1989). Besides the important role in melanisation, the ProPO system is involved in cellular communication between different haemocytes (Söderhäll and Cerenius 1992). Its components stimulate several cellular defence reactions such as phagocytosis, nodule formation and encapsulation (Johansson and Söderhäll 1989).

2.3.6 Phagocytosis

Phagocytosis is a process carried out by haemocytes (phagocytes) that have the capacity to recognize and ingest molecules, such as bacteria, spores, or senescent cells of the organism. This immunity process has also been preserved during evolution, some authors suggest this mechanism as a precursor of vertebrate innate immunity (Mudlarz *et al.* 2006). In the freshwater crab, *Parachaeraps bicarinatus*, and the European green crab, *Carcinus maenas*, phagocytes are the main cells that participate in the elimination of circulating particles in the hemocoel (Vazquez *et al.* 2009). Phagocytes of the American lobster, *Homarus americanus*, recognize only gram-negative but not gram-positive bacteria (Mori and Stewart 2006). On the contrary, phagocytes of the blue crab, *Callinectes sapidus,* eliminate both gram-negative and gram-positive bacteria (Cassels *et al.* 1986). Other studies of the opsonic effect of cell-free haemolymph lectins on the phagocytosis exerted by circulating haemocytes of the kuruma prawn, *Metapenaeus japonicus*, and the freshwater prawn, *Macrobrachium rosenbergii*, revealed that phagocytosis involves a double mechanism for foreign material recognition (Vazquez *et al.* 2009): one of

these mechanisms is determined by lectin-specificity for O- and N-acetylated carbohydrates and the second mechanism seems to be unspecific, although both allow for the phagocytosis of pathogen agents (Kondo *et al.* 1992). The phagocytosis activity of crustacean can be evaluated by flow cytometry analysis (Lee *et al.* 2001), determination of reactive oxygen production (Song and Hsieh 1994) or nitric oxide syntheses activity (Chang *et al.* 2003).

2.3.7 Nodule formation

The nodule formation reaction in crustacean occurs when the body cavity is invaded by a large number of micro-organisms, in excess of those that can be removed by phagocytosis. The micro-organisms become entrapped in several layers of haemocytes, and becomes melanised by host phenoloxidase activity (Söderhäll and Cerenius 1992).

2.3.8 Encapsulation

Encapsulation is a multi-cellular response to eliminate foreign particles that cannot be destroyed by humoral mechanisms by killing pathogens or, at least, restricting their movement and growth in the hemocoel cavity (Vazquez *et al.* 2009). Encapsulation in crayfish, *Astacus leptodactylus* showed semi-granular haemocyte aggregation and the presence of adhesive factors surrounding particles larger than 10 μm diameter, such as helminthes and fungal spores (Durliat 1985; Persson *et al.* 1987). Encapsulation can work as a protective barrier preventing pathogens from entering the muscle or the hemocoel. A typical capsule consists of 5–30 compact layers of haemocytes, without intercellular spaces (Rather and Vinson 1983).

2.4 IMMUNOSTIMULANTS IN CRUSTACEAN AQUACULTURE

In aquaculture, the use of immunostimulants, environmentally-friendly preventive approaches, to stimulate the non-specific immune system, improving the health of cultured organisms, and reducing mortalities is becoming increasingly important. Immunostimulant is defined as "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens" (Bricknell and Dalmo 2005). In aquaculture an immunostimulant could be defined as an agent that is used to stimulate the immune mechanisms and promote resistance against harmful micro-organisms

(Smith *et al.* 2003b). Many different types of substances are currently known to act as immunostimulants which are often grouped based on their functions or origin (Anderson 1992; Secombes 1994). Immunostimulants applied in aquaculture can be divided into five main groups depending on their sources: live bacteria; dead bacteria (bacterins or bacterial antigen); glucans; peptidoglycans; and lipopolysaccharides (LPS) (Smith *et al.* 2003b). Recently, mannan oligosaccharide (MOS) has been reported to show immune stimulation potential in several fish and crustacean species (Craig and McLean 2003; Staykov 2004; Zhou and Li 2004; Hossu *et al.* 2005; Staykov *et al.* 2005c, 2005a; Bogut *et al.* 2006; Culjak *et al.* 2006; Daniels *et al.* 2006; Dimitroglou *et al.* 2006; Staykov *et al.* 2006; Dimitroglou *et al.* 2007; Genc *et al.* 2007a; Terova *et al.* 2009). MOS is also known as a prebiotic applied in animal husbandry, poultry and aquaculture industry (Bongers and van der Huevel 2003; Patterson and Burkholder 2003; Gibson *et al.* 2004; Yousefian and Amiri 2009; Ringo *et al.* 2010). Smith *et al.* (2003b) summarised the immunostimulants which have been successfully used in improving crustaceans against infectious micro-organisms in laboratory conditions (Table 2.4).

The application methods for immunostimulants include immersion, as a dietary supplement or by injection. The application method could be an important factor affecting the success of the treatment in enhancing protection against disease (Smith *et al.* 2003b). Many authors have reported that administration by injection is the most efficacious (Sakai 1999), however, it is extremely labor intensive and costly. In addition, this method often represents an additional stress to the cultured animal. Administration methods of immersion or as a dietary component has proved to be the preferred option within commercial shellfish farms (Smith *et al.* 2003b).

Table 2.4: Immunostimulants reported to stimulate the crustacean immune system

Immunostimulants	Crustacean species	Nature of study	Reference
(i) Live bacteria			
Vibrio spp.	*Penaeus monodon*	In vivo challenge	(Teunissen *et al.* 1998)
V harveyi (Strain PDEX)	*Penaeus monodon*	In vitro assay	(Alabi *et al.* 2000)
(ii) Killed bacteria			

Aerococcus viridans var. *homari*	*Homarus americanus*	Survival rate	(Keith *et al.* 1992)
V. vulnificans	*Penaeus monodon*	Phenoloxidase and Superoxide determination	(Sung *et al.* 1996)
V. harveyi (strains, BP03, BP04, BP05, and IN7)	*Penaeus indicus*	Survival rate	(Alabi *et al.* 1999)
Vibrio spp. (ANM 708) *Photobacterium* spp. (AAC 727)	*Macrobrachium rosenbergii*	Survival rates	(Vici *et al.* 2000)
(iii) Glucan			
β-1,6-/β-1,3-glucan from *Saccharomyces cerevisiae*	*Penaeus monodon*	Phenoloxidase determination and survival rates	(Sung *et al.* 1994)
β-1,6-/β-1,3-glucan from *Saccharomyces cerevisiae*	*Penaeus monodon*	Stress tolerance, growth and survival rates	(Song *et al.* 1997)
β-1,6-/β-1,3-glucan from *Saccharomyces cerevisiae*	*Litopenaeus vannamei*	Survival rates and growth studies	(Scholz *et al.* 1999)
β-1,6-/β-1,3-glucan from *Laminaria digitata*	*Penaeus monodon*	In vitro assay	(Sritunyalucksana *et al.* 1999)
β-1,6-/β-1,3-glucan from *Schizophyllum commune*	*Penaeus monodon*	Survival rate and in vitro assay	(Chang *et al.* 2000)
(iv) Peptidoglycans			
From *Brevibacterium lactofermentum*	*Penaeus monodon*	Survival rate and growth	(Boonyaratpalin *et al.* 1995)
From *Bifdobacterium thermophilum*	*Penaeus japonicus*	Survival rates and phagocytosis assay	(Itami *et al.* 1998)
Commercial formulation	*Penaeus monodon*	In vitro assay	(Sritunyalucksana *et al.* 1999)

(v) Lipopolysaccharides			
From *Escherichia coli* serotype 055:135	*Penaeus monodon*	In vitro assay	(Sritunyalucksana *et al.* 1999)
From *Pantea agglomerans*	*Penaeus japonicus*	Survival rates and in vitro assay	(Takahashi *et al.* 2000)

Among the above immunostimulants, the immune stimulatory effects of beta glucan have been most well-studied (Sakai 1999). Recently, the roles of mannan oligosaccharides as prebiotic and immunostimulant is also receiving heightened attention in aquaculture (Ringo *et al.* 2010).

2.4.1 Beta glucan

Beta glucans are polymers of glucose, one of three polymer classes of polysaccharides (Hong *et al.* 1994). They are now becoming an alternative approach to antibiotic treatments in the prevention of diseases in aquaculture species (Bachere 2003; Soltanian *et al.* 2007) and are widely used to enhance resistance of fish and crustacean against a wide range of diseases (Soltanian *et al.* 2007). Beta glucans are considered as microbial surface antigens and have ability to activate defensive cellular functions in invertebrates (Campa-Córdova *et al.* 2002). The immunostimulants can stimulate cellular function, induce degranulation and activate proPO through reacting with beta glucan binding protein, an endogenous protein found in crustaceans (Vargas-Albores and Yepiz-Plascencia 2000).

In crustacean aquaculture, the protective role of beta glucan as immunostimulant has been well-proven (Table 2.5). However, the best effectiveness of beta glucan application was only obtained when applied at the appropriated concentrations. Most studies reported that the oral administration of beta glucan at a level of 0.2% is the most effective for *Peanaus monodon* (Liao *et al.* 1996; Chang *et al.* 1999; Chang *et al.* 2000; Chang *et al.* 2003; Suphantharika *et al.* 2003). However, at higher levels, namely in the range of 0.4 – 0.8%, beta glucan did not show any efficacy in immune response of *P. monodon* (Suphantharika *et al.* 2003). Inefficacy of beta glucan when applied at high concentration dose (15 mg/L) was also observed in *Macrobrachium*

rosenbergii post-larvae, but the most effective in enhancement of protection against pathogens for *M. rosenbergii* post-larvae was obtained at a dose of 10 mg/L of beta glucan suspension (Misra *et al.* 2004).

2.4.2 Mannan oligosaccharides (MOS)

Mannan oligosaccharide is glucomannoprotein complexes derived from the cell wall of the yeast, *Saccharomyces cerevisiae* (Sweetman and Davies 2007). A common commercial product which contained about 25% of MOS is Bio-Mos (Alltech Inc., USA). MOS is considered to be a pre-biotic immunostimulant which can activate non-immune responses of cultured species (Daniels *et al.* 2006). This compound can also promote the efficiency of the digestive tract by increasing the regularity, height and integrity of gut villi (Hooge 2004) and stimulate the growth and activity of beneficial bacteria while inhibit the growth of harmful pathogenetic bacteria in digestive tract (Daniels *et al.* 2006). In livestock industry, MOS is able to block fimbriae of pathogenic bacteria, and thus able to prevent their adhesion to the mucous epithelium (Kogan and Kocher 2007). Due to these properties, MOS is now used as an immunostimulant and a growth promoter supplemented in diets for both terrestrial and aquatic cultured organisms.

Table 2.5: Use of beta glucan as immunostimulants in crustacean species

Crustacean species	Applied method	Results	Reference
Penaeus monodon	Dietary component	Improved growth and survival; enhanced the immune system; improved resistant ability to white spot syndrome virus -infection; enhanced haemocyte phagocytic activity, cell adhesion and superoxide anion production. Increased phenoloxidase and nitroblue tetrazolium reaction	(Sung *et al.* 1994; Liao *et al.* 1996; Song *et al.* 1997; Chang *et al.* 1999; Sritunyalucksana *et al.* 1999; Chang *et al.* 2000; Chang *et al.* 2003; Suphantharika *et al.* 2003)

Litopenaeus vannamei	Immersion	Increased superoxide anion generation and superoxide dismutase activity	(Campa-Córdova *et al.* 2002)
Macrobrachium rosenbergii	Immersion	Increased lysosomal enzyme, acid phosphatase activities	(Misra *et al.* 2004)
Artemia	Dietary component	Increased resistant to pathogens *Vibrio campbellii* and *V. proteolyticus;*	(Marques *et al.* 2006; Soltanian *et al.* 2007)

The use of MOS to improve survival and health status of livestock and poultry industries is well documented (Sweetman and Davies 2007). MOS was used as dietary supplementation to change intestinal morphology in rabbits, resulting in longer villi, and thus increasing absorption surface (Mourão *et al.* 2006). Dietary MOS enhanced growth and survival (Rozeboom *et al.* 2005) and increased concentrations of immunoglobulin of pigs (Funderburke 2002). In aquaculture, although the efficacies of MOS have been proved in some finfish species, the use of MOS for crustaceans is rather limited. The use of MOS as immunostimulant and prebiotic has been recently reviewed by Ringo *et al.* (2010) (Table 2.6).

Table 2.6: Efficacies of dietary mannan oligosaccharides (MOS) in aquatic animals

Species (g)	Administration	Results	Reference
Atlantic salmon (*Salmo salar)* (200 ± 0.6 g)	10 g kg^{-1} - 4 months	Reduced oxygen consumption; increased protein and energy concentration in the whole body	(Grisdale-Helland *et al.* 2008)
Channel catfish (*Ictalurus punctatus*) (16 g)	2 g kg^{-1}- 4 weeks	No effect on growth performance, haematology or immune function and survival against *Edwardsiella ictaluri*	(Welker *et al.* 2007)
Cobia (*Rachycentron canadum*) larvae	0.2% - 13 days post hatch	Increased larval survival and microvilli alignment; reduced supranuclear vacuoles	(Salze *et al.* 2008a)
European lobster	*Artemia*	Increased larval survival	(Daniels *et al.*

(*Homarus gammarus*)	*nauplii*-enrichment		2006)
European sea bass (*Dicentrarchus labrax*) (33.7 ± 7.7 g)	20 and 40 g kg^{-1} – 67 days	Increased growth; reduced lipid vacuolization and reduced the presence of *Vibrio alginolyticus* on head kidney	(Torrecillas *et al.* 2007)
Rainbow trout (*Oncorhynchus mykiss*) (30 g)	2 g kg^{-1} – 90 days	Increased growth, survival and antibody titre and lysozyme activity	(Staykov *et al.* 2007)
Rainbow trout (37.5 ± 1 g)	1.5, 3 or 4.5 g kg^{-1} – 90 days	1.5 g kg^{-1} increased growth rate 1.5 g and 3 g kg^{-1} increased intestinal villi	(Yilmaz *et al.* 2007)
Rainbow trout	2 g kg^{-1} – 8 weeks	Increased absorptive surface in the posterior gut region; increased microvilli density and microvilli length	(Dimitroglou *et al.* 2008)
Rainbow trout (13.2 g)	4 g kg^{-1} – 12 weeks	Increased growth; increased haemolytic and phagocytic activities; increased mucus weight; increased survival against *Vibrio anguillarum*	(Rodrigues-Estrada *et al.* 2008)
Red drum (*Sciaenops ocellatus* L.) (500 g)	10 g kg^{-1} – 3 weeks	Increased protein and organic apparent digestibility coefficient values; reduced lipid apparent digestibility coefficient	(Burr *et al.* 2008)
Gulf sturgeon (*Acipenser oxyrinchus desotoi*) (130 g)	3 g kg^{-1} – 5 weeks	None effect on growth performance, feed conversion and gross gastrointestinal morphology	(Pryor *et al.* 2003)
Hybrid tilapia (9.8 g)	1.5, 3 and 4.5 g kg^{-1} – 80 days	None effect on growth parameters and body indices; dry matter and protein contents of fillets increased with	(Genc *et al.* 2007b)

		increasing rates of MOS	
Nile tilapia (*Oreochromis niloticus*) (13.6 ± 0.7 g)	2, 4, 6, 8 and 10 g kg)1 – 45 days	None effect on haematological parameters; reduced daily feed consumption with increasing level of MOS	(Sado *et al.* 2008)
Nile tilapia (0.82 g)	2, 4, and 6 g kg^{-1} – 3 weeks	Increased weight, length and average daily growth of fish fed 4, and 6 g kg^{-1} MOS; increased survival against *Streptococcus agalactiae*	(Samrongpan *et al.* 2008)
Tiger shrimp (*Penaeus semisulcatus*) (0.34 g)	1.5, 3 and 4.5 g kg$^-$1 – 48 days	3 g kg^{-1} increased growth, feed conversion and survival; no detrimental effect was noted on hepatopancreas tissue	(Genc *et al.* 2007a)

2.5 APPROACHES TO EVALUATE THE PERFORMANCE AND HEALTH STATUS OF CRUSTACEAN

Techniques used to evaluate efficacy of immunostimulants/prebiotics supplemented in the diet for aquatic animals included the measurement of survival and specific growth rate (Craig and McLean 2003; Staykov *et al.* 2005a, 2005b; Culjak *et al.* 2006; Daniels *et al.* 2006). Further, the physiological indicators of crustacean including wet and dry hepatosomatic indices (McClain 1995a, 1995b), wet and dry tail muscles to body weight ratios and moisture level in hepatopancreas and tail muscle (Castell and Budson 1974; Fotedar 1998b; Sang and Fotedar 2004) was also used to evaluate the health status and stress level of crustacean affected by feed additive. The improvement of immune system capacity of crustacean by immunostimulants were examined by measurement of several immunological indicators including total haemocyte count, differentiate haemocyte count, haemolymph clotting time, bacteraemia (Tsvetnenko *et al.* 1999; Fotedar *et al.* 2001; Fotedar *et al.* 2006), neutral red retention time of lysosomal membrane (Lowe *et al.* 1995; Hauton *et al.* 1998, 2001; Nicholson 2003; Hauton and Smith 2004; Song *et al.* 2007). In addition, the technique to evaluate the digestive tract health of aquatic animal stimulated by immunostimulants/prebiotic such as total bacteria in the digestive tract, microvilli structure, absorption surface area and epithelium layer and

epidermis cell (Dimitroglou *et al.* 2005; Dimitroglou *et al.* 2007; Sweetman and Davies 2007; Dimitroglou *et al.* 2008) and digestive enzyme activities (Biesiot and Capuzzo 1990; Furne *et al.* 2005; Perera *et al.* 2005) were used.

2.5.1 Specific growth rate and biomass

Measurement of absolute weight gains over time periods and calculating specific growth rate are the simplest way to assess the growth (Busacher *et al.* 1990). Growth in crustaceans is widely used to access the efficacy of dietary supplementation (Gu *et al.* 1996). Growth is one of the useful indicators of crustacean health and condition because it integrates all of the biotic and abiotic variables acting on the aquatic animal and reflects secondary effects of chronic stress due to nutrient deprivations (Larkin 1978; Fotedar 1998b). The growth of marron expressed as specific growth rate in various culture environments, from aquaria to battery culture system or in intensive crayfish culture system varies between 0.5 to 1.1%/day (Morrissy 1976, 1979, 1990; Morrissy *et al.* 1990; Jussila and Evans 1996a; Fotedar *et al.* 1997; Fotedar 2004). However, in culture of crustaceans, specific growth rate may not yield a reliable measure of nutritional effectiveness of a test diet as the animals also derive their nutrition by cannibalizing other animals. Further, stocking density of the animal may influence the specific growth rate. Therefore, harvested biomass incorporates a measure of survival is more reliable parameter in the measurement of effectiveness of a diet (Fotedar 1998b; Verhoef *et al.* 1998; Verhoef and Austin 1999a, 1999b).

2.5.2 Physiological indicator

Hepatosomatic indices and moisture levels

Wet and dry hepatosomatic indices and moisture content of hepatopancreas have been used as indicators of nutritional and health status of crustacean (Evans *et al.* 1992; Mannonen and Henttonen 1995; McClain 1995a, 1995b; Fotedar 1998b; Sang and Fotedar 2004). The inverse linear relationship between hepatopancreas moisture content and total energy content in marron was reported by Jussila and Mannonen (1997). Hepatosomatic indices (ratios of wet and dry weight of hepatopancreas to total body wet weight) together with moisture content were used to describe the condition of wild capture lobster. Those parameters was also used to estimate the difference between growth rate among wild capture lobster, *Jarus lalandii*, populations, and the differences in conditions of rock lobster (*Jasus edwardsii*) under

different feeding treatments (Cabib *et al.* 1982; Cockcroft 1997; Musgrove 1997). In noble crayfish (*Astacus astacus*), energy reserved in hepatopancreas depleted during ovarian maturation reflected in an increase in moisture content of the hepatopancreas (80% comparing to 60 - 70% in reproductively inactive animal) (Huner *et al.* 1990).

Tail muscles to body weight ratios and tail moisture levels

Tail muscle is considered as a storage site of energy reserves, and tail muscles to body weight ratio and tail moisture level can be used as condition index (Fotedar 1998b). Starvation increased the whole body moisture contend of redclaw crayfish (*Cherax quadricarinatus*) juveniles. The water content in the crayfish deceased when the feeding rate increased and during starvation they catabolized tissue protein to meet their metabolic requirements (Gu *et al.* 1996). Tail moisture content of western king prawn (*Penaeus latisulcatus*) decrease with the increase of rearing salinity (Sang and Fotedar 2004).

2.5.3 Immunological indicators

Total haemocyte count (THC)

Total haemocyte count (THC) has been used as a common indicator of the immune capacity of crustacean. The reduction on THC reflects the stress and the weakness in immune capacity of the crustacean. Rearing conditions, nutrition conditions, handling and transportation stress have been shown to affect marron THC (Jussila *et al.* 1997; Evans *et al.* 1999; Jussila *et al.* 1999). Lower THC was observed in marron reared in intensive culture system than in earthen ponds and in communal tanks. Starvation, initiation of handling and transportation stress were reported to cause a decrease in marron haemocyte numbers (Evans *et al.* 1999; Jussila *et al.* 1999). The THC of marron ranges from 2.3 to 10.7 x 10^6 cells/mL depending on rearing condition as in Table 2.7. Handling during post-harvest also reduced THC of western rock lobster (*Panulirus cygnus*) (Jussila *et al.* 1997). The changes in THC of crustacean related to many stress conditions and could be used in assessing stress, health and immune statuses in crustaceans (Durliat 1985; Smith *et al.* 1995; Jussila *et al.* 1997; Evans *et al.* 1999; Jussila *et al.* 1999; Jussila *et al.* 2001).

Table 2.7: THC of marron in different rearing conditions

Rearing condition	Marron THC ($\times 10^6$ cells/mL)	Reference
Farmed and wild population	2.3 - 5.3	(Evans *et al.* 1992)
Semi-intensive farm pond	6.5 – 8.5	(Jussila 1997b)
Intensive crayfish culture system	2.3 - 5.3	(Jussila 1997b)
Farmed and acclimated for 14 days in an aquarium	2.1 – 4.0	(Evans *et al.* 1999; Jussila *et al.* 1999)
Farmed and acclimated for 30 days in an aquarium	4.3	(Jussila *et al.* 1999)
Communal tank	9.7 – 10.7	(Jussila 1997b)

Differential haemocyte count (DHC)

Beside THC, differential haemocyte count (DHC) has also been considered as immunological indicator of crustaceans (Jussila *et al.* 1997; Fotedar *et al.* 2001; Fotedar *et al.* 2006). The proportion of granular cells in western rock lobster (*Panulirus cygnus*) ranged from 5.1% to 13.1% (Jussila *et al.* 1997), comparative levels as observed by Fotedar *et al.* (2001) (6.6%) and Fotedar *et al.* (2006) (11.4%). Whereas, hyaline cells were comprised 29.1% to 37.0% and semi-granular cells were comprised 51.1% - 62.9% of the total haemocyte counts of the western rock lobster (Jussila *et al.* 1997). Differently, Fotedar *et al.* (2006) reported the proportions of semi-granular cells and hyaline cells were 50.0% - 71.3% and 22.3% - 37.8%, respectively. However, hyaline cells and semi-granular cells form most of the haemocytes in the haemolymph of the western rock lobster (*Panulirus cygnus*) and the correlation between these two haemocyte types is high (Jussila *et al.* 1997; Fotedar *et al.* 2006). In the American lobster (*Homarus americanus*) and California spiny lobster (*Panulirus interruptus*) hyaline cells formed the highest proportion of haemocytes (Hearing and Vernick 1967; Hose *et al.* 1990).

Similar to the THC, various factors such as environmental stressors and animal conditions have been reported to affect the proportion of different haemocytes. The proportion of granular cells in western rock lobster has been reported to be reduced by air exposure (Fotedar *et al.* 2001). Moribund lobsters were shown to have lower

proportion of granular cells than healthy lobsters (Jussila *et al.* 1997). The proportion of hyalinocytes peaks around ecdysis while granulocytes are more numerous in intermolt in penaeid shrimp (Hose *et al.* 1992). Changes in the proportion of different haemocyte types in crustaceans have been also shown to be sex dependant (Sequeira *et al.* 1995) , disturbance (Jussila *et al.* 2001) and holding duration (Fotedar *et al.* 2006). These studies suggested the possibility of using different haemocyte counts as an indicator of stress or conditions in crustaceans.

Jussila *et al.* (2001) reported that in western rock lobster, proportion of hyalinocytes correlated positively with clotting time while proportion of semigranulocytes correlated negatively with clotting time. However, Fotedar *et al.* (2006) did not find a significant correlation between clotting time and proportion of any haemocyte types in western rock lobster kept in storage tanks.

Bacteramia

Bacteria have been found in marron haemolymph but bacterial infections have not been reported to affect haemocyte numbers of marron (Jussila 1997b). In other crustaceans, bacterial and fungal infections have been shown to affect their THC. Bacterial and fungal infections caused a decline in American lobster THC (Stewart *et al.* 1967) and in signal crayfish (*Pacifastacus leniusculus*) THC (Persson *et al.* 1987). The possibility of using bacteraemia as stress and immune indicators has been suggested (Fotedar *et al.* 2001; Fotedar *et al.* 2006).

Haemolymph clotting time

Haemolymph clotting time of three crayfish species (*Astacus astacus*, *A. leptodactylus* and *Orconectes limosus*) from Europe ranged from 4.27 to 5.76 min, in which haemolymph was placed on a watchglass and the clot formation was examined with a glass rod (Gondko *et al.* 1981). Haemolymph clotting time of western rock lobster ranged from 52.7 to 56 seconds when using a capillary tube to measure haemolymph clotting time (Jussila *et al.* 2001).

Short-term stressors such as handling and exercise have been shown to shorten haemolymph clotting times of western rock lobster (Jussila *et al.* 2001) while Fotedar *et al.* (2006) reported that western rock lobster haemolymph clotting time increased

significantly over the holding period in storage tanks. There is inverse relationship between haemolymph clotting time and THC demonstrated by the less the number of haemocytes, the longer the clotting time (Durliat and Vranckx 1983; Jussila *et al.* 2001). Haemolymph clotting time has been reported to be changed by the presence of bacteria or environmental pollutants (Newman and Feng 1982; Smith and Johnston 1992). Jussila *et al.* (2001) suggested that haemolymph clotting time is easy to measure and could be used as an indicator of stress in crustaceans.

Neutral red retention time of lysosomal membranes

Lysosomes are located in the semi-granular and granular cells of many marine invertebrates and are released by degranulation process of haemocytes during immune response (Ratcliffe *et al.* 1985; Sung and Sun 1999). Once entering into the plasma, the proteolytic enzymes that exist in lysosomes are released, and then assist in the breakdown of foreign material through membrane destabilization (Hauton *et al.* 2001; Faurschou and Borregaard 2003). Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is a cytotoxic weak base and is used in the neutral red retention assay (NRR) as a biomarker to test the structural integrity of lysosomal membranes. The color disappears when lysosomal membranes are damaged and proteolytic enzymes are released (Lowe and Pipe 1994). It has been demonstrated that the NRR time could be successfully used as indicator of tress condition and immunological status of European lobster (*Homarus gammarus)* (Hauton and Smith 2004), Chinese shrimp *Fenneropenaeus chinensis* (Yao *et al.* 2008), and blacklip abalone (*Haliotis rubra*) (Song *et al.* 2007).

CHAPTER 3: SIZE DEPENDENT PHYSIOLOGICAL, IMMUNOLOGICAL AND GUT- MORPHOLOGICAL CHARACTERISTICS OF MARRON, *CHERAX TENUIMANUS* (SMITH, 1912) – A BASE LINE DATA

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3.1 INTRODUCTION

Marron, *Cherax tenuimanus* (Smith 1912) is one of the most important crayfish species for the aquaculture industry in Western Australia (O'brien and Davies 2000). Various studies have been conducted to understand the biology (Semple *et al.* 1995), physiology (Jussila 1997b; Jussila and Evans 1998), nutritional requirements (O'Brien 1995; Fotedar 1999; O'Brien and Davies 2000) and culture techniques (Whisson 1995; Jussila and Evans 1996a, 1996b) of marron. However, there is a lack of published information on the basic physiological and immunological characteristics and gut morphology of farm raised marron. This research is a first step in collecting the base line data on physiological and immunological parameters and gut morphology of different size classes of marron raised in pond based commercial farm environment.

3.2 MATERIALS AND METHODS

3.2.1 Experimental animal

Three size classes (mean ± S.D.) of marron (20 small: 7.14 ± 2.08 g/ind.; 15 medium: 37.33 ± 2.73 g/ind. and 10 large: 139.67 ± 6.69 g/ind.) were used in this research. The marron were supplied by Aquatic Resource Management Pty Ltd, Western Australia and shipped to the Curtin Aquatic Research Laboratory (CARL). Each group of marron was placed in the tank (800 mm diameter, 500 mm high, 250 L capacity) provided with aerated, recirculating filtered freshwater and acclimated to the culture conditions for 10 days. During the acclimation periods, the marron were starved for the first three days and then provided with the commercial feed (26% protein) at 1.5% body weight per day. At the end of the acclimation period, physiological parameters, immunological parameters and gut micrograph of marron were determined.

3.2.2 Data collection

Three maron from each tank were sampled for measurement of physiological and immunological parameters and gut morphology.

Physiological parameters

Organosomatic indices of the marron were measured by applying the method described by Sang and Fotedar (2004). Organosomatic indices of three randomly sampled marron from each tank were measured at the end of the acclimation period. The tail muscle (i.e. the complete mass of muscle in the abdomen of the marron) and hepatopancreas (all lobes of the hepatopancreas) of the marron were weighed to determine the wet hepatosomatic index (HSIwet) and wet tail muscle index (TMIwet) using the following formulae:

HSIwet = Whwet x 100/W, where Whwet is weight of wet hepatopancreas (g) and W is total weight of marron; TMIwet = Wtwet x 100/W, where Wtwet is weight of wet tail muscle.

The whole hepatopancreas and tail muscle were then dried for 24 h at 105°C. The hepatopancreas moisture content (HM%) and tail muscle moisture content (TM%), dry hepatosomatic index (HSIdry) and dry tail muscle index (TMIdry) were calculated using the following formulae:

HM% = 100 x (Whwet – Whdry)/Whwet, where Whdry is weight of dry hepatopancreas (g); TM% = 100 x (Wtwet – Wtdry)/Wtwet, where Wtdry is weight of dry tail muscles (g); HSIdry = Whdry x100/W; TMIdry = Wtdry x 100/W.

The osmoregulatory capacity of the marron was determined following the method described by Sang and Fotedar (2004). The haemolymph osmolality of three sampled marron from each tank was measured at the end of the experiment. Fifty µL of haemolymph was extracted from the pericardial cavity through the intersegmental membrane between the cephalothorax and the first abdominal segment using 0.5 mL syringe containing 0.1 mL of precooled (100C) of 0.1% glutaraldehyde (fixative) in 0.2 M sodium cacodylate (buffer) solution. The osmolality of the mix solution was measured using a Cryoscopic Osmometer – Osmom 030 (Gonotec, Inc, Germany). The osmolality of the blank fixative and buffer mix was also measured and the haemolymph osmolality was calculated using following equation:

Haemolymph osmolality = 3 x Osmolality of total mix – 2 x Osmolality of fixative and buffer mixture.

In addition, the osmolality of the culture media was also determined. The osmoregulatory capacity was calculated as the difference between haemolymph osmolality and medium osmolality.

Immunological parameters

Total (THC) and granular cells (GCs) were conducted as per the established procedure for western rock lobster (Fotedar *et al.* 2001). The bases of the fifth thoracic legs of three marron from each culture tank were cleaned with 70% alcohol. A 0.2 mL aliquot of haemolymph was withdrawn into a 1-mL sterile syringe containing 0.2 mL of 1% glutaraldehyde (fixative) in 0.2M sodium cacodylate (buffer) solution and dispensed into an Eppendorf tube kept on ice. Total haemocyte counts for individual marron were estimated with a haemocytometer (Neubauer, Germany) under 100-fold magnification. 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 mm3). For GCs, one drop of haemolymph solution was placed on a slide and smeared. The smear was air dried and fixed in 70% methanol for 10 min. Fixed smears were stained with May-Grunwald and Giemsa (Merck, Darmstadt, Germany) (10 min in each) and mounted with coverslip. Total of around 200 cells were counted on each slide. The granular cells among the total 200 cells were distinguished on the basis of the larger cell size, smaller pale nucleus, and larger number of eosinophillic granules in the cytoplasm, and their proportion was determined.

Bacteraemia assessment were performed based on the established procedure (Fotedar *et al.* 2001). A 0.05-mL aliquot of haemolymph was withdrawn into a sterile syringe and quickly placed in form of small drops on a nutrient agar (NA) plate and smeared; the plate was carefully inverted and kept in the incubator chamber at temperature of 25°C for 24 h. Colony forming units (CFU) were counted for each plate and CFU/mL calculated for each sample on the basis of a total volume of 0.05 mL/plate. The CFU/mL were ranked 1 (1–250 CFU/mL) to 12 (2751–3000 CFU/mL), and a

final rank of 13 was assigned to those samples in which the colonies were too numerous for an accurate count.

Haemolymph clotting time was determined following the protocol described by Fotedar *et al.* (2001). Haemolymph withdrawn into a sterile syringe was dispensed into an Eppendorf tube. A 30-μL aliquot was quickly transferred into another tube and drawn into a capillary tube. The tube was repeatedly inverted until the haemolymph stopped moving, and the time was noted as haemolymph clotting time.

Gut micrograph assessment

Guts of three marron from each treatment at the end of culture period were dissected and prepared for scanning electron microscope (SEM) following the procedure described by Dunlap and Adaskaveg (1997) with some modification. The guts of marron were immersed in 3% glutaraldehyde in 0.1 M cacodylate buffer over night. The guts were then washed in 3 changes of the cacodylate buffer and 3 changes in distill water for 5 min per change. After that, the guts were immersed in 2% OsO_4 for 2 h following by 3 washes in the distill water for 5 min per wash. The samples were then dehydrated using a series of 50%, 75%, 95% ethanol solutions for 5 min and finally 3 times in 100% ethanol for 5 min per change. The samples were then chemically dried by washing in a series of 50%, 75% and 100% (twice) hexamethyldisilizane (HMDS) in ethanol solutions for 5 min per change. Finally, the samples were dried at room temperature and mounted on a stub using carbon tape, coated with gold and viewed under a pressure scanning electron microscope (LX 30). The images obtained from SEM were described for distribution, densities and abnormal syndrome of the villi in the gut.

3.2.3 Data analysis

SPSS v. 15 was used to analyse the collected data. Results were presented as mean ± SE. ANOVA (analysis of variance) and LSD (least significant difference) post hoc tests were used to determine significant differences among sized groups of marron All significant tests were at $P < 0.05$ level.

3.3 RESULTS

TMIwet and TMIdry were significantly higher (P < 0.05) and TM% was significantly lower (P < 0.05) in larger marron than other sizes. HSIwet, HM%, HSIdry and osmoregulatory capacities were independent of size class (Table 3.1). THC was significantly higher in medium-sized marron (Figure 3.1) whereas GCs was independent of any size class (Figure 3.2). Bacteraemia was lower and haemolymph clotting time was significantly longer in the smaller marron than others (Figure 3.3 and 3.4).

Gut micrographs of marron showed that the midgut of marron has prominent rough areas (Fig 3.5 A and B) while the hindgut with longitudinally round folds except for the rectum area where longitudinal sharp folds dominate. The distribution of villi is all around hindgut area including rectum (Figure 3.5 C, D, E, F,G) whereas no villi are found in the midgut in all marron (Figure 3.5 A,B). The villi are present in groups of 4 to 8 in a straight line. Villi of rectum area appear finer and shorter than villi of other parts of hindgut in all marron. Density of villi in hindgut is approximately 43 villi per $20\mu m^2$ in the small marron whereas it is about 35 and 32 villi per $20\mu m^2$ in medium and larger marron, respectively. Villi of medium marron appear longer than other size classes.

Table 3.1: Physiological parameters of marron at different size classes

Parameters	Marron weight (mean ± S.E.) (g)		
	7.14 ± 2.08	37.33 ± 2.73	139.67 ± 6.69
HSIwet	5.63 ± 0.23[a]	5.94 ± 0.48[a]	5.38 ± 0.48[a]
TMIwet	22.50 ± 0.48[a]	23.93 ± 0.13[ab]	25.27 ± 1.13[b]
HM%	71.15 ± 2.34[a]	68.92 ± 1.27[a]	69.22 ± 1.23[a]
TM%	82.52 ± 0.31[a]	80.00 ± 0.90[b]	79.72 ± 0.24[b]
HSIdry	1.63 ± 0.18[a]	1.86 ± 0.21[a]	1.66 ± 0.18[a]
TMIdry	3.93 ± 0.06[a]	4.78 ± 0.20[b]	5.13 ± 0.28[b]
Osmoregulatory capacity (mOsm/kg)	485.67 ± 45.05[a]	452.33 ± 49.54[a]	509.00 ± 6.43[a]

Mean in the same row with different superscript letters are significantly different (P<0.05).
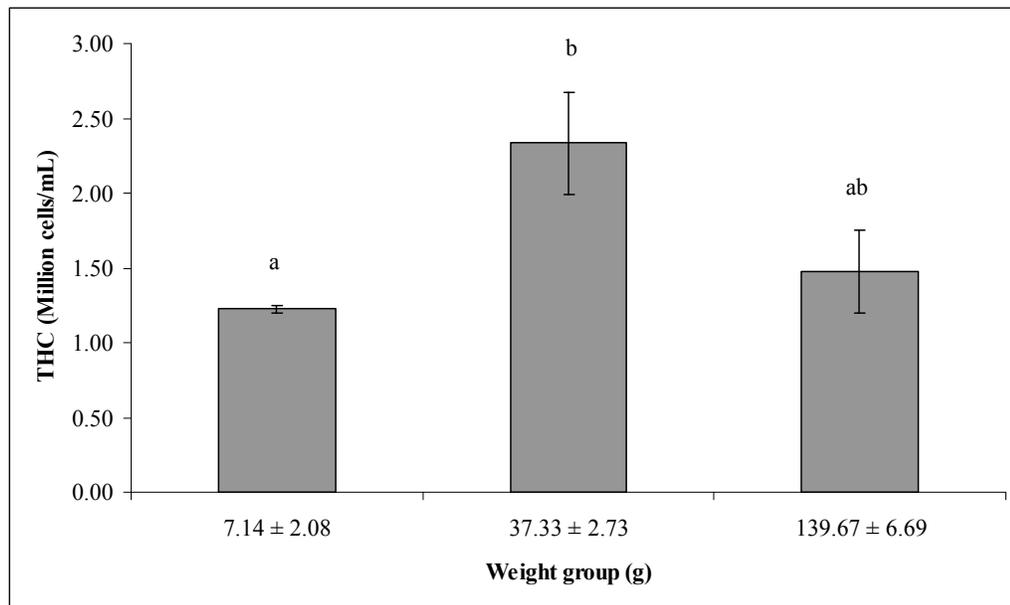
Figure 3.1: Total haemocyte count of marron.

Different letters above the bars denote significant differences at P < 0.05 in mean values
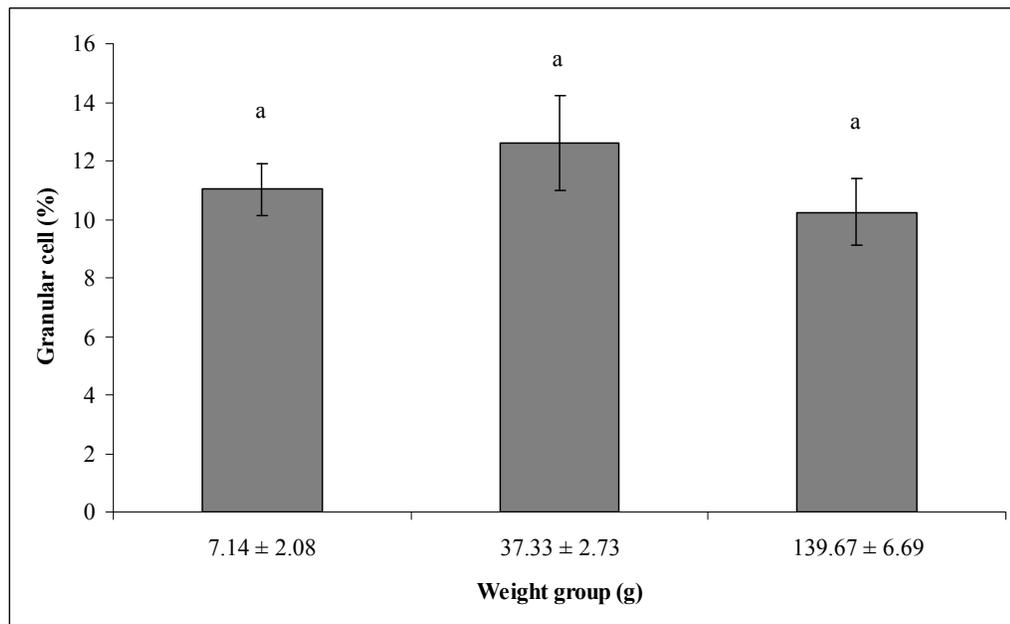
Figure 3.2: Proportion of granular cell of marron.*

* No significant differences (P > 0.05) were observed among three groups
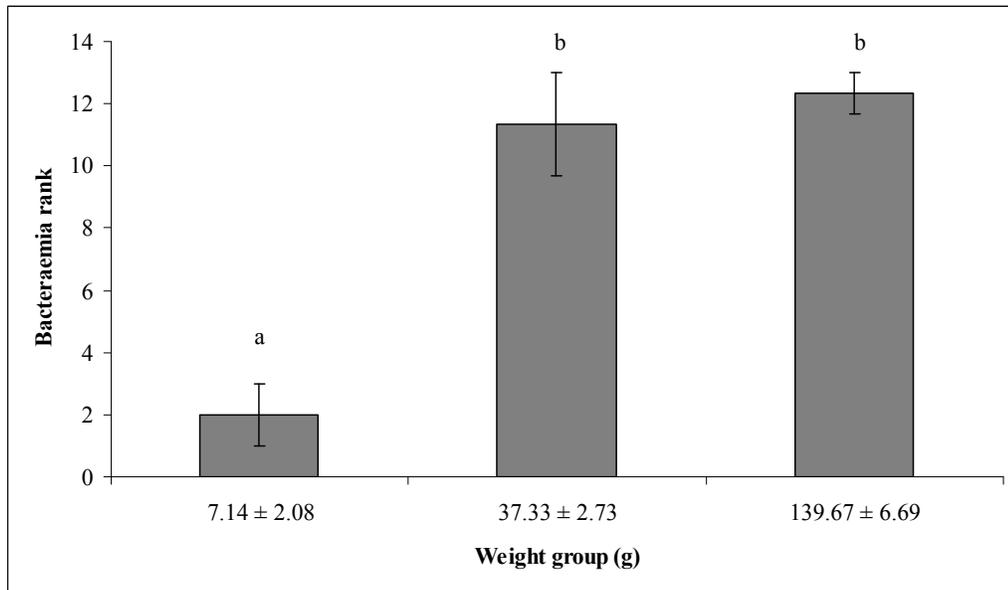
Figure 3.3: Bacteraemia rank of marron.

Different letters above the bars denote significant differences at P < 0.05 in mean values
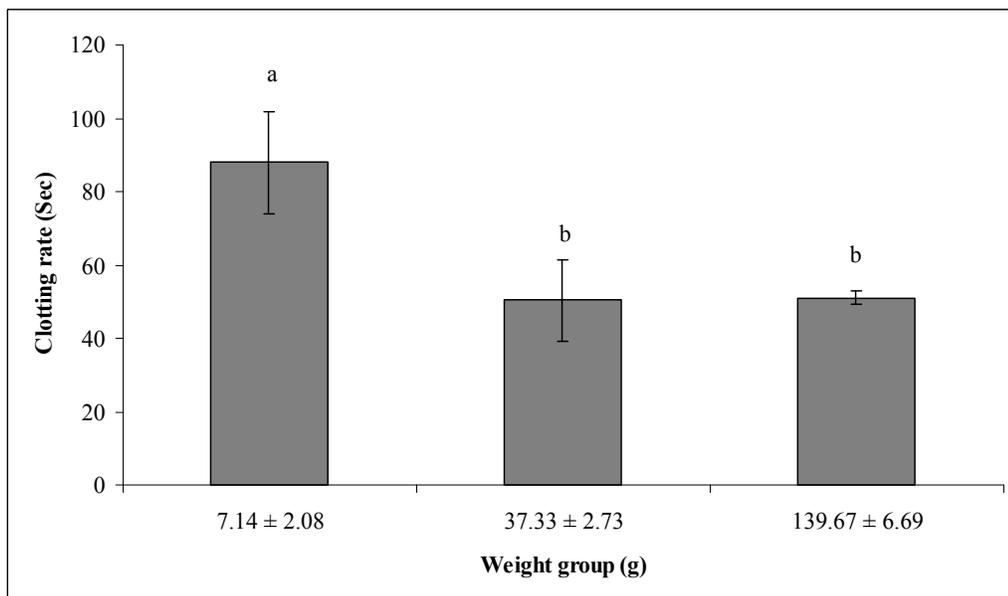
Figure 3.4: Haemolymph clotting time of marron.

Different letters above bars denote significant differences at P < 0.05 in mean values

Note:
A: midgut of medium size;
B: midgut of large size;
C: hindgut of medium size;
D: hindgut of large size;
E: rectum of medium size;
F: rectum of large size;
G: hindgut of small size
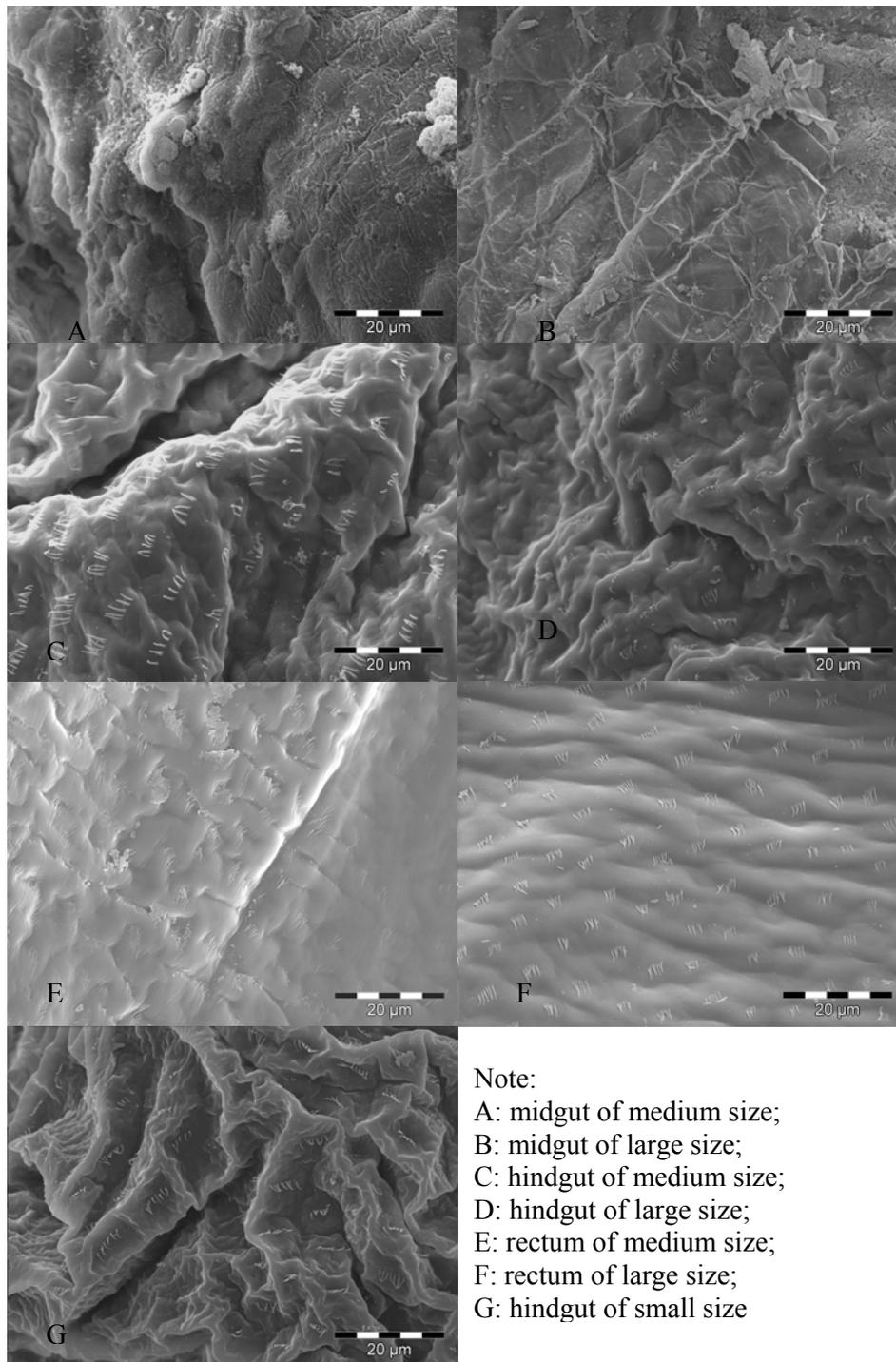
Figure 3.5: Scanning electron microscopic image of different sized marron digestive tracks.

3.4 DISCUSSION

This study is the first attempt to compare the physiological, immunological parameters and gut morphology of different-size farmed marron. The hepatopancreas, the main digestive gland in crustaceans, responsible for both the absorbing of nutrients from the digestive tract and excreting fluids also enables the breakdown of nutrients. It is also the main energy reserve in crustaceans. The size and moisture content of hepatopancreas is affected by nutrient status, developmental stage, growth and molting (Alderman & Polglase, 1988; Huner, Könönen, & Lindqvist, 1990; Jussila & Mannonen, 1997) and could be used to determine the condition of a crustacean (Huner *et al.* 1990; Evans *et al.* 1992). These results demonstrated that hepatosomatic indices of marron from the commercial farm are independent of size. However, higher TMIdry and TMIwet in the larger marron indicate that the larger marron store higher level of energy in tail muscles. This is in consistent with the statement that combined evaluation of other indicators such as moisture content of the hepatopancreas and tail muscle, the wet and dry hepatosomatic indices and the wet and dry weight of tail muscle to body weight ratios are an effective method to evaluate the condition of crustacean. The use of only one index may not accurately reflect the condition of crustacean (Fotedar 1998b). Osmoregulatory capacity (OC) is used as a tool in monitoring the physiological condition and the effect of stress in crustaceans (Castille and Lawrence 1981; Dall 1981; Ferraris *et al.* 1987; Henryon and Purvis 2000; Lignot *et al.* 2000). This research is the first record of the haemolymph osmolality of marron under farming condition. The haemolymph osmolality of marron ranged from 462 to 519 mOsm/kg (given the OC ranging from 452 to 509 mOsm/kg) and was not size dependent. The haemolymph osmolality of marron is lower than that of marine crustacean such as western king prawn (*Penaeus latisulcatus*) (> 664 mOsm/kg) (Sang and Fotedar 2004), tiger prawn (*P. semisulcatus*) (930 mOsm/kg) (Clark 1992) and white leg prawn (*P. vannamei*) (Castille and Lawrence 1981). The OC of marron from the current study is in contradiction with the OC of penaeid prawn which changes according to development stages of the animal (Chen and Lin 1998; Lignot *et al.* 1998; Lignot *et al.* 1999).

Total haemocyte count (THCs), proportion of granular cells, bacteraemia rank and haemolymph clotting time has been used as the immunological and health indicator

for the western rock lobster*, Panulirus cygnus* (Jussila *et al.* 1997; Söderhäll 1997). Low number of haemocytes and a low proportion of granular cells are indicative of stressed marron (Jussila *et al.* 1997; Fotedar *et al.* 2001). The results revealed that medium-sized marron have higher immune ability indicated by higher THC and lower clotting rate of haemolymph. The presence of bacteria in the haemolymph of both stressed and healthy crustaceans have been reported (Haskell *et al.* 1975; Scott and Thune 1986; Fotedar *et al.* 2001). While Jussila (1997b) discovered bacterial infection is common in the haemolymph of the marron and the noble crayfish (*Astacus astacus*) reared in the stress environment of an intensive crayfish culture system. Low bacteria levels in haemolymph are considered to be harmless to the host marron (Jussila 1997b), but higher levels can lead to a decrease in the immune resistance of crayfish (Alderman and Polglase 1988). In the current study, longer commercial culture environmental variables may lead to higher stress as shown by increase in bacteraemia in larger marron. This in turn leads to longer exposure and thusresponse to environmental stressors.

Microvilli in the gut of red swamp crayfish (*Procambarus clarkia*) have four roles: (1) to assist in irrigation of the hindgut, (2) to protect the cuticular layer, (3) to assist smooth movement of feces and (4) to assist intake of environmental water (Chisaka *et al.* 1999). The current study is just the primary observation on the gut morphology of marron and can be used as a base line study to compare the future research on the health of the marron digestive track. The observations obtained are insufficient to discuss any relationship between the distribution and density of microvilli and the role of different region of the digestive track.

CHAPTER 4: EFFECTS OF DIETARY MANNAN OLIGOSACCHARIDE ON SURVIVAL, GROWTH, PHYSIOLOGICAL CONDITION AND IMMUNOLOGICAL RESPONSES OF MARRON, *CHERAX TENUIMANUS* (SMITH, 1912)

4.1 INTRODUCTION

Rapid growth, high survival and disease resistance are important components in the aquaculture industry. Antibiotics have been included in aquaculture feeds worldwide at the sub-therapeutic concentration as a standard practice because of their positive effects on the weight gain, feed utilization and prevention of mortality of the target species (Gatlin 2002). Recently, the possible stimulation of antibiotics on the development of resistant bacterial strains in both animals and humans, with associated risks to human health, have been the subjects of controversy (Genc *et al.* 2007a). In aquaculture, the application of antibiotics or other chemicals is expensive and undesirable as it risks the contamination of both the environment and the final product (Capone *et al.* 1996). The repeated application of antibiotics, in the long term, also encourages the spread of drug resistant pathogens. Thus, there is a great need to maximize the immune competence of the aquatic stock whilst minimizing the use of antibiotics.

Besides the development of vaccines, dietary supplements including immunostimulants such as probiotics and prebiotics have received increased attention in aquaculture. Prebiotics are classified as non-digestible food ingredients that beneficially affect the host by stimulating growth or activity of a limited number of bacteria in the digestive tract (Genc *et al.* 2007a). Development of prebiotics in aquaculture is in its infancy, compared with the progress that has been made in the development of prebiotics for poultry (Patterson and Burkholder 2003). Although many immunostimulants for example, synthetic chemical, bacterial derivatives, polysaccharides including mannan oligosaccharide (MOS), animal and plant extracts, hormone and cytokine (Sakai 1999) have been used, very little information on the application of immunostimulant is available in crayfish aquaculture.

Bio-Mos (Alltech, USA) is MOS derived from the outer cell wall of yeast (*Saccharomyces cerevisiae*) which has been applied as a prebiotic widely in animal husbandry (Genc *et al.* 2007a). In aquaculture, the effects of MOS on growth performances and immune status have been investigated for several fish species (Staykov *et al.* 2007; Torrecillas *et al.* 2007; Welker *et al.* 2007; Salze *et al.* 2008a). Recently, a penaeid shrimp, *Penaeus semisulcatus*, has been reported to show an increased survival and growth when MOS was applied in their diet (Genc *et al.* 2007a) while information on other crustacean including marron is limited.

Marron, *Cherax tenuimanus* (Smith 1912) are endemic to southwestern Australia and has been one of the most important species for the aquaculture industry in Western Australia (O'brien and Davies 2000). Although various studies have been conducted on marron biology (Semple *et al.* 1995), physiology (Jussila 1997b; Jussila and Evans 1998), nutritional requirements (O'Brien 1995; Fotedar 1999; O'Brien and Davies 2000) culture techniques (Whisson 1995; Jussila and Evans 1996a, 1996b), only a limited number of studies were carried out on feed additives, and there is only one record on the use of dietary MOS to increase the bacterial and stressor resistant ability of marron (Sang *et al.* 2009).

In addition to traditional performance indicators of feed additives such as growth and survival, measures of crustacean health can include physiological parameters such as organosomatic indices, moisture content and osmoregulartory capacity (Sang and Fotedar 2004). Immune–physiological parameters such as total number of haemocytes (THC), proportion of different haemocyte types (DHC), bacteraemia and hemolymph clotting time have also been used as indicators of immunological and health status of crustacean (Smith *et al.* 1995; Jussila *et al.* 1999; Fotedar *et al.* 2001; Fotedar *et al.* 2006; Sang *et al.* 2009). This study aims to determine the effects of dietary MOS on the survival, growth, physiology and immunology of marron.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of test diets

Six different isonitrogenous and isocaloric diets supplemented with 0 (C) (control), 0.05% (D1), 0.1% (D2), 0.2% (D3), 0.4% (D4) and 0.8% (D5) of MOS (Bio-Mos, Alltech, USA) were formulated and prepared. The main ingredients of Bio-Mos are brewers dried yeast and dried *Saccharomyces cerevisiae* fermentation soluble containing minimum of 30% protein, 1.4% crude fat and maximum of 13% crude fibre. All feed ingredients used in this trial were supplied by Specialty Feeds Pty Ltd in Western Australia. The proximate compositions of the ingredients and supplements were used as a basis to form the required formulas using the software FeedLIVE version 1.52 (Table 4.1).

All of the dry feed ingredients and supplements were passed through a 100-μm mesh sieve in order to obtain a uniform particle size and then thoroughly mixed with a predetermined amount of freshwater. The mixed ingredient was then passed through a mince mixer to obtain pellets of 1 mm diameter. The pellets were dried in the direct sunlight for 6 h and then were allowed to cool at room temperature for 30 min followed by packing in plastic containers.

Analysis of moisture content, dry matter, crude protein and ash of the diets were performed according to the standard methods of the Association of Official Analytical Chemists (AOAC 1995). Gross energies of diets were determined using a bomb calorimeter (Calorimeter C2000, Crown Scientific, Germany). All formulated feed samples were analyzed in triplicate.

4.2.2 Experiment design

Marron with initial weight of 4.44 ± 0.20 g (mean ± S.E.) were supplied by Aquatic Resource Management Pty Ltd, Western Australia, shipped to the Curtin Aquatic Research Laboratory (CARL) and stocked in tanks provided with aerated, recirculating filtered freshwater and acclimated to the culture conditions for 2 weeks. During the acclimation period, marron were fed a commercial diet supplied by Enviroplus, Australia (26% protein, 47 – 50% carbohydrate, 9% lipid and 8.9% ash) at the rate of 15 % total weight day^{-1}.

Table 4.1: Experimental diet formulations

Ingredients	C (%)	D1 (%)	D2 (%)	D3 (%)	D4 (%)	D5 (%)
Fish oil	3.20	3.20	3.20	3.20	3.20	3.20
Soybean meal	10.15	10.15	10.14	10.14	10.14	10.11
Fishmeal	33.78	33.80	33.83	33.83	33.86	34.01
Wheat flour	49.35	49.25	49.21	49.08	48.85	48.33
Ascorbic acid	0.05	0.05	0.05	0.05	0.05	0.05
Betaine	1.20	1.20	1.20	1.20	1.20	1.20
Calcium ascobate	0.02	0.02	0.02	0.02	0.02	0.02
Premix	0.15	0.15	0.15	0.15	0.15	0.15
Cholesterol	0.25	0.25	0.25	0.25	0.25	0.25
Wheat starch	1.85	1.85	1.85	1.85	1.85	1.85
Bio-Mos	0.00	0.05	0.10	0.20	0.40	0.80
Total	100	100	100	100	100	100

Plastic cylindrical tanks (800 mm diameter, 500 mm high, 250 L capacity) were used as culture units in the experiment. Sufficient PVC pipes (Ø42 mm) and oyster net (2a = 10 mm) were placed in each tank to provide shelters for marron. The tank was supplied with aerated recirculating freshwater (originated from bore-well and aged for a month) at a rate of approximately 3 L min^{-1}. The water in each tank was independently filtered through both mechanical and biological filters.

Eighteen culture tanks were used for the trial. After acclimation, intermolt marron were randomly distributed among the culture tanks at the density of 15 marron tank^{-1}. Each random block of three tanks was fed one of the above diets so that each diet was represented in three replicates.

Marron were reared for 16 weeks. The food was provided at the rate of 5 – 6% body weight every second day. Uneaten food and faeces were siphoned before feed was provided. The amount of water lost during siphoning was added into each tank to retain the water level.

Water quality parameters such as pH, total ammonium, nitrite and nitrate were monitored weekly using pH meter WP-80, TPS Company and chemical test kits (Aquarium Pharmaceuticals, INC). Dissolved oxygen was measured by CyberScan DO300 Waterproof Dissolved Oxygen Meter. The number of marron in each tank was checked every 4 weeks by removing the shelter and manual counting. The individual weight was measured to two decimal places using a balance SHIMADZU AW 220 (LabComerce Inc, USA) every 4 weeks. Physiological and immunological parameters were measured at 8 weeks and at the end of culture period.

4.2.3 Data collection

Survival of marron in all tanks was measured using the following calculation:

$$S = 100 \times (n_t/n_o)$$

Where: S is the survival rate; n_t is the number of marron at time t and n_o is the number of marron at the commencement of the experiment.

Biomass was calculated as:

$$B = W1 + W2 + \ldots + Wn$$

where as Wi is the wet weight of marron (i) and n is the total number of marron in one tank. Biomass increment (BI%) was calculated as:

$$BI\% = 100 \times (\text{final biomass} - \text{initial biomass})/\text{initial biomass}.$$

All marron used for analysis of physiological and immunological parameters were in the intermolt stage. Molt stage was determined based on the developing setae on the pleopods as described by Turnbull (1989).

Organosomatic indices of marron were measured by applying the method of (Sang and Fotedar 2004). Organosomatic indices of three randomly sampled marron from each tank were measured at the end of the experimental period. The tail muscle (i.e. the complete mass of muscle in the abdomen of marron) and hepatopancreas (all lobes of the hepatopancreas) of marron were weighed to determine the wet hepatosomatic index (HSIwet) and wet tail muscle index (TMIwet) using the following formulae:

HSIwet = Whwet x 100/W, where Whwet is weight of wet hepatopancreas (g) and W is total weight of marron; TMIwet = Wtwet x 100/W, where Wtwet is weight of wet tail muscle.

The whole hepatopancreas and tail muscle were then dried to a constant weight at 105°C for 24 h. The hepatopancreas moisture content (HM%) and tail muscle moisture content (TM%), dry hepatosomatic index (HSIdry) and dry tail muscle index (TMIdry) were calculated using the following formulae:

HM% = 100 x (Whwet – Whdry)/Whwet, where Whdry is weight of dry hepatopancreas (g); TM% = 100 x (Wtwet – Wtdry)/Wtwet, where Wtdry is weight of dry tail muscles (g); HSIdry = Whdry x100/W; TMIdry = Wtdry x 100/W.

The osmoregulatory capacity of marron was determined following the method of Sang and Fotedar (2004). The haemolymph osmolality of three sampled marron from each tank was measured at the end of the experiment. Fifty µL of haemolymph was extracted from the pericardial cavity through the intersegmental membrane between the cephalothorax and the first abdominal segment using 0.5 mL syringe containing 0.1 mL of precooled (10°C) of 0.1% glutaraldehyde (fixative) in 0.2 M sodium cacodylate (buffer) solution. The osmolality of the mix solution was measured using a Cryoscopic Osmometer – Osmom 030 (Gonotec, Inc, Germany). The osmolality of the blank fixative and buffer mix was also measured and the haemolymph osmolality was calculated using following equation:

Haemolymph osmolality = 3 x Osmolality of total mix – 2 x Osmolality of fixative and buffer mixture.

In addition, the osmolality of the culture media was also determined. The osmoregulatory capacity was calculated as the difference between haemolymph osmolality and medium osmolality.

Total haemocyte count (THC) and granular cells (GCs) were counted as per the established procedure for western rock lobster (Fotedar *et al.* 2001). The base of the fifth thoracic leg of three marron from each culture tank were cleaned with 70% alcohol. A 0.2-mL aliquot of haemolymph was withdrawn into a 1-mL sterile syringe containing 0.2 mL of 1% glutaraldehyde (fixative) in sodium cacodylate (buffer)

solution (0.2M) and dispensed into an Eppendorf tube kept on ice. THC for individual marron was estimated with a haemocytometer (Neubauer, Germany) under 100-fold magnification. 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). For GCs, one drop of haemolymph solution was placed on a slide and smeared. The smear was air dried and fixed in 70% methanol for 10 min. Fixed smears were stained with May-Grunwald and Giemsa (Merck, Darmstadt, Germany) (10 min in each) and mounted with coverslip. Total of around 200 cells were counted on each slide. The GCs among the total 200 cells were distinguished on the basis of the larger cell size, smaller pale nucleus, and larger number of eosinophillic granules in the cytoplasm, and their proportion was determined.

Bacteraemia assessment were performed based on the established procedure (Fotedar *et al.* 2001). A 0.05 mL aliquot of haemolymph was withdrawn into a sterile syringe and quickly placed in form of small drops on a nutrient agar (NA) plate and smeared; the plate was carefully inverted and kept in the incubator chamber at temperature of 25°C for 24 h. Colony forming units (CFU) were counted for each plate and CFU mL^{-1} calculated for each sample on the basis of a total volume of 0.05 mL plate^{-1}. The CFU mL^{-1} were ranked 1 (1–250 CFU mL^{-1}) to 12 (2751–3000 CFU mL^{-1}), and a final rank of 13 was assigned to those samples in which the colonies were too numerous for an accurate count.

Haemolymph clotting time was determined following the protocol described by Fotedar *et al.* (2001) with some modification. Haemolymph withdrawn into a sterile syringe was dispensed into an Eppendorf tube. A 30 μL aliquot was quickly transferred into another tube and drawn into a capillary tube. The tube was repeatedly inverted until the haemolymph stopped moving, and the time was noted as haemolymph clotting time.

4.2.4 Data analysis

SPSS v. 15 was used to analyse the data. Results were presented as mean ± SE. The normality of data was assessed by the Shapiro-Wilk test (Winer 1991) and the homogeneity of variance was assessed by Levene test (Winer 1991) prior to the

analysis. Normally distributed data were subjected to one-way analysis of variance (ANOVA) using post-hoc LSD test for data of homogenous variance, and Tamhane's test (Winer 1991) for data of non-homogenous variance. All percentage data was subjected to the arcsine transformation prior to analysis. All significant tests were at $P < 0.05$ level.

4.3 RESULTS

4.3.1 The test diets and water quality parameters

There was no significant difference ($P > 0.05$) in the gross energy, crude protein, moisture content, dry matter and ash content among six formulated diets (Table 2). During the course of the trial, water temperature ranged between 19.2 and $21.7^{\circ}C$, pH was between 7.6 and 8.3, oxygen was ≥ 6 ppm, NH_2 and NH_3 were below detectable levels.

Table 4.2: Proximate nutrient components of the test diets

Diets	Energy ($J\,kg^{-1}$)	Crude protein ($g\,kg^{-1}$)	Ash ($g\,kg^{-1}$)	Dry matter ($g\,kg^{-1}$)	Moisture content ($g\,kg^{-1)}$)
C	18.8 ± 0.05	346.4 ± 0.3	61.6 ± 1.7	945.6 ± 2.5	54.4 ± 2.6
D1	18.8 ± 0.02	344.1 ± 0.2	60.2 ± 3.5	940.1 ± 2.9	59.9 ± 2.9
D2	18.8 ± 0.02	348.6 ± 1.4	59.8 ± 3.6	941.3 ± 3.7	58.7 ± 3.7
D3	18.8 ± 0.12	346.6 ± 1.6	61.6 ± 1.6	946.0 ± 2.8	54.0 ± 2.8
D4	18.8 ± 0.06	348.1 ± 1.1	60.4 ± 0.8	941.3 ± 1.1	58.7 ± 1.1
D5	18.8 ± 0.02	345.7 ± 2.6	61.1 ± 3.5	946.1 ± 3.4	53.9 ± 3.4

4.3.2 Survival and growth

After 28 days, the survival of marron fed C ($86.67 \pm 3.85\%$) was significantly lower ($P < 0.05$) than marron fed D3 ($97.78 \pm 2.22\%$). After 56 days of rearing, survival of marron fed C ($77.78 \pm 2.22\%$) and D1 ($71.11 \pm 2.22\%$) were significantly lower ($P < 0.05$) than marron fed D2 ($88.89 \pm 2.22\%$), D3 ($97.78 \pm 2.22\%$) and D4 ($91.11 \pm 2.22\%$). After 112 days, survival of marron fed C ($68.89 \pm 2.22\%$), D1 ($62.22 \pm 5.88\%$) and D5 ($68.89 \pm 5.88\%$) were significantly lower ($P < 0.05$) than marron fed D3 ($95.56 \pm 2.22\%$) and D4 ($84.44 \pm 2.22\%$) (Figure 4.1).

The weight of marron was not significantly different (P > 0.05) among marron fed different diets. However, the biomass increments were higher (P < 0.05) in marron fed D3 and D4 compared to marron fed C, D1 and D5. The lowest biomass increments (P < 0.05) were observed in marron fed diets C, D1 and D5 (Figure 4.2).
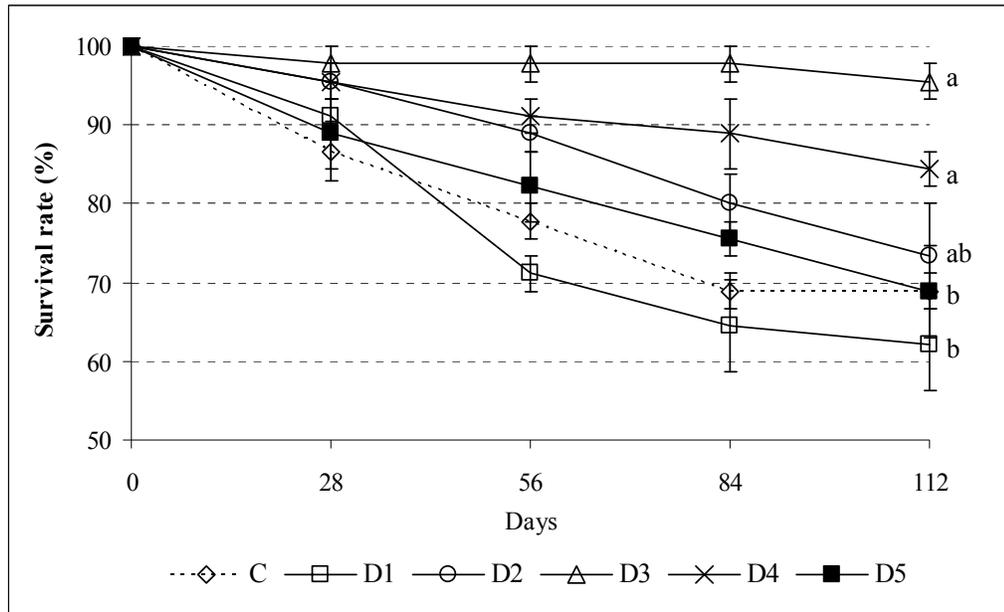
Figure 4.1: Survival of marron fed the different Bio-Mos supplemented diets (mean ± S.E.).

Different letters denote significant difference (P < 0.05).
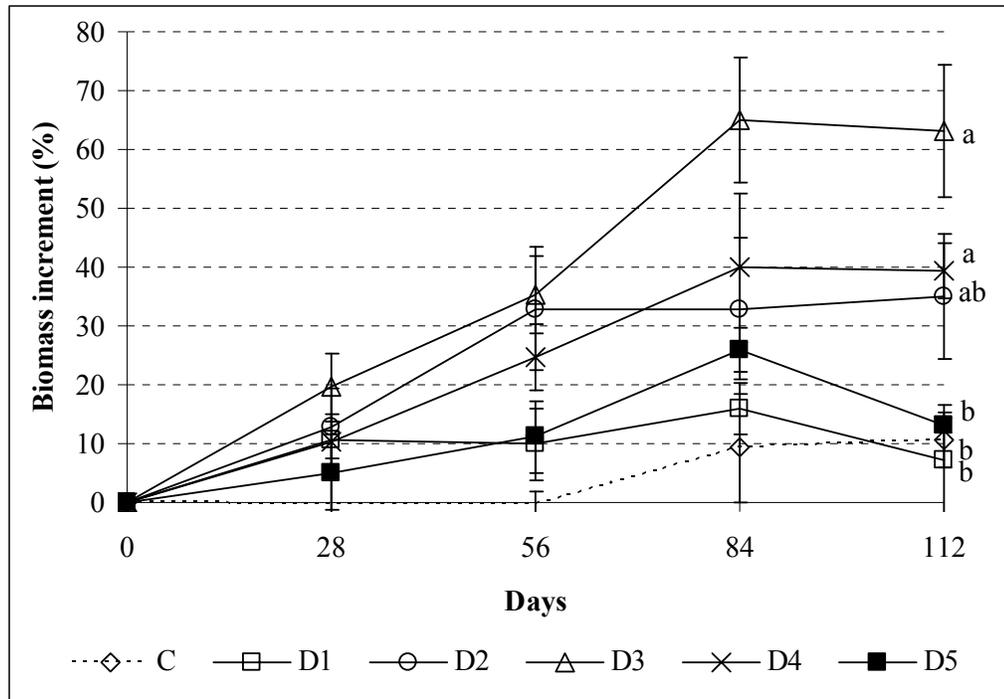
Figure 4.2: Total biomass increment of marron fed the different Bio-Mos supplemented diets (Mean ± S.E.).

Different letters denote significant difference (P < 0.05).

4.3.3 Physiological parameters

After 112 days of rearing, there was no significant difference (P > 0.05) in the HSIwet and HSIdry of marron fed different diets. However, the TMIwet and TMIdry of marron fed C was the lowest (P < 0.05) compared to any of the diets supplemented with MOS. Among marron fed MOS diets, TMIwet of marron fed D1 was the highest which was significantly higher (P < 0.05) than marron fed D2. HM% and TM% did not differ (P > 0.05) among studied groups (Table 4.3).

Marron fed C, D1 and D2 did not produce any change in HSIwet while marron fed diets D3, D4 and D5 had a significant increase (P < 0.05) after 56 and then decrease in HSIwet after 112 days of rearing. HSIdry of marron fed D1, D3 and D4 increased significantly after 56 days of rearing. TM% of marron fed D1, D3 and D4 reduced significantly (P < 0.05) after 56 days of rearing while after 112 days TM% was reduced in the remaining diets. The reduced trend in TM% continued for marron fed D3 and D4 till 112 days. HM% of marron fed D1 decreased significantly (P < 0.05) after 112 days of rearing while it was not affected by other diets. After 56 days,

TMIwet and TMIdry of marron fed any diet increased significantly ($P < 0.05$). From day 56 to 112, TMIwet of marron fed C reduced significantly ($P < 0.05$) and TMIdry increased significantly ($P < 0.05$) in marron fed D2, D3 and D4 (Table 4.3).

After 112 days, OC of marron fed D2, D3, D4 were significantly higher ($P < 0.05$) than marron fed C. OC of marron fed D5 decreased significantly ($P < 0.05$) after 56 days and increased again after 112 days while OC of marron fed other diets did not change during the rearing period (Table 4.3).

Table 4.3: Physiological parameters of marron fed different Bio-Mos supplemented diets

Parameters	Day	C	D1	D2	D3	D4	D5
HSIwet	0	$_1$5.6 ± 0.23^a	$_1$5.6 ± 0.23^a	$_1$5.6 ± 0.23^a	$_1$5.6 ± 0.23^a	$_1$5.6 ± 0.23^a	$_1$5.6 ± 0.23^a
	56	$_1$6.7 ± 0.50^{ab}	$_1$6.5 ± 0.13^a	$_1$6.2 ± 0.37^a	$_2$7.2 ± 0.38^{ab}	$_2$7.4 ± 0.44^{ab}	$_2$7.9 ± 0.59^b
	112	$_1$6.3 ± 0.70^a	$_1$6.5 ± 0.52^a	$_1$5.8 ± 0.71^a	$_{12}$6.9 ± 0.58^a	$_{12}$6.6 ± 0.36^a	$_1$5.9 ± 0.04^a
TMIwet	0	$_1$22.5 ± 0.48^a	$_1$22.5 ± 0.48^a	$_1$22.5 ± 0.48^a	$_1$22.5 ± 0.48^a	$_1$22.5 ± 0.48^a	$_1$22.5 ± 0.48^a
	56	$_2$27.1 ± 0.15^a	$_2$28.1 ± 2.40^a	$_2$27.3 ± 1.10^a	$_2$29.8 ± 0.87^a	$_2$27.6 ± 0.76^a	$_2$29.7 ± 1.33^a
	112	$_1$23.1 ± 0.32^a	$_2$29.8 ± 1.21^b	$_{12}$26.2 ± 1.44^c	$_2$27.2 ± 1.19^{bc}	$_2$26.9 ± 0.78^{bc}	$_2$27.3 ± 0.49^{bc}
HM%	0	$_1$71.1 ± 2.34^a	$_1$71.1 ± 2.34^a	$_1$71.1 ± 2.34^a	$_1$71.1 ± 2.34^a	$_1$71.1 ± 2.34^a	$_1$71.1 ± 2.34^a
	56	$_1$65.8 ± 4.06^a	$_{12}$64.9 ± 2.30^a	$_1$67.3 ± 5.12^a	$_1$68.2 ± 2.96^a	$_1$65.5 ± 2.16^a	$_1$68.9 ± 3.21^a
	112	$_1$65.2 ± 0.71^a	$_2$62.5 ± 2.75^a	$_1$60.9 ± 4.36^a	$_1$62.9 ± 3.78^a	$_1$66.3 ± 0.97^a	$_1$61.8 ± 2.82^a
TM%	0	$_1$82.5 ± 0.31^a	$_1$82.5 ± 0.31^a	$_1$82.5 ± 0.31^a	$_1$82.5 ± 0.31^a	$_1$82.5 ± 0.31^a	$_1$82.5 ± 0.31^a
	56	$_1$80.9 ± 0.75^a	$_2$77.9 ± 1.03^b	$_1$80.5 ± 0.81^a	$_2$80.5 ± 0.60^a	$_2$79.2 ± 0.32^{ab}	$_1$80.4 ± 0.91^a
	112	$_2$76.7 ± 0.34^a	$_2$76.7 ± 0.95^a	$_2$75.5 ± 0.59^a	$_3$76.1 ± 0.15^a	$_3$75.6 ± 0.64^a	$_2$75.9 ± 1.25^a
HSIdry	0	$_1$1.6 ± 0.18^a	$_1$1.6 ± 0.18^a	$_1$1.6 ± 0.18^a	$_1$1.6 ± 0.18^a	$_1$1.6 ± 0.18^a	$_1$1.6 ± 0.18^a
	56	$_1$2.3 ± 0.36^a	$_2$2.3 ± 0.18^a	$_1$2.1 ± 0.39^a	$_2$2.3 ± 0.29^a	$_2$2.5 ± 0.14^a	$_2$2.5 ± 0.34^a
	112	$_1$2.1 ± 0.22^a	$_2$2.4 ± 0.05^a	$_1$2.3 ± 0.46^a	$_2$2.5 ± 0.08^a	$_2$2.2 ± 0.11^a	$_{12}$2.2 ± 0.17^a
TMIdry	0	$_1$3.9 ± 0.06^a	$_1$3.9 ± 0.06^a	$_1$3.9 ± 0.06^a	$_1$3.9 ± 0.06^a	$_1$3.9 ± 0.06^a	$_1$3.9 ± 0.06^a

	56	$_25.2 \pm 0.17^{a}$	$_26.7 \pm 0.26^{b}$	$_25.3 \pm 0.31^{ab}$	$_25.8 \pm 0.26^{ab}$	$_25.7 \pm 0.20^{ab}$	$_25.8 \pm 0.42^{ab}$
	112	$_25.4 \pm 0.08^{a}$	$_26.9 \pm 0.54^{b}$	$_36.4 \pm 0.29^{b}$	$_36.7 \pm 0.10^{b}$	$_36.6 \pm 0.24^{b}$	$_26.6 \pm 0.46^{b}$
OC $(mOsm\ kg^{-1})$	0	$_1486 \pm 45^{a}$	$_1486 \pm 45^{a}$	$_1486 \pm 45^{a}$	$_1486 \pm 45^{a}$	$_1486 \pm 45^{a}$	$_1486 \pm 45^{a}$
	56	$_1468 \pm 29^{ab}$	$_1456 \pm 2^{ab}$	$_1475 \pm 9^{ab}$	$_1447 \pm 27^{ab}$	$_1510 \pm 55^{a}$	$_2386 \pm 26^{b}$
	112	$_1441 \pm 30^{a}$	$_1484 \pm 5^{ab}$	$_1519 \pm 18^{bc}$	$_1488 \pm 2^{bc}$	$_1528 \pm 6^{c}$	$_1481 \pm 29^{ab}$

Data in the same row having different superscript letters (a, b, c…) and data in the same column of each parameter having different subscript number (1,2,3..) are significantly different at $P < 0.05$.

4.3.4 Immunological parameters

After 112 days, marron fed C had significantly higher bacteraemia than marron fed other diets. Bacteraemia of marron fed C and D3 significantly increased ($P < 0.05$) after 112 days of culture whereas bacteraemia in marron fed the other diets did not show any significant changes. Among marron fed MOS diets, the lowest bacteraemia was observed in marron fed D4 which was significantly lower ($P < 0.05$) than marron fed D1, D3 and D5 (Table 4.4).

Haemolymph clotting time of marron fed different diets reduced significantly ($P < 0.05$) after 56 days of rearing. Haemolymph clotting time of marron fed D3 was reduced significantly ($P < 0.05$) from the day 56 to the day 112 of culture whereas that of marron fed other diets remained unchanged. After 56 days, haemolymph clotting rate of marron fed D5 was lower ($P < 0.05$) than marron fed C. At day 112, haemolymph clotting times of marron fed D3 and D4 were lower ($P < 0.05$) than that of marron fed other diets (Table 4.4).

There was an increasing trend in all diet groups in THCs over time. The THC of marron fed D2 and D4 was significantly increased ($P < 0.05$) after 56 days. For marron fed the other diets, a significant increase in THC was observed at day 112 of rearing. THC of marron fed C was significantly lower compared to marron fed D1, D2, D3 and D4 after 112 days of rearing (Table 4.4).

The GCs of marron fed the MOS supplemented diets of equal and less than 0.4% significantly increased after 56 days and was unchanged until day 112, except for marron fed D3. After 112 days, GCs were lower ($P < 0.05$) in marron fed C and D1 compared to marron fed other diets. The highest GCs were in marron fed D4 after 112 days of rearing (Table 4). Relationship between GCs and bateraemia rank was expressed as: Bacteraemia rank = -0.6072 x GCs + 15.827 with $R^2 = 0.6472$.

Table 4.4: Immunological parameters of marron fed different Bio-Mos supplemented diets

Parameters	Day	C	D1	D2	D3	D4	D5	FM (7.14 ± 2.08 g)
Bacteraemia rank	0	$_1$2.0 ± 1.00[a]	$_1$2.0 ± 1.00[a]	$_1$2.0 ± 1.00[a]	$_1$2.0 ± 1.00[a]	$_1$2.0 ± 1.00[a]	$_1$2.0 ± 1.00[a]	
	56	$_{12}$3.0 ± 1.00[a]	$_1$3.0 ± 1.15[a]	$_1$3.0 ± 0.58[a]	$_1$1.3 ± 0.33[a]	$_1$2.3 ± 0.88[a]	$_1$2.0 ± 1.00[a]	
	112	$_2$6.7 ± 1.20[a]	$_1$4.0 ± 1.00[b]	$_1$2.3 ± 0.33[bc]	$_2$4.0 ± 0.58[b]	$_1$1.0 ± 0.00[c]	$_1$3.7 ± 0.67[b]	2.0 ± 1.00
Hemolypmh clotting time	0	$_1$89.7 ± 4.91[a]	$_1$89.7 ± 4.91[a]	$_1$89.7 ± 4.91[a]	$_1$89.7 ± 4.91[a]	$_1$89.7 ± 4.91[a]	$_1$89.7 ± 4.91[a]	
	56	$_2$53.7 ± 2.33[a]	$_2$51.7 ± 1.76[ab]	$_2$49.0 ± 2.65[ab]	$_2$51.7 ± 3.33[ab]	$_2$46.0 ± 2.08[ab]	$_2$45.7 ± 2.96[b]	
	112	$_2$46.0 ± 2.08[a]	$_2$47.7 ± 0.33[a]	$_2$44.7 ± 1.86[a]	$_3$36.0 ± 1.73[b]	$_2$38.0 ± 1.15[b]	$_2$50.7 ± 3.28[a]	88.0 ± 14.01
THC (million cell/mL)	0	$_1$1.4 ± 0.20[a]	$_1$1.4 ± 0.20[a]	$_1$1.4 ± 0.20[a]	$_1$1.4 ± 0.20[a]	$_1$1.4 ± 0.20[a]	$_1$1.4 ± 0.20[a]	
	56	$_1$1.7 ± 0.22[a]	$_1$2.0 ± 0.14[a]	$_2$2.5 ± 0.49[ab]	$_1$1.8 ± 0.17[a]	$_2$3.1 ± 0.44[b]	$_1$2.0 ± 0.31[a]	
	112	$_2$3.4 ± 0.16[a]	$_2$4.0 ± 0.24[bc]	$_3$4.3 ± 0.09[b]	$_2$4.4 ± 0.18[b]	$_2$4.1 ± 0.24[bc]	$_2$3.7 ± 0.19[ac]	1.22 ± 0.03
GCs (%)	0	$_1$11.0 ± 0.87[a]	$_1$11.0 ± 0.87[a]	$_1$11.0 ± 0.87[a]	$_1$11.0 ± 0.87[a]	$_1$11.0 ± 0.87[a]	$_1$11.0 ± 0.87[a]	
	56	$_{12}$14.8 ± 1.74[a]	$_2$18.3 ± 1.23[bc]	$_2$19.5 ± 1.20[c]	$_2$18.6 ± 1.46[bc]	$_2$23.7 ± 0.04[d]	$_1$13.1 ± 1.41[a]	
	112	$_2$16.7 ± 1.25[a]	$_2$18.4 ± 0.61[ab]	$_2$19.8 ± 0.37[bc]	$_3$22.2 ± 0.54[de]	$_2$23.4 ± 0.25[e]	$_2$20.4 ± 0.40[cd]	11.0 ± 0.86

Data in the same row having different superscript letters (a, b, c…) and data in the same column of each parameter having different subscript number (1,2,3..) are significantly different at P < 0.05.

4.4 DISCUSSION

The results of the present study indicate that the application of dietary MOS at 0.2 to 0.4% can significantly improve the survival and increase the total biomass production of marron. Further, the mean survival (75.5 ± 3.14%) of marron in the present experiment was higher than obtained before in an intensive system (Jussila and Evans 1996a). Similarly, the inclusion of MOS in the diet has also enhanced the survival of rainbow trout (*Oncorhynchus mykiss*) (Staykov *et al.* 2007) and cobia (*Rhycocentrum cadidum*) (Salze *et al.* 2008a). The positive effect of dietary MOS on total biomass production was also demonstrated in rainbow trout (*Oncorhynchus mykiss*) (Staykov *et al.* 2007) and common carp (*Cyprinus carpio* L) (Staykov *et al.* 2005c). However, MOS supplementation at 0.15, 0.3 and 0.45% did not change the survival of tiger prawn, *Penaeus semisulcatus* (Genc *et al.* 2007a). In the current study, the growth of marron fed different diets was similar or slower than reported before (Tsvetnenko *et al.* 1995; Jussila and Evans 1996a) and dietary MOS did not enhance the growth of marron. Null effect of MOS on growth was also reported in channel catfish (Welker *et al.* 2007) while the positive effects of MOS on growth have been reported for cobia larvae (Salze *et al.* 2008a), rainbow trout (Staykov *et al.* 2007) and European seabass (*Dicentrarchus labrax*) (Torrecillas *et al.* 2007).

The growth of marron in the current experiment was considered lower than commercial growth rates, however, the physiological parameters of marron in the control group were similar to that of marron from the commercial farm (Fotedar 1998b). Organosomatic indices and moisture contents of hepatopancreas and tail muscles have successfully been used as indicators of crustacean condition (Mannonen and Henttonen 1995; McClain 1995b; Jussila 1997b). While molt stages is one of the factors influencing the physiological condition of crayfish (Jussila 1997), marron used for analysis in the current experiment were in the same molt stage. Our results therefore have suggested that the application of MOS did improve the physiological condition of marron as shown by the changes in some of the organosomatic indices such as TMIwet, TMIdry and in osmoregulatory capacity. The significantly higher TMIwet and TMIdry observed in marron fed MOS indicated that MOS stimulated the storage of energy in the tail muscle and could thus have reduced the impact of stress (Fotedar *et al.* 1999). In addition, higher OC of marron fed 0.1, 0.2 and 0.4% MOS supplemented diets in the current experiment could be an indirect

sign of the health stimulation by dietary MOS. The higher OC also reduces the impact of water-borne pollutants, environmental stressors and pathological agents (Lignot *et al.* 2000). However, further research is required to validate the impacts of dietary MOS on the OC marron when they are exposed to various pollutants.

Low THCs is a possible indicator of a poor health condition in crayfish (Jussila *et al.* 1997). The results from this study indicate that marron are healthier when they are fed up to 0.4% of MOS supplemented diets. In addition, the increased trend in THCs of marron in all treatments after 56 days of culture indicates that marron in the current culture system were healthier than in the farm where they originated. Moreover, the inclusion of 0.2 to 0.4% of MOS in their diets is the most effective supplementation level to alter their differential haemocyte counts. The GCs play an important function in the crustacean immune system and thus their proportion in crustacean hemolymph can be used as a health indicator (Söderhäll 1997). In the present experiment marron fed the diets with MOS supplemented level of equal or more than 0.1% showed higher GCs than marron in other groups, thus indicating healthier condition of those marron (Jussila *et al.* 1997; Fotedar *et al.* 2001).

The presence of bacteria in the haemolymph of both stressed and healthy crustaceans have been reported by Cornick and Stewart (1966); Haskell *et al.* (1975) and Fotedar *et al.* (2001). Marron in the present study had lower bacteraemia rank when fed MOS supplemented diets compared to the control diet. Low bacteria levels in haemolymph are considered to be harmless to the host crayfish (Jussila 1997b). Sang *et al.* (2009) reported that supplementation of MOS at 0.2 to 0.4% results in an increased ability of marron to resist the bacterial infection. The high level of bacteraemia resulted in lower THCs and the lower GCs in marron fed control diet leading to a decrease in the immune resistance of marron (Alderman and Polglase 1988). The presence of bacteria in the hemolymph may lead to haemocytes participating in phagocytosis and encapsulation in order to remove or destroy bacteria, therefore decreasing THCs (Fontaine and Lightner 1974; Söderhäll *et al.* 1986). Higher THCs and also higher proportion of GCs of marron fed MOS has been shown to improve crayfish immune system and thus also lower bacteraemia rank (Johansson *et al.* 2000).

Shorter haemolymph clotting time of marron fed MOS showed that the MOS improves primary defense mechanism of the host's defense against foreign microbes

in marron. The higher the number of hyaline cells in the haemolymph, the shorter the time required for the haemolymph to clot (Fotedar *et al.* 2006). The higher THCs of marron fed the 0.2% and 0.4% MOS diets, resulted in a higher number of hyaline cells and thus a shorter clotting time.

The physiological and immunological evidences from this experiment show that the MOS inclusion of 0.2 to 0.4% in the diet is the most appropriate of the tested concentrations to improve the performance of marron.

CHAPTER 5: PREBIOTIC MANNAN OLIGOSACCHARIDE DIET
IMPROVES HEALTH STATUS OF THE DIGESTIVE SYSTEM OF
MARRON, *CHERAX TENUIMANUS* (SMITH, 1912)

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5.1 INTRODUCTION

Increasing economical and social concern to reduce the use of antibiotics and other therapeutic chemicals used in aquaculture has encouraged more environmentally friendly approaches to improve growth and disease control (Hansen and Olafsen 1999). Two main groups to fulfill this role are probiotics and prebiotics (Gatesoupe 1999; Torrecillas *et al.* 2007).

Prebiotics have been defined as selectively fermented ingredients that induce positive changes in the composition and/or activity in the gastrointestinal microbiota (Gibson *et al.* 2004). Those compounds help to alter the microbial community in the intestine to one dominated by beneficial bacteria (Manning and Gibson 2004). Some of the more common prebiotics used include fructooligosaccharide (FOS), transgalactooligosaccharide (TOS), and inulin (Burr *et al.* 2005). Recently, one prebiotic named mannan oligosaccharide (MOS) derived from the cell wall of *Saccharomyces cerevisiae* has been used in poultry (Hooge 2004), animal husbandry (Rozeboom *et al.* 2005; Mourão *et al.* 2006) and aquaculture (Torrecillas *et al.* 2007). This compound has been shown to affect gut health by reducing pathogen adsorption and immune modulation (Staykov *et al.* 2007). In aquaculture, incorporation of MOS in the diet leads to the improvement of the gut health of sea bream, *Diplodus sargus* (Dimitroglou *et al.* 2005), sole, *Solea senegalensis* (Sweetman and Davies 2007) and rainbow trout, *Oncorhynchus mykiss* (Dimitroglou *et al.* 2008) by making the gut microvilli more uniform and longer. Supplementation of MOS in the diet, however, did not induce any change in the hepatopancreas of tiger shrimp, *Penaeus semisulcatus* (Genc *et al.* 2007a). Despite the potential benefits to health and performance noted in some aquatic species, the use of MOS in crustacean aquaculture has been poorly studied and no information is available for marron, *Cherax tenuimanus*.

The marron has been an important aquaculture species in Western Australia for over 30 years (O'brien and Davies 2000). Although various studies have been conducted on nutrient requirements of marron (O'Brien 1995; Fotedar 1998b; Fotedar *et al.* 1999; Fotedar 2004), there is very limited information on the effects of any dietary supplements on marron. This study aimed to evaluate the effects of MOS (Bio-Mos, Aqua Grade, Alltech, USA) supplementation on the gut microbiota and histology of marron.

5.2 MATERIALS AND METHODS

Marron juveniles (4.44 ± 0.20 g – mean $\pm$ S.E.) were purchased from Aquatic Resource Management Pty Ltd, Western Australia and shipped to the Curtin Aquatic Research Laboratory (CARL). The marron were stocked into tanks provided with aerated, recirculating filtered freshwater and acclimated to culture conditions for 2 weeks. During the acclimation period, the marron were fed a commercial diet on every alternate day, supplied by Enviroplus, Australia (26% protein, 47-50% carbohydrate, 9% fats and 8.9% ash) at the rate of 3% total bodyweight.

Eighteen cylindrical plastic tanks (800 mm diameter, 500 mm high, 250 L capacity) were used for the experiment. Sufficient PVC pipes and oyster net of appropriate sizes were placed in each tank to provide shelter for the marron. The tank was supplied with aerated recirculating freshwater at a rate of approximately 3L min^{-1}. The water in each tank was filtered through both mechanical and biological filters. During the experiment, water temperature ranged between 19.2 and 21.7°C, and pH was between 7.58 and 8.26. The marron from the acclimation tanks were randomly distributed among the culture tanks at the density of 15 marron per tank.

Six isonitrogenous and isocalorific test diets supplemented with 0%, 0.05%, 0.1%, 0.2%, 0.4% and 0.8% mannan oligosaccharide (MOS) (Bio-Mos, Alltech, USA) were formulated. Feed ingredients and supplements used to formulate the diets were supplied by Specialty Feeds Pty Ltd in Western Australia except for Bio-Mos which was supplied by Alltech, USA. Diets were formulated with the FeedLIVE software package, version 1.52. All the dry feed ingredients and supplements were passed through a 100 µm mesh sieve, thoroughly mixed with a reasonable amount of freshwater and passed through a mince mixer to obtain pellets (1 mm diameter). The

pellets were dried in direct sunlight for 6 h, allowed to cool at room temperature for 30 min then packed and stored. Analyses of the dry matter, crude protein and ash of the diets were performed according to the standard methods of the Association of Official Analytical Chemists (AOAC 1995). There was no significant difference (P > 0.05) in gross energy, crude protein, moisture and ash content among the formulated diets with different supplemented levels of Bio-Mos (Table 5.1).

Each randomized blocks of three tanks were fed one of the test diets for 16 weeks so that each diet was represented in triplicate. The supplemented experimental and control diets were initially provided at the rate of 10% body weight every second day which was reduced to 5-6% depending on the amount of uneaten food left in the tanks. Uneaten food and faeces were siphoned before feed was provided. The amount of water lost during siphoning was added into each tank to retain the water level.

At the end of culture period, guts of 5 marron from each tank were dissected out and weighed to four decimal places (Wgut) and placed in a sterile test tube. To determine the total bacteria, five mL of PBS (Phosphate Buffered Saline) was added to the tube and homogenized using a glass rod and homogenizer to obtain a solution. Several serial 10-fold (10x, 100x, 1000x, 10000x) dilutions were prepared using PBS solution. Aliquots of each diluted homogenized solution were incubated on a Nutrient Agar plate (0.1 mL/plate, replicated in 3 plates for each dilution, for 24 h at 25°C in order to count colonies. Similarly, diluted solutions were incubated in Thiosulfate Citrate Bile Sucrose Agar (TCBS) plates for 46 h to count total *Vibrio* spp. Only those plates having between 20 and 200 colonies were included for the bacterial counts.

To visually assess the condition of marron guts, 5 marron from each treatment were dissected at the end of the culture period and prepared for scanning electron microscopy (SEM) following the procedures of Dunlap and Adaskaveg (1997) with some modification. Mounted specimens were viewed under a pressure scanning electron microscope (LX 30) and described in terms of distribution, density and abnormalities among gut villi.

Histological analysis of marron gut was performed following the method described by Genc *et al*. (2007a). At the end of the trial, guts of marron were dissected and fixed in 4% buffer formalin for 24 h. After dehydrating by passing the tissue through a series of alcohol solutions of 70, 85 and 98%, the samples were vacuum embedded in paraffin. The histological sections (4 – 5 µm) were stained for general morphological purposes with hematoxylin and eosin (H&E). The samples were photographically analysed and documented using an Olympus BX 50 microscope at 40x magnification.

Table 5.1: Formulation and proximate nutrient components of the test diets for marron

Ingredients and proximate nutrient components	D1 (%)	D2 (%)	D3 (%)	D4 (%)	D5 (%)	D6 (%)
Fish oil	3.20	3.20	3.20	3.23	3.23	3.23
Soybean meal	10.15	10.15	10.14	10.14	10.14	10.11
Fishmeal	33.78	33.80	33.83	33.83	33.86	34.01
Wheat flour	49.35	49.25	49.21	49.08	48.85	48.33
Ascorbic acid	0.05	0.05	0.05	0.05	0.05	0.05
Betaine	1.20	1.20	1.20	1.20	1.20	1.20
Calcium ascorbate	0.02	0.02	0.02	0.02	0.02	0.02
Premix	0.15	0.15	0.15	0.15	0.15	0.15
Cholesterol	0.25	0.25	0.25	0.25	0.25	0.25
Wheat starch	1.85	1.85	1.85	1.85	1.85	1.85
Bio-Mos	0.00	0.05	0.10	0.20	0.40	0.80
Energy (calories/gm)	4487.67	4501.67	4501.00	4495.00	4486.00	4485.33
Crude protein (%)	34.64	34.41	34.86	34.66	34.81	34.57
Dry matter (%)	94.56	94.01	94.13	94.60	94.13	94.61
Moisture content (%)	5.44	5.99	5.87	5.40	5.87	5.39
Ash (%)	6.16	6.02	5.98	6.16	6.04	6.11

The normality of data were assessed by the Shapiro-Wilk test (Winer 1991) and the homogeneity of variance was assessed by Levene (Winer 1991) test prior to analysis. Normal data were subjected to one-way analysis of variance using post-hoc LSD test

for data of homogenous variance, and Tamhane's test (Winer 1991) for data of non-homogenous variance. Non-normal data were subjected to non-parametric testing using Kruskal-Wallis H test (Winer 1991). Differences were considered significant at 5% level of probability.

5.3 RESULTS

At the end of 16 weeks, total bacteria counts in the guts of marron were significantly affected by different Bio-Mos supplemented levels. The highest concentration of bacteria was in marron fed 0.4% Bio-Mos and the lowest was in marron fed 0.05% Bio-Mos. Marron fed 0.1%, 0.2% and 0.4% Bio-Mos had more bacteria in the gut than the marron fed 0%, 0.05% and 0.8% Bio-Mos. Differences among treatments were significant ($P < 0.05$) except between marron fed 0% and 0.8% Bio-Mos (Table 5.2).

Vibrio spp. counts from marron guts was significantly higher ($P < 0.05$) in the gut of marron fed 0.1% Bio-Mos than other diets. Marron fed 0%, 0.05%, 0.2%, 0.4% and 0.8% Bio-Mos in the diet showed no difference ($P > 0.05$) in total *Vibrio* spp. in the gut after 112 days of culture. Total bacteria/total *Vibrio* ratio was the highest in marron fed 0.4% Bio-Mos, while diets with 0%, 0.2% and 0.8% Bio-Mos had similar gut bacteria/*Vibrio* ratios. The lowest total bacteria/*Vibrio* spp. ratios were in the marron fed 0.05% and 0.1% Bio-Mos (Table 5.2). The linear relationship between total bacteria and *Vibrio* spp. in the gut of marron was discontinuous, difficult to interpret and rather weak ($R^2 = 0.0193$).

Scanning electron micrographs of the middle section of the hindgut of marron fed the different diets are shown in Figure 5.1. Villi in the gut were present in groups of 3 to 11 villi. The villi distribution was uniform on the inner surface of the gut. Villi of marron fed Bio-Mos supplemented diets seem to be longer than villi of marron fed the control diet. The number of villi per group of marron fed 0% Bio-Mos was significantly lower than marron fed all Bio-Mos supplemented diets. Marron fed 0.2% and 0.4% Bio-Mos had the highest number of villi per group followed by marron fed 0.05%, 0.1% and 0.8% Bio-Mos. Marron fed 0%, 0.05%, and 0.8% Bio-Mos had a significantly lower density of villi ($P < 0.05$) than the marron fed 0.1%, 0.2% and 0.4% Bio-Mos (Table 5.3).

Table 5.2: Bacteria in the gut of marron fed different Bio-Mos supplemented diets (mean ± SE)

Diets*	Total bacteria (million CFU/g)	*Vibrio* (million CFU/g)	Bacteria/*Vibrio* ratio
D1	a974.50 ± 80.66	a0.51 ± 0.09	a2,033.34 ± 384.54
D2	b702.09 ± 71.53	a2.78 ± 0.61	bc293.51 ± 97.34
D3	c2,449.51 ± 58.86	b9.99 ± 1.56	c259.77 ± 47.48
D4	d1,905.06 ± 84.93	a1.46 ± 0.58	a1,816.93 ± 670.60
D5	e3,283.90 ± 72.33	a0.82 ± 0.05	d4,013.93 ± 244.57
D6	a1,216.90 ± 124.64	a1.34 ± 0.39	abc1,144.84 ± 462.75

Value in the same column of each parameter having different subscript letters are

significantly different at P < 0.05.

* D1: Bio-Mos free, D2: 0.05 % Bio-Mos, D3: 0.10% Bio-Mos, D4: 0.20% Bio-Mos, D5: 0.40% Bio-Mos, D6: 0.80% Bio-Mos

Microscopy of transverse sections of marron gut showed that animals fed 0.2%, 0.4% and 0.8% had a thicker epithelium layer than the marron fed 0%, 0.05% and 0.1% Bio-Mos (Figure 5.2). In addition, the density of epidermal cells in the gut surface of marron fed 0.2%, 0.4% and 0.8% Bio-Mos was higher than that in marron fed 0%, 0.05% and 0.1% Bio-Mos (Figure 5.3).

Table 5.3: Villi in the hindgut of marron fed different Bio-Mos supplemented diets

Diets*	Number of villi/group	Villi density (villi/100 μm^2) (mean ± SE)
D1	a6.13 ± 0.04	a9.67 ± 0.38
D2	b7.44 ± 0.20	a10.93 ± 0.60
D3	b7.68 ± 0.20	b13.56 ± 0.48
D4	c8.85 ± 0.61	b14.63 ± 0.94
D5	bc8.22 ± 0.27	b14.04 ± 0.27
D6	bc8.00 ± 0.26	a10.41 ± 0.72

Value in the same column of each parameter having different subscript letters are significantly

different at P < 0.05.

* D1: Bio-Mos free, D2: 0.05 % Bio-Mos, D3: 0.10% Bio-Mos, D4: 0.20% Bio-Mos, D5: 0.40% Bio-Mos, D6: 0.80% Bio-Mos
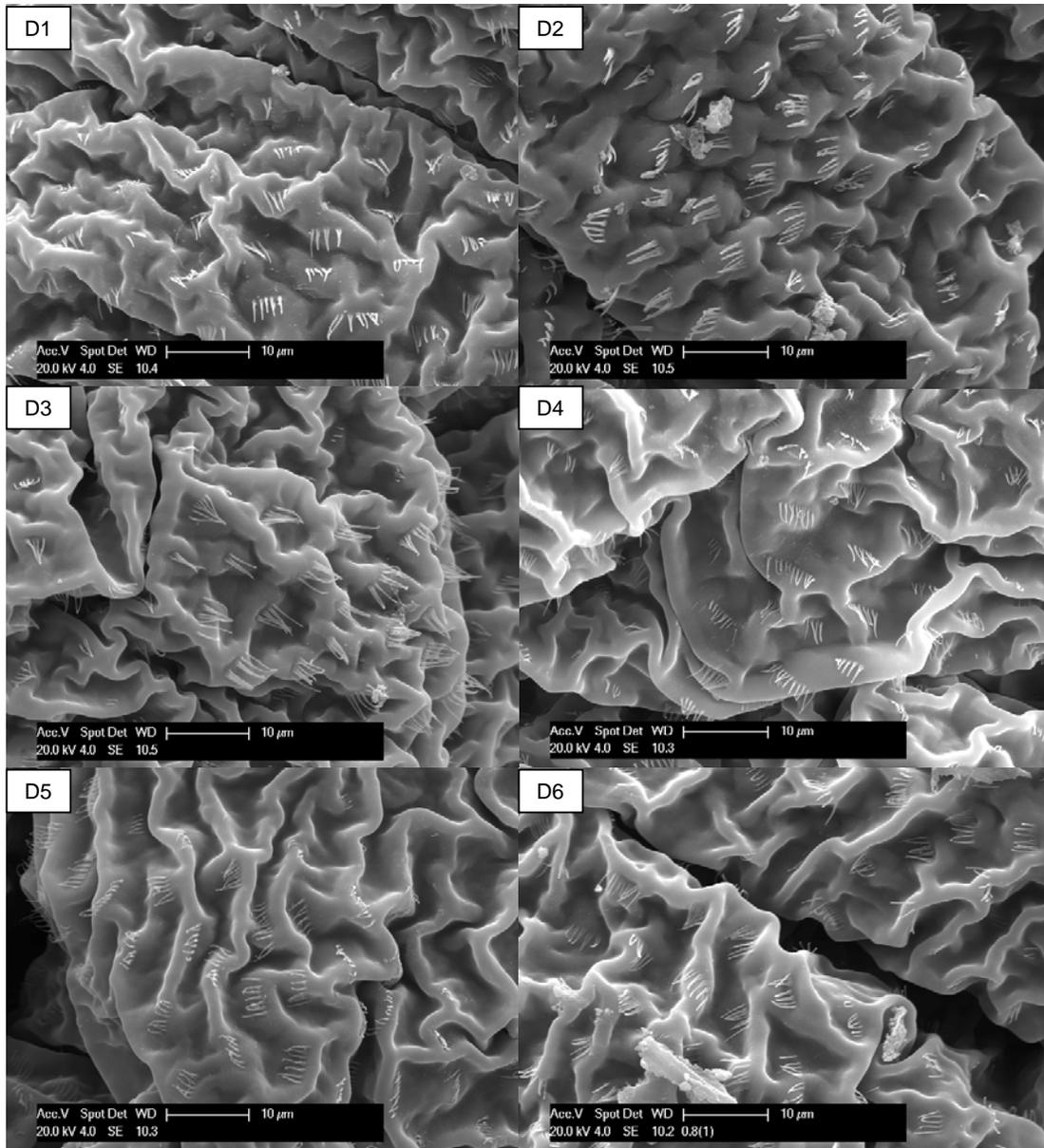
Figure 5.1: Gut micrographic of marron fed different Bio-Mos supplemented diets.

D1: Bio-Mos free; D2: Bio-Mos - 0.05%; D3: Bio-Mos - 0.1%; D4; Bio-Mos - 0.2%; D5; Bio-Mos - 0.4%; D6: Bio-Mos - 0.8%.

.
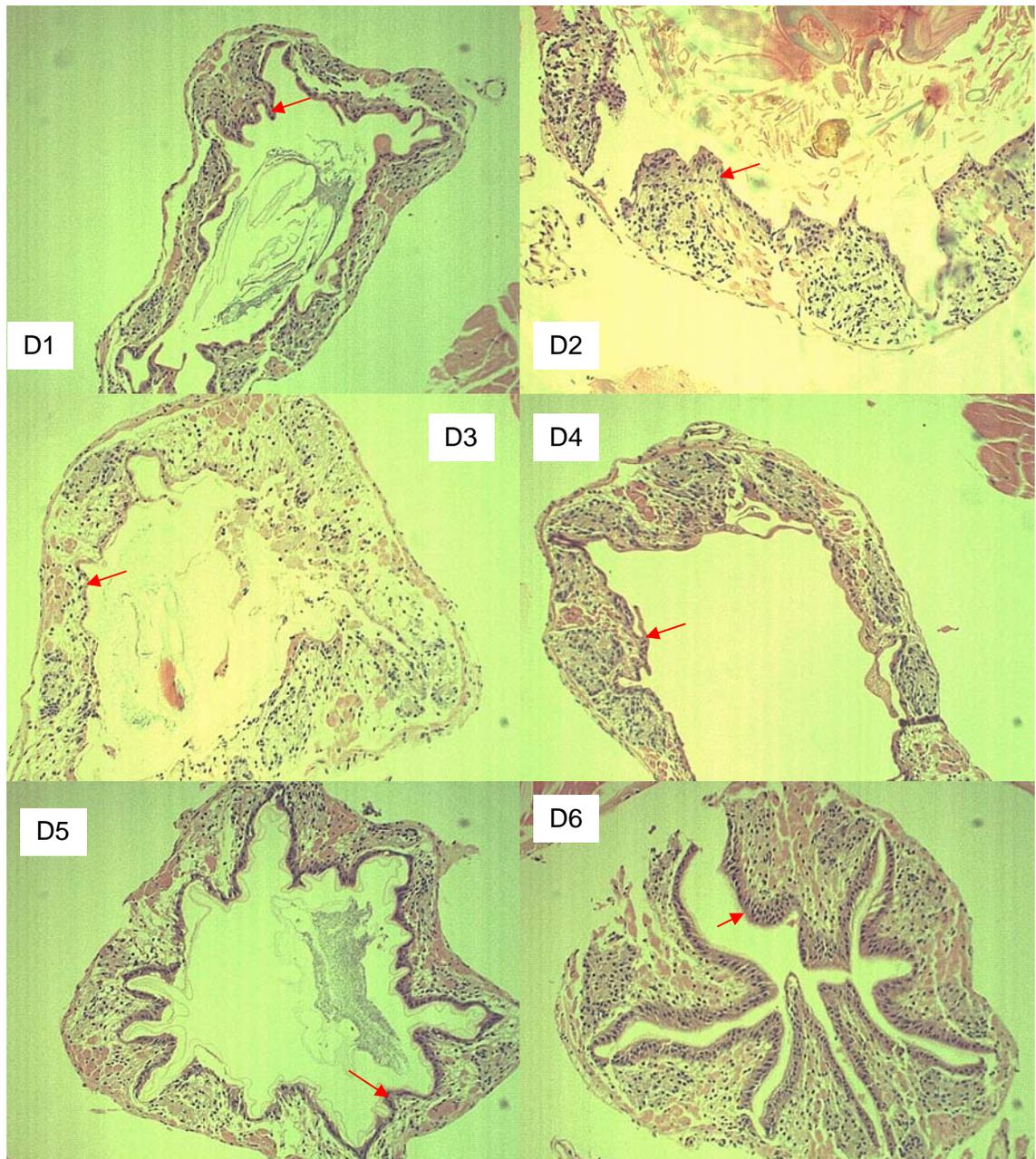
Figure 5.2: Transversal sections of the gut of marron, fed the different Bio-Mos supplemented diets at 100X magnifications.

Sections were all taken from the same general area of the gut, H&E stained. The arrow points the epithelium layer of the gut. D1: Bio-Mos free; D2: Bio-Mos - 0.05%; D3: Bio-Mos - 0.1%; D4; Bio-Mos - 0.2%; D5; Bio-Mos - 0.4%; D6: Bio-Mos - 0.8%.
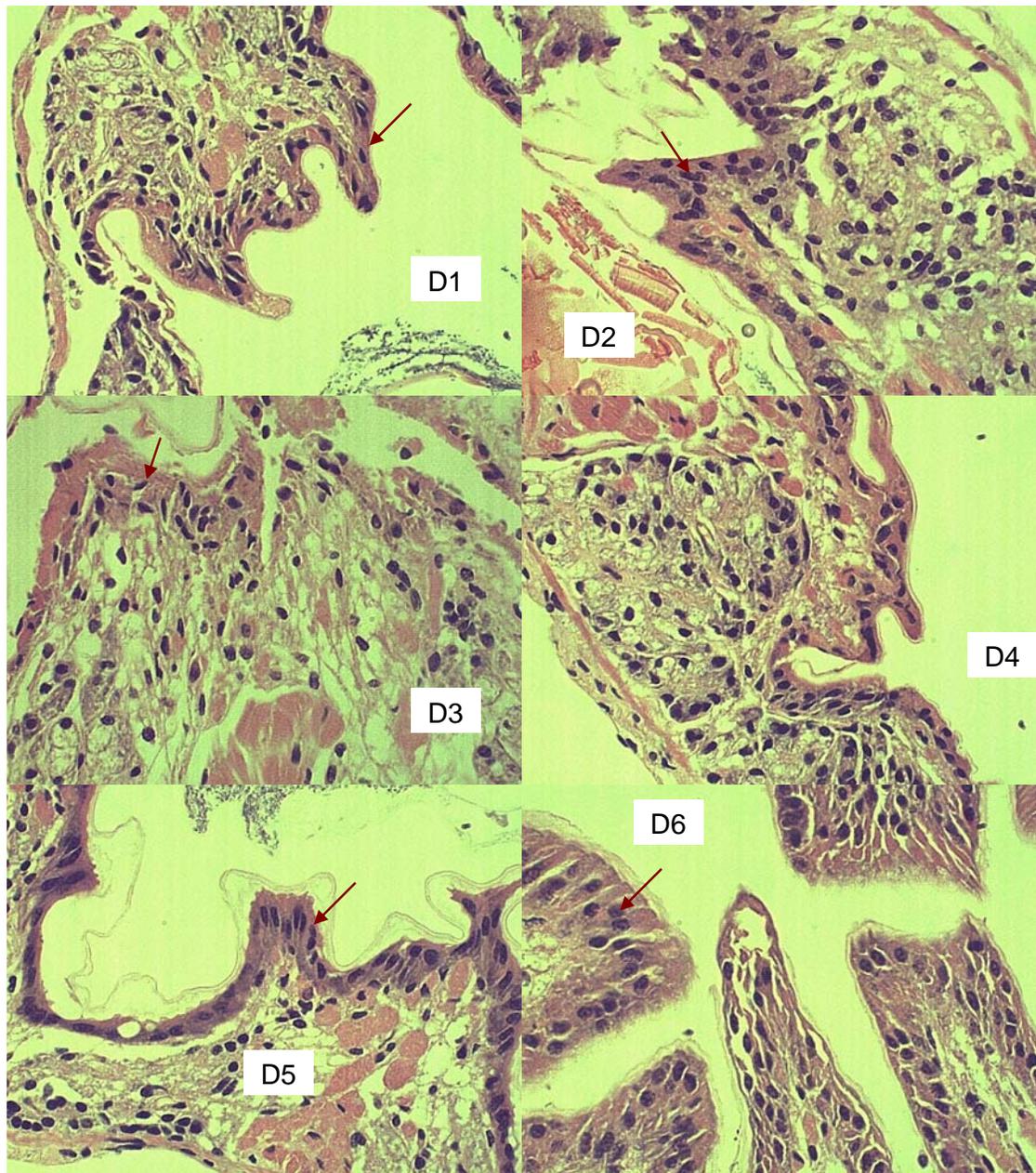
Figure 5.3: Transversal sections of the gut of marron, fed the different MOS supplemented diets (H&E stained, 400X magnification).

(The arrow points the epidermis cell of the inner gut lining). D1: Bio-Mos free; D2: Bio-Mos - 0.05%; D3: Bio-Mos - 0.1%; D4; Bio-Mos - 0.2%; D5; Bio-Mos - 0.4%; D6: Bio-Mos - 0.8%.

5.4 DISCUSSION

The increased concern on the use of antibiotics and chemicals in aquaculture has promoted research toward the safe alternative products to improve the growth, food conversion efficiency, health and disease resistance of the host (Daniels *et al.* 2006). Prebiotics are indigestible carbohydrates which stimulate the growth and activity of beneficial bacteria in the intestine and can activate the innate immune responses of cultured organisms when used as dietary supplements (Hooge 2004). Prebiotics have also increased the efficiency of the digestive tract in many organisms through increasing the regularity, height and integrity of the gut villi (Hooge 2004). Evidence has shown that microbiota in the digestive tract stimulated by prebiotics play an integral role in growth, digestion, immunity, and disease resistance in poultry (Patterson and Burkholder 2003) and in humans (Gibson and Roberfroid 1995). The use of prebiotics in aquaculture is rather limited (Gatlin 2002) and this work is the first attempt to investigate the role of prebiotic, mannan oligosaccharide through Bio-Mos, on the gut health of marron.

Total bacteria in the marron gut increased with increasing dietary Bio-Mos up to 0.8%. However, the incidence of *Vibrio* spp. was lowest in 0.4% Bio-Mos fed marron. Furthermore, at the application rate of 0.4% Bio-Mos, non-pathogenic bacteria in the digestive tract dominated over *Vibrio* spp. The same microbial profile of marron fed the control and the 0.8% Bio-Mos diets indicate that high level of Bio-Mos may actually reverse the beneficial effects of MOS in the diet. The current results suggest that 0.4% is the most helpful concentration of Bio-Mos in marron diets, results more or less consistent with previous studies of Bio-Mos included at 0.24% into diets for Jian carp, *Cyprinus carpio* (Zhou and Li 2004). Recent work by Dimitroglou *et al*. (2008) showed that Bio-Mos reduced potentially pathogenic *Micrococcus* spp., *Staphylococcus* spp., *Aeromonas/Vibrio* spp. and other unidentified Gram + bacteria and increased beneficial *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacter* spp. in the guts of rainbow trout (*Oncorhynchus mykiss*).

Results from current experiment also imply that inclusion of 0.2 to 0.4% Bio-Mos in the diet increases the density, and possibly the length, of villi in the gut. More villi increase gut irrigation, help to protect the cuticle layer and/or smooth movement of

faeces (Chisaka *et al.* 1999). Protection provided by gut villi thickens gut epithelium and increases the density of epidermis cells compared to marron fed MOS-free diets. A thick epithelium and high density of epidermis cells improves gut transport, assimilation, nutrient storage (Ferna´ ndez *et al.* 2002). These results are in total agreement with previous studies showing that Bio-Mos increases microvilli density and length in sea bream, *Diplodus sargus* (Dimitroglou *et al.* 2005), sole, *Solea senegalensis* (Sweetman and Davies 2007) and rainbow trout (Dimitroglou *et al.* 2008).

In conclusion, the use of MOS, Bio-Mos, can improve digestive tract health of marron by increasing the number of beneficial bacteria, gut villi density and epithelium thickness, especially when supplemented at levels of 0.2% to 0.4% in the diet.

CHAPTER 6: DIETARY SUPPLEMENTATION OF MANNAN OLIGOSACCHARIDE IMPROVES THE IMMUNE RESPONSES AND SURVIVAL OF MARRON, *CHERAX TENUIMANUS* (SMITH, 1912) WHEN CHALLENGED WITH DIFFERENT STRESSORS

6.1 INTRODUCTION

Bacterial and viral diseases are known to be the major constraints in aquaculture industry in recent years (Scholz *et al.* 1999) and are impeding the development and sustainability of the industry throughout the world (Bondad-Reantaso *et al.* 2005). Thus, the need to control the loss due to disease outbreaks in aquaculture is becoming increasingly important. In most cases, water is disinfected before use and antibiotics are used to protect cultured species from microbial infections (Vadstein 1997). Such practices have led to promote the spread of antibiotic-resistant pathogens in both cultured species and in the environment (Gatlin 2002; Kesarcodi *et al.* 2008). In addition, the application of such practices is expensive and undesirable due to having harmful effects to the environment and human health (Capone *et al.* 1996).

In addition, the global demand for safe food has prompted the search for natural, alternative growth promoters to be used in aquatic animal feeds. Recently, immunostimulants such as probiotics and prebiotics have showed as preventive and environmentally-friendly alternatives to antibiotics in aquaculture, especially for fishes and crustacean (Campa-Córdova *et al.* 2002; Bachere 2003; Soltanian *et al.* 2007). However, the application of prebiotics, classified as non-digestible food ingredients that beneficially affect the host by stimulating growth and/or activity of a limited number of bacteria in the intestine, is in its infancy with crustacean, compared with the progress that has been made in the development of prebiotics for poultry (Patterson and Burkholder 2003). Thus, there is a increased attention on the use of prebiotics as a key strategy to maintain and expand the aquaculture industries in the recent years (Genc *et al.* 2007a).

As a common prebiotic, mannan oligosaccharide (MOS) derived from the cell wall of *Saccharomyces cerevisiae,* has recently been used in poultry, husbandry and

aquaculture as a diet supplement (Torrecillas *et al.* 2007). This compound has been shown to affect gut health by pathogen adsorption and immune modulation in the target species. In finfish aquaculture, incorporation of MOS in the diet has promoted the resistance ability to bacteria infection and stressors of common carp (*Cyprinus carpio*) (Staykov 2004), rainbow trout *(Salmo gairdneri irideus G.)* (Staykov *et al.* 2006), channel catfish (*Ictalurus punctatus*) (Welker *et al.* 2007), rainbow trout (*Oncorhynchus mykiss*) (Staykov *et al.* 2007) and sea bass (*Dicentrarchus labrax*) (Torrecillas *et al.* 2007). In crustacean aquaculture the use of MOS has been studied by Genc *et al.* (2007a) on *Penaeus semisulcatus*.

Marron (*Cherax tenuimanus* Smith) is one of the world's largest freshwater crayfish found in Australia and capable of reaching 2 kg in weight (Morrissy 2000). Native to Western Australia, it has been recognised as a potential aquaculture species due to its attractive attributes (Rouse and Kartamulia 1992). Recently, marron was introduced into South Africa, Zimbabwe, Japan, USA, China and the Caribbean as a commercial aquaculture species (Morrissy *et al.* 1990). As a result of the national and international interest in marron farming, its distribution has been widely extended (Bryant and Papas 2007) and research is in progress related to its aquaculture potential. The area of nutrient manipulations in the formulated diets in order to improve the marron productivity has attracted many researchers (Tsvetnenko *et al.* 1995; Fotedar *et al.* 1997; Fotedar 1998b; Fotedar *et al.* 1999; O'Brien and Davies 2000; Fotedar 2004). Despite the available information on the macronutrient requirements of marron, the use of supplemented elements in the diet to improve the health status and immunity of marron is still unknown and there is no record on the use of MOS in the diet of marron.

Air exposure has been used as a common stressor to evaluate the health of crab (*Cancer pagurus*) (Lorenzon *et al.* 2008), lobster (*Panulirus cygnus*) (Fotedar *et al.* 2001), western king prawn (*Penaeus latisulcatus*) and brown tiger shrimp (*Penaeus esculentus*) (Sang and Fotedar 2005). NH3 at toxic level has been proved to alter the physiological and immunological status of different crustacean such as mud crabs (*Scylla serrata*) (Romano and Zeng 2007a), blue swimmer crabs (*Portunus pelagicus*) (Romano and Zeng 2007b), etc. However, the effects of this stressor on marron have not been investigated. The present paper aims to evaluate the

effectiveness of MOS in enhancing the survival and immunity of marron when exposed to bacteria, NH_3 and air. Total haemocyte count (THC), different haemocyte count (DHC), haemolymph clotting time, bacteraemia and lysosomal membrane stability of haemocyte are used as tools to evaluate the immune responses of marron.

6.2 MATERIALS AND METHODS

6.2.1 Test diets

Mannan oligosaccharide (Bio-Mos, Alltech Inc, USA) which is derived from the cell wall of *Saccharomyces cerevisiae*, containing minimum of 30% protein, 1.4% crude fat and maximum of 13% crude fiber was supplemented at two levels of 0.2% and 0.4% to a basal control diet (0% Bio-Mos). The concentrations of Bio-Mos at 0.2% and 0.4% are the most effectiveness on performance of marron. All test diets contained approximately 34% crude protein, 8% crude lipid, 6% ash which is considered nutritionally adequate for the growth of juvenile marron (Fotedar 2004). The proximate compositions of the ingredients (supplied by Specialty Feeds Pty Ltd, Western Australia) and Bio-Mos (supplied by Alltech Inc, USA) were used as a basis to form the required diets using the software FeedLIVE version 1.52 (Table 6.1). The pellets of 1 mm diameter were prepared by mixing the feed ingredients through a mince mixer and then drying them in the direct sunlight for six h. The dried pellets were then allowed to cool at a room temperature for 30 min prior to packaging and storing in a dark room.

6.2.2 Experimental animals

Marron were supplied by Aquatic Resource Management Pty Ltd, Western Australia and shipped to the Curtin Aquatic Research Laboratory (CARL). The marron were stocked in tanks provided with aerated, recirculating filtered freshwater and were then acclimated to the culture conditions for 2 weeks. During the acclimation period, the marron were fed with a commercial diet supplied by Enviroplus, Australia (26% protein, 47-50% carbohydrate, 9% fats and 8.9% ash) at a rate of 3% total biomass per two days.

6.2.3 Culture system

Plastic cylindrical tanks (800 mm diameter, 500 mm high, and 250 L capacity) were used for the trials. Sufficient PVC pipes and oyster net of appropriate sizes were

placed in each tank to provide shelters for the marron. The tank was supplied with aerated recirculating freshwater at a rate of approximately 3L min^{-1}. The water in each tank was filtered through both mechanical and biological filters.

Table 6.1: Ingredients of the test diets

Ingredients	Test diets		
	Control	0.2% Bio-Mos	0.4% Bio-Mos
	(%)	(%)	(%)
Fish oil	3.20	3.23	3.23
Soybean meal	10.15	10.14	10.14
Fishmeal	33.78	33.83	33.86
Wheat flour	49.35	49.08	48.85
Ascorbic acid	0.05	0.05	0.05
Betaine	1.20	1.20	1.20
Calcium ascobate	0.02	0.02	0.02
Premix	0.15	0.15	0.15
Cholesterol	0.25	0.25	0.25
Wheat starch	1.85	1.85	1.85
Bio-Mos	0.00	0.20	0.40
Total	100	100	100

6.2.4 Feeding and challenge of marron with various stressors

A total of 3 independent trials were conducted one after the other. Each trial involved the exposure of the selected stressor to marron under laboratory conditions.

Challenge with bacteria - Trial 1

Total 9 culture tanks were used for this trial. Each tank was stocked with 15 marron of 10.44 ± 0.20 g (mean ± S.E.). Each of three test diets was randomly assigned to three tanks, giving three replicates per diet. The marron were provided the food every two days at the initially feeding rate 5% of the body weight which was thereafter adjusted for each tank according to feeding response. Uneaten food and faeces were removed before every feeding.

Preparation of bacteria stock solution: Stock solution of *Vibrio. mimicus* (isolated from blisters of dead yabby, *Cherax albidus*) was obtained from the Department of Agriculture, Western Australia. The concentration of stock solution was approximately 0.53×10^6 CFU/mL.

Challenge test was initiated on day 30 of the feeding trial. Six marron from each test-diet tank were injected through the base of the fifth thoracic leg with 20 μL bacteria stock solution. The injected marron were marked using the polish before releasing back into their original tanks to avoid repeat sampling. The infected marron were monitored for survival, total haemocyte count, differential haemocyte count, haemolymph clotting time, bacteraemia and lysosomal membrane stability after 24, 48 and 96 h of injection.

Exposure to air during simulated transport - Trial 2

Juvenile marron (4.44 ± 0.20 g – mean $\pm$ S.E.) after routine acclimation were distributed to 9 independent tanks at a density of 15 marron per tank. Each random block of three tanks was provided with one diet so that each test diet was represented in triplicate. After 112 days of culture, the marron were subjected to a simulated live transport. From each dietary group, 12 marron (4 from each replicate) were tagged with nail polish and put in a polystyrene box (60 x 40 x 30 cm) for 36 h. The box had two ice-gel bags covered by a foam layer. Marron were placed on this foam and then covered by another layer of foam. The box was then covered by a lid and sealed. After 36 h in the box, marron were placed back into their culture medium for 6 h and then examined for total haemocyte count, granular cell and haemolymph clotting time.

Exposed to NH₃ - Trial 3

Marron (94 ± 2.17 g - mean $\pm$ S.E) were stocked into 6 tanks at a density of 8 marron per tank. Three tanks were fed with the Bio-Mos-free diet and the three remaining tanks were fed with the 0.4% Bio-Mos supplemented diet for 42 days. The marron in each tank were then exposed to NH_3 by adding NH_4Cl into the culture media to obtain NH_4^+ concentration of 20 mgl^{-1} which is equal to a concentration of 1.37 mgl^{-1} of NH_3 at the temperature of 16.8^oC and the pH of 8.4 (Trussell 1972). Survival, total haemocyte count, differential haemocyte count, haemolymph clotting time,

bacteraemia and osmoregulatory capacity after 1 day, 3 days and 7 days of NH_3-exposure was measured.

6.2.5 Data collection

Survival

Survival rate of marron in all tanks was measured using the following calculation:

$$S = 100 \text{ x } (n_t/n_o)$$

Where: S is the survival rate; n_t is the number of marron at time t and n_o is the number of marron at the commencement of the challenge.

Total haemocyte count (THC) and differential haemocyte count (DHC)

Total and differential haemocyte counts were conducted as per the established procedure for rock lobster (Fotedar *et al.* 2001). The base of the fifth thoracic leg of each marron from each culture tank was cleaned with 70% alcohol. A 0.2 mL aliquot of haemolymph was withdrawn into a 1-mL sterile syringe containing 0.2 mL of anticoagulant (1% glutaraldehyde in 0.2M sodium cacodylate) and dispensed into an Eppendorf tube kept on ice. Total haemocyte counts for individual marron were estimated with a haemocytometer (Neubauer, Germany) under 100× magnification, from the anticoagulant /haemolymph mixture. 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).

For differential haemocyte counts, one drop of haemolymph/anticoagulant mixture was placed on a slide and smeared. The smear was air dried and fixed in 70% methanol for 10 min. Fixed smears were stained with May-Grunwald and Giemsa (10 min in each) (Bancroft and Stevens 1977) and mounted with a coverslip. Approximately 200 cells were counted on each slide. The granular cells were distinguished on the basis of the larger cell size, smaller pale nucleus, and larger number of eosinophillic granules in the cytoplasm, and their proportion to overall haemocytes was calculated. Hyaline cells had round shape, big nucleus and little or no cytoplasm; while semi-granular cells had a longer shape, big nucleus and little or no cytoplasm.

Bacteraemia assessment

Bacteraemia assessment were performed based on the establised procedure (Fotedar *et al.* 2001). A 0.05 mL aliquot of haemolymph was withdrawn into a sterile syringe and quickly placed in form of small drops and smeared on a nutrient agar (NA) plate (Atlas and Parks 1997); the plate was carefully inverted before putting into the incubator chamber at temperature of 25°C for 24 h. Colony forming units (CFU) were counted for each plate and CFU/mL calculated for each sample on the basis of a total volume of 0.05 mL/plate. The CFU/mL were ranked 1 (1–250 CFU/mL) to 12 (2751–3000 CFU/mL), and a final rank of 13 was assigned to those samples in which the colonies were too numerous for an accurate count.

Haemolymph clotting time

Haemolymph clotting time was determined following the protocol described by Fotedar, *et al.* (2001) with some modification. Haemolymph withdrawn into a sterile syringe was dispensed into an Eppendorf tube. A 30-μL aliquot was quickly transferred into another tube and drawn into a capillary tube. The tube was repeatedly inverted until the haemolymph stopped moving, and the time was noted as haemolymph clotting time.

Neutral red retention assay

The lysosomal membrane stability in haemocytes of marron was assessed using neutral red retention assay based on an established protocol (Hauton *et al.* 1998) with some modification. A stock solution of neutral red was produced by dissolving 10 mg of neutral red powder in 1 mL of dimethyl sulphoxide. The working solution (dye concentration 0.02 mg/mL) was prepared by diluting 10 μL of the stock solution with 5 mL of artificial saline water. A 0.2 mL aliquot of marron haemolymph sample was transferred into an Eppendorf tube containing 0.2 mL artificial saline water and gently mixed. The mixture of haemolymph sample and artificial saline water was placed onto a microscope slide treated with a poly-L-lysine solution to enhance cell adhesion. The sample was immediately placed in a 10°C incubator for 15 min to allow the haemocytes to attach onto the slide. The slide was removed from the incubator and the excess haemolymph was removed. Forty μL of neural red working solution was added to the slide and the sample was covered with a coverslip. The slide was then returned to the incubator. Every 15 min the slide was taken out and the

sample was examined using a microscope. The time at which 50% of the haemocytes had started to lose dye from their lysosomes was recorded as the neutral red retention time of the marron lysosomal membrane.

6.2.6 Data analysis

A multiple comparison test - Least significant different (LSD) and an Independent-sample T test were used to examine the significant difference among treatments using the SPSS version 15.0. Percent data were normalized using an arcsin transformation before analysis. For significant difference, it was required that P < 0.05. One way ANOVA was analyzed and results were expressed as mean ± standard error in the figures and tables.

6.3 RESULTS

6.3.1 Trial 1 - Challenge with bacteria

Survival

Bio-Mos supplemented diets resulted in lower (P < 0.05) marron mortalities when injected with *V. mimicus* (Figure 6.1) compared to marron fed the control diet.

Total and differential haemocyte count

There was no significant difference ($P > 0.05$) in mean THCs of marron fed any of the diets before challenging with the *V. mimicus*. At 24 h after the challenge, the mean THCs of the marron fed the control diet was significantly lower ($P < 0.05$) while mean THCs of the marron fed 0.2% Bio-Mos and 0.2% Bio-Mos diets remained unchanged. After 24 h of bacterial challenge, mean THCs was significantly lower ($P < 0.05$) in the marron fed the control diet than in the marron fed the Bio-Mos diets. However, at 48 h after the challenge, there was no significant difference ($P > 0.05$) in mean THCs of the marron fed any of the test diets. After 96 h of the challenge, the highest THC was in marron fed 0.4% Bio-Mos diet (Table 6.2).
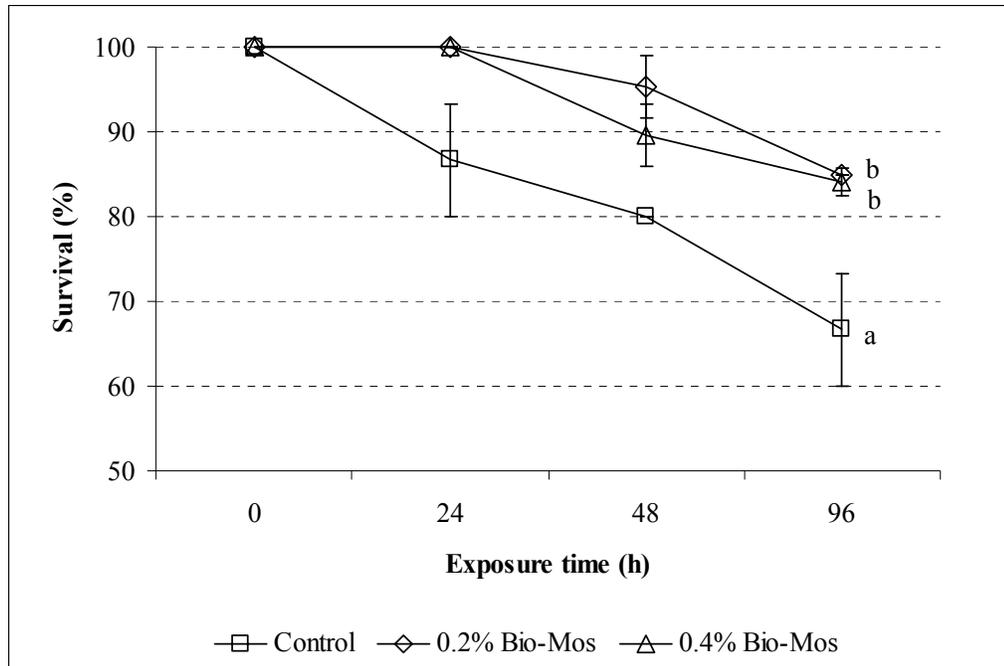
Figure 6.1: Mean ± SE survival of marron challenged with *Vibrio mimicus*. Different letters indicate significantly different means at each time at P < 0.05 (n = 18).

Before the *V. mimicus* injection, mean proportion of granular cells was significantly lower ($P < 0.05$) in the marron fed the control diet than in the marron fed the Bio-Mos diets. At 24 h after the injection, there were decreases in mean proportions of granular cells of marron in all treatments but the significant decline ($P < 0.05$) was only found in marron fed the control diet resulting in the significantly lower proportion of granular cells in the marron fed the control diet. Semi-granular cells were not significantly different between the marron fed the control and the Bio-Mos diets and the proportion of these cells remained unchanged during the *V. mimicus* challenge. However, the proportion of hyaline cells showed the opposite trend than granular cells during the challenge (Table 6.2).

Haemolymph clotting time

There was no significant difference (P > 0.05) in mean haemolymph clotting time of the marron fed any of the diets before and after the *V. mimicus* injection (Table 6.2).

Table 6.2: Mean ± SE immune parameters of marron challenged with *Vibrio mimicus*

Parameters	Time after infection (h)	Test diets		
		Control	0.2% Bio-Mos	0.4% Bio-Mos
THCs (million cells/mL)	0*	$_{1}$3.26 ± 0.47[a]	$_{1}$3.66 ± 0.04[a]	$_{1}$3.77 ± 0.2[a]
	24	$_{2}$1.73 ± 0.04[a]	$_{1}$4.56 ± 0.44[b]	$_{1}$3.67 ± 0.45[b]
	48	$_{12}$2.85 ± 0.62[a]	$_{1}$3.70 ± 1.08[a]	$_{1}$3.33 ± 0.26[a]
	96	$_{12}$2.30 ± 0.54[a]	$_{1}$2.91 ± 0.41[a]	$_{1}$3.90 ± 0.11[b]
Granular cells (%)	0*	$_{13}$5.40 ± 0.57[a]	$_{1}$14.64 ± 0.81[b]	$_{1}$20.09 ± 2.20[c]
	24	$_{2}$1.86 ± 0.33[a]	$_{1}$13.11 ± 3.32[b]	$_{12}$13.35 ± 2.93[b]
	48	$_{3}$6.86 ± 0.60[a]	$_{1}$10.78 ± 0.69[b]	$_{12}$14.05 ± 1.34[c]
	96	$_{12}$3.73 ± 1.0[a]	$_{1}$14.18 ± 2.54[b]	$_{2}$11.70 ± 1.78[b]
Semi-granular cells (%)	0*	$_{1}$8.80 ± 0.16[a]	$_{1}$7.38 ± 1.74[a]	$_{12}$9.95 ± 2.19[a]
	24	$_{1}$6.47 ± 2.63[a]	$_{1}$13.80 ± 3.09[a]	$_{1}$4.88 ± 2.57[a]
	48	$_{1}$10.73 ± 2.0[a]	$_{1}$10.81 ± 2.58[a]	$_{2}$15.04 ± 1.73[a]
	96	$_{1}$7.16 ± 2.51[a]	$_{1}$7.05 ± 3.28[a]	$_{1}$5.92 ± 0.38[a]
Hyaline cells (%)	0*	$_{12}$85.79 ± 0.73[a]	$_{12}$77.97 ± 2.48[ab]	$_{1}$69.96 ± 4.37[b]
	24	$_{2}$91.66 ± 2.70[a]	$_{1}$73.08 ± 0.25[b]	$_{2}$81.76 ± 3.90[b]
	48	$_{1}$82.4 ± 1.68[a]	$_{2}$78.4 ± 1.94[ab]	$_{1}$70.90 ± 1.13[b]
	96	$_{12}$89.1 ± 2.26[a]	$_{2}$78.75 ± 0.74[b]	$_{2}$82.37 ± 2.16[b]
Vibrio rank	0*	$_{1}$1.00 ± 0.00[a]	$_{1}$1.00 ± 0.00[a]	$_{1}$1.00 ± 0.00[a]
	24	$_{2}$2.33 ± 0.33[a]	$_{1}$1.33 ± 0.33[a]	$_{1}$1.00 ± 0.00[a]
	48	$_{3}$10.00 ± 0.00[a]	$_{1}$1.00 ± 0.00[b]	$_{1}$1.67 ± 0.67[b]
Haemolymph clotting time (s)	0*	$_{1}$74.00 ± 3.51[a]	$_{1}$72.33 ± 21.53[a]	$_{1}$72.67 ± 10.27[a]
	24	$_{1}$66.33 ± 5.17[a]	$_{1}$57.33 ± 6.06[a]	$_{1}$59.67 ± 1.76[a]
	48	$_{1}$72.67 ± 9.06[a]	$_{1}$58.67 ± 10.68[a]	$_{1}$44.67 ± 4.98[a]
	96	$_{1}$65.33 ± 13.2[a]	$_{1}$66.33 ± 2.96[a]	$_{1}$53.33 ± 3.28[a]
NRRT (min)	0*	> 120	> 120	> 120
	24	$_{1}$23.33 ± 3.33[a]	$_{1}$26.67 ± 3.33[a]	$_{1}$26.67 ± 3.33[a]
	48	$_{1}$15.00 ± 0.00[a]	$_{2}$55.00 ± 7.64[b]	$_{2}$53.33 ± 1.67[b]
	96	$_{2}$40.00 ± 5.00[a]	$_{3}$111.67 ± 4.4[b]	$_{3}$93.33 ± 8.82[b]

Different superscript letters in the same row indicate significantly different means at P < 0.05. Means for each immune parameter in any one column not preceded by the same subscript numbers are significantly different at P < 0.05. 0 is the time before infection.*

Bacteraemia

Vibrio spp. in the haemolymph of marron fed the control diets significantly increased (P < 0.05) after 24 and 48 h of *V. mimicus* injection while there was no significant difference in haemolymph *Vibrio* spp. of marron fed the Bio-Mos diets before and after the injection. The significant difference (P < 0.05) in the *Vibrio* spp. numbers in the haemolymph of marron fed the control and the Bio-Mos diets was observed after 48 h of infection (Table 6.2).

Neutral red retention time

Before *V. mimicus* injection, NRR times of marron lysosomal membranes in all treatment groups were more than 120 min. Twenty four h after the injection, the mean NRR times of lysosomal membranes in all the injected marron sharply decreased to less than 30 min. At 48 h after the injection, while the mean NRR time of the marron fed the control diet still declined, the lysosomal membrane stability of the marron fed the Bio-Mos started recovering, especially the mean NRR time of the marron fed the Bio-Mos diets increased significantly (P < 0.05). At 96 h after the injection, the lysosomal membrane stability of the marron fed the control diet began to recover but the mean NRR time of the lysosomal membrane was significantly shorter (P < 0.05) than the mean NRR time of the marron fed the Bio-Mos diets. Lysosomal membrane stability of the marron fed the 0.2% Bio-Mos diet recovered significantly faster (P < 0.05) than that of the marron fed the control diet (Table 6.2).

6.3.2 Trial 2 - Exposure to air during simulated transport

Survival

After 36 h of simulated transport, no mortalities were observed in any group.

Total and differential haemocyte count

THCs of marron fed control diets significantly declined (P < 0.05) after 36 h of transportation while it remained unchanged in the marron fed Bio-Mos diets. Before subjected to simulated transportation stress, granular cells proportion was lower in the marron fed the control than the Bio-Mos diets and it did not change after the exposure to the transport stressor in marron fed any of the diets (Table 6.3).

Table 6.3: Mean ± SE immune parameters of marron challenged after subjected to simulated live transportation

Parameters	Time	Test diets		
		Control	0.2% Bio-Mos	0.4% Bio-Mos
THCs (million cells/mL)	BF	$_1$3.39 ± 0.16^a	$_1$4.43 ± 0.18^b	$_1$4.06 ± 0.24^b
	AT	$_2$2.56 ± 0.24^a	$_1$3.57 ± 0.42^b	$_1$4.11 ± 0.42^b
Granular cells (%)	BF	$_1$16.46 ± 1.25^a	$_1$22.19 ± 0.53^b	$_1$23.41 ± 0.25^b
	AT	$_1$15.79 ± 0.98^a	$_1$21.12 ± 0.34^b	$_1$22.27 ± 1.42^b
Haemolymph clotting time (min)	BF	$_1$46.00 ± 2.08^a	$_1$36.00 ± 1.73^b	$_1$38.00 ± 1.15^b
	AT	$_2$69.00 ± 1.00^a	$_2$43.00 ± 1.66^b	$_1$35.33 ± 3.18^c

Different superscript letters in the same row indicate significantly different means at P < 0.05. Means for each immune parameter in any one column not preceded by the same subscript numbers are significantly different at P < 0.05. BF: before and AT: after transportation stress.

Haemolymph clotting time

The haemolymph clotting time of marron fed the control and 0.2% Bio-Mos diets significantly reduced after the simulated transportation and it was unchanged in the marron fed 0.4% Bio-Mos diet (Table 6.3).

6.3.3 Trial 3 - Exposed to NH3

Survival

After 7 days of exposure to NH_3, survival of marron fed 0.4% Bio-Mos diet was significantly higher (P< 0.05) than that of marron fed the control diets (Figure 6.2).

Total and differential haemocyte count

THCs and DHC of marron fed the control and 0.4% Bio-Mos diets were not significantly different before exposure to NH_3. After 1, 3 and 7 days, exposure, THCs of marron fed 0.4% Bio-Mos diet was higher (P < 0.05) than marron fed the control diets whereas there was no difference (P > 0.05) among the proportions of granular cells, semi-granular and hyaline cells when marron were fed different diets. THCs of marron fed either the control or Bio-Mos supplemented diets significantly reduced after 1, 3 and 7 days of exposure while the proportion of different cells

remained unchanged until the day 7 when the proportion of granular cells decreased and the proportion of hyaline cells increased (Table 6.4).
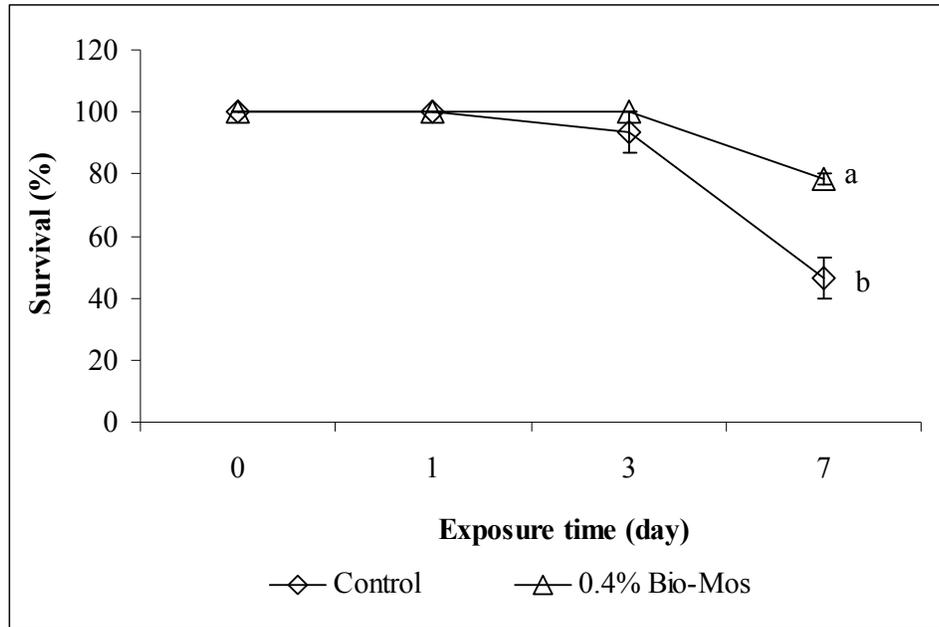
Figure 6.2: Mean ± SE survival of marron challenged with NH3.
Different letters indicate significantly different means at each time at P < 0.05 (n = 21).

Haemolymph clotting time

When exposed to NH_3, haemolymph clotting time of marron fed either the control or 0.4% Bio-Mos diets significantly increased. After 3 days of exposure, haemolymph clotting time of marron fed 0.4% Bio-Mos diet was shorter than marron fed the control diet (Table 6.4).

Bacteraemia

Bacteraemia of marron fed control diets significantly increased (P < 0.05) after 1 day exposure to NH_3 whereas there was no change in bacteraemia of marron fed 0.4% Bio-Mos diets during 7 day of NH_3 exposure (Table 6.4).

Table 6.4: Mean ± SE immune parameters of marron exposed to NH3

Parameters	Exposure time(day)	Diets	
		Control	0.4% Bio-Mos
THCs (million cells/mL)	0[*]	$_1 9.87 \pm 0.91^a$	$_1 11.10 \pm 1.74^a$
	1	$_2 4.42 \pm 0.42^a$	$_2 6.27 \pm 0.20^b$
	3	$_3 1.67 \pm 0.24^a$	$_3 2.87 \pm 0.20^b$
	7	$_3 1.11 \pm 0.06^a$	$_3 2.22 \pm 0.03^b$
Granular cells (%)	0[*]	$_1 21.27 \pm 1.03^a$	$_1 25.66 \pm 3.93^a$
	1	$_1 21.59 \pm 0.87^a$	$_1 20.54 \pm 2.65^a$
	3	$_1 24.91 \pm 6.62^a$	$_1 29.13 \pm 5.42^a$
	7	$_2 13.60 \pm 2.75^a$	$_2 12.90 \pm 0.43^a$
Semi-granular cells (%)	0[*]	$_1 0.39 \pm 0.39^a$	$_1 0.42 \pm 0.20^a$
	1	$_1 0.38 \pm 0.11^a$	$_1 0.47 \pm 0.25^a$
	3	$_1 0.58 \pm 0.35^a$	$_1 0.47 \pm 0.36^a$
	7	$_2 2.06 \pm 0.47^a$	$_1 0.81 \pm 0.32^a$
Hyaline cells (%)	0[*]	$_1 78.33 \pm 0.64^a$	$_1 73.93 \pm 4.04^a$
	1	$_1 78.02 \pm 0.94^a$	$_1 71.98 \pm 2.53^a$
	3	$_1 74.50 \pm 6.33^a$	$_1 70.38 \pm 5.23^a$
	7	$_1 84.33 \pm 2.91^a$	$_2 86.28 \pm 0.75^a$
Vibrio rank	0[*]	$_1 3.00 \pm 1.00^a$	$_1 2.33 \pm 0.88^a$
	1	$_2 10.00 \pm 1.52^a$	$_1 2.33 \pm 1.33^b$
	3	$_2 7.66 \pm 0.66^a$	$_1 4.00 \pm 1.52^a$
	7	$_2 10.33 \pm 1.45^a$	$_1 6.00 \pm 1.00^b$
Haemolymph clotting time (s)	0[*]	$_1 41.00 \pm 9.02^a$	$^1 39.17 \pm 4.47^a$
	1	$_{12} 80.50 \pm 11.62^a$	$_{12} 55.17 \pm 7.33^a$
	3	$_2 86.33 \pm 6.87^a$	$_{12} 61.66 \pm 2.31^b$
	7	$_2 101.17 \pm 24.31^a$	$_2 72.50 \pm 7.40^a$

*Different superscript letters in the same row indicate significantly different means at P < 0.05. Means for each immune parameter in any one column not preceded by the same subscript numbers are significantly different at P < 0.05. 0 *is the time before exposure.*

6.4 DISCUSSION

The results of current study indicate that, application of dietary Bio-Mos can significantly improve the marron's ability to resist stressors such as bacterial infection, exposure to NH_3 and live transport indicated by the higher survival and positive immune responses. Similar effects have been described for seabass (Torrecillas *et al.* 2007), cobia larvae (Salze *et al.* 2008a) when they were fed dietary Bio-Mos. Improved survival of marron when exposed to stressors is a direct consequence of enhanced health status and positive immune responses of marron as it has been shown in other aquaculture species (Welker *et al.* 2007). The similar outcome is also suggested by improvement in the health of digestive system of animals fed Bio-Mos (Genc *et al.* 2007a; Staykov *et al.* 2007; Salze *et al.* 2008a). Our unpublished data have further confirmed that better condition of digestive system, improved immune indicators for better health status of the marron is achieved when fed 0.2% and 0.4% Bio-Mos supplemented diets. Bio-Mos supplementation in the diets has also been previously reported to promote resistance to bacteria infection and stressor of common carp (*Cyprinus carpio*) (Staykov 2004), rainbow trout *(Salmo gairdneri irideus* G.*)* (Staykov *et al.* 2006), channel catfish (*Ictalurus punctatus*) (Welker *et al.* 2007), rainbow trout (*Oncorhynchus mykiss*) (Staykov *et al.* 2007) and sea bass (*Dicentrarchus labrax*) (Torrecillas *et al.* 2007).

Total number of haemocytes, proportion of haemocyte types, bacteraemia and haemolymph clotting time have been used as indicators of health of crustaceans (Durliat and Vranckx 1983; Evans *et al.* 1992; Smith *et al.* 1995; Jussila 1997a; Jussila *et al.* 1997; Evans *et al.* 1999; Jussila *et al.* 1999; Fotedar *et al.* 2001; Jussila *et al.* 2001; Fotedar *et al.* 2006). Recently, neutral red retention (NRR) assay has also been used to assess the immune status of crustaceans by measuring the dye retention time of lysosomal membranes (Yao *et al.* 2008). Neutral red is a cytotoxical weak base (Lowe and Pipe 1994) and has been successfully used in the NRR assay as a biomarker to test the stability of lysosomal membranes in aquatic animal (Lowe *et al.* 1995; Hauton *et al.* 1998, 2001; Nicholson 2003; Hauton and Smith 2004; Song *et al.* 2007).

In decapod crustaceans, life cycle, food intake, disease outbreaks, pollutants and environmental stress affect the circulating haemocyte count of crustaceans both in

quantity and quality (Persson *et al.* 1987; Le Moullac and Haffner 2000; Liu and Chen 2004). In the present study, all marron tested were in the intermolt stage, thus considered to have similar physiological and immunological status. Our unpublished data have shown that Bio-Mos supplemented in diets has improved the immune response of marron when it was applied for the duration of 112 days. In addition, dietary Bio-Mos has been proved to have effects on immune indicators of rainbow trout (*Oncorhynchus mykiss*) after 42 days (Staykov *et al.* 2007) and sea bass (*Dicentrarchus labrax*) after 72 days (Torrecillas *et al.* 2007) application. The changes in the THCs and DHCs of the marron after exposure to stressors in the current trials imply that bacterial infection, NH_3 and air exposure during simulated live transportation could influence the circulating haemocyte counts of marron. After the *V. mimicus* injection, THCs and proportion of granular cells of marron fed the control diet dropped significantly. The same observations have been reported in black tiger prawn (*Penaeus monodon*) (van de Braak *et al.* 2002) Chinese shrimp (*Fenneropenaeus chinensis*) (Yao *et al.* 2008)   when they are injected with *V. anguillarum*. The decline in THC after *V. mimicus* injection could be associated with haemolymph locomotion to the injection site and haemocyte lysis as a result of defence activities (Omori *et al.* 1989; van de Braak *et al.* 2002). However, THC of marron fed the Bio-Mos diets did not decrease after the *Vibrio* injection. Sequeira et al. (1996) found that *P. japonicus* haemocytes have the capacity to proliferate, and the proliferation rate can be increased three times when the shrimp are injected with the immunostimulant lipopolysaccharide. Our previous study (Sang *et al.* 2010a) also showed that THC in marron increased significantly after feeding with Bio-Mos. In the present study, the incorporation of Bio-Mos in diets may stimulate and increase the proliferation rate of marron haemocytes to compensate for the loss of haemocytes due to the *V. mimicus* infection, resulting in the THC proportions to remain constant.

The present study also reveals that the THCs of marron were lower when marron were exposed to NH_3 at the concentration of 1.37 mg L^{-1}. Circulating haemocytes of several species of crustacean are affected by extrinsic factors like temperature, pH, salinity, dissolved oxygen and ammonia (Cheng and Chen 2001; Cheng *et al.* 2002; Liu *et al.* 2004). The decrease in THC is frequently reported in crustacean exposed to stress condition. *Litopenaeus stylirostris* following exposure to ammonia at 3.0 mg L^{-1} decreased its THC by 30% (Le Moullac and Haffner 2000), Chinese mitten-handed

crab (*Eriocheir sinensis*) reduced the THC indicating suppressed resistance when exposure to ammonia (Hong *et al.* 2007). These reductions in THC relates to the accumulation of haemolymph ammonia and urea, and causes the catabolism of haemocyanin and protein to amino acids (Chen and Cheng 1993). However, the marron fed Bio-Mos diets was healthier shown by lowered reduction rate (80%) of THC comparing to the marron fed the control diet (88.75%) after 7 days of exposure to the stressor.

Air-exposure has been demonstrated to reduce the health status of rock lobster (*Panulirus cygnus*) (Fotedar *et al.* 2001). The trial 2 showed that the marron fed Bio-Mos diets are healthier than marron not fed Bio-Mos indicated by the higher THC after 36 hour of simulated live transport. It is also inferred from current results that the significant reduction in the THCs of marron fed the diets without Bio-Mos supplementation when challenged with bacteria and NH_3 exposure has negatively affected the ability to combat the bacteria infection (Johansson *et al.* 2000; Misra *et al.* 2004), thus, resulting in the higher bacteraemia.

Crustacean haemolymph clotting involves aggregation of the circulating haemocytes and the gelation of the plasma by factors released from the haemocytes (Durliat 1985). Haemolymph clotting time has been reported to be changed by the presence of bacteria (Newman and Feng 1982; Aono *et al.* 1993), environmental pollutants (Smith and Johnston 1992) or holding period in storage tanks and live transportation (Fotedar *et al.* 2006). In the current experiment, haemolymph clotting time of marron was not affected by *V. mimicus* injection. Although Durliat and Vranckx (1983) found a relationship between haemolymph clotting time and number of haemocytes in lobsters, the authors stated that haemocyte numbers could not be explained by the changes in haemolymph clotting time. To date, little is known about the mechanism by which the changes in crustacean haemolymph clotting time are induced. However, challenging with NH_3 and air exposure has affected the haemolymph clotting time of marron. Thus, further research needs to be conducted to define the mechanism in which pollutants, nitrogen metabolites and air exposure stressors affect the clotting time of marron. Marron fed the Bio-Mos diet showed the shortest clotting time after 3 days of NH_3 exposure and 36 h of air-exposure indicating the ability of Bio-Mos to

improve health status initial immune defense activity of marron under these stress conditions.

Neutral red is a cytotoxical weak base (Lowe and Pipe 1994) and has been successfully used in the NRR assay as a sensitive indicator of lipid membrane integrity in cases of bacterial infection and immunostimulants toxicity in shellfish (Lowe *et al.* 1995; Hauton *et al.* 1998, 2001; Nicholson 2003; Hauton and Smith 2004; Song *et al.* 2007). Lysosomes found within the semi-granular and granular haemocytes are polymorphic, hydrolytic enzyme-containing organelles that have the capacity to take up and retain neutral red dye. The dye disappears when lysosomal membranes are damaged (Lowe and Pipe 1994) and unhealthy cells lose neutral red dye at a faster rate than healthy cells due to a decrease in cellular membrane stability (Borenfreund and Puerner 1985; Harding *et al.* 2004). In this study, NRR assay was successfully developed using detection of lysosomes of haemocytes in the marron. Without any stimulation, the NRR time of marron lysosomal membrane in the current experiment was more than 120 min.

The injection of *V. mimicus* has altered the stability of the lysosomal membrane of marron as it showed a rapid decline in NRR time. Similarly, NRR time of Chinese shrimp was significantly shortened after *Vibrio* injection (Yao *et al.* 2008). In marron fed the diet without Bio-Mos, the decrease in lysosomal membrane stability is related with the decrease in THC, granular cell count (GC) and semi-granular cell count (SGC) after the injection. The similar phenomenon was found in freshwater prawn (*Macrobrachium rosenbergii*) (Sahoo *et al.* 2007), oysters (*Ostrea edulis* and *Crassostrea gigas*) and the scallop (*Pecten maxima*). Hauton *et al.* (2001) suggesting that GC and SGC might degranulate at first, then following by the lysosome lyses to defense against pathogen in marron initial acute infection (Yao *et al.* 2008). After 48 h of the injection, the lysosomal membrane stability recovery was significantly faster in marron fed the Bio-Mos diets than in those fed the control diet, indicating that Bio-Mos as supplementation in diets could have improved the resistance of marron against *V. mimicus* by fastening the recover rate of lysosomal membrane stability.

In conclusion, immunological responses to dietary Bio-Mos supplementation have shown supplementation of Bio-Mos improves the survival of the marron, improves

the health status and increases marron's defense ability to bacterial infection and other stress conditions. Further research needs to be conducted to clarify the mechanisms of Bio-Mos action(s) in the health and immunity improvements of marron.

CHAPTER 7: EFFECTS OF DIETARY BETA - 1,3 - GLUCAN ON THE GROWTH, SURVIVAL, PHYSIOLOGICAL AND IMMUNE RESPONSE OF MARRON, *CHERAX TENUIMANUS* (SMITH, 1912)

7.1 INTRODUCTION

Microbial disease is a major threat to the sustainability of aquaculture (Bachere 2003). For microbial pathogen resistance, invertebrates are entirely dependent on non-specific immune mechanisms to cope with infection as they lack the specific immunological "memory" that is found in fish and warm-blooded animal (Soltanian *et al.* 2007). The use of specific biological compounds (immunostimulants) that enhance immune responses of the target organisms, rendering animals more resistant to disease may be an excellent preventive tool against infections by pathogenic organisms (Anderson 1992).

The distribution of marron (*Cherax tenuimanus*) in Western Australia has been extended as far east as Esperance and as far north as Geraldton and global interest in marron farming has led to the species being introduced into South Africa, Zimbabwe, Japan, USA, China and the Caribbean as well as several Australian states (Morrissy *et al.* 1990). Like all freshwater crayfish, marron can have several small epibionts attached to their exoskeleton and gills. Two such epibionts are *Epistylis* and *Temnocephala*. These are symptomatic of poor water quality and result in poor growth rates, particularly in unaerated ponds containing excessive organic matter (Morrissy *et al.* 1990). This infection reduced appeal of marron to consumer. Although there is no report on the losses in marron aquaculture caused by bacterial infection, the threat to consumers of organism infected marron needs to be considered.

Beta - 1,3 - glucan, soluble carbohydrates from the cell walls of yeast *Saccharomyces cerevisiae,* is known to have a potent stimulatory effect on the immune system of mammals, fish and crustaceans. In aquaculture, β- 1,3 glucan have successfully been used to enhance the resistance of fish and crustacean against bacterial or viral infection (Sakai 1999). In culture of crustaceans, the addition of beta glucan through diet significantly enhanced the survival and the resistance of postlarvae; juveniles

(Chang *et al.* 1999; Chang *et al.* 2003) and adult of *Penaeus monodon* (Chang *et al.* 2000) to *Vibrio damsela*, *V. harveyi* and white spot syndrome virus infection. Survival and the responses against vibriosis infection of *Macrobrachium rosenbergii* postlarvae are also improved by applying β- 1,3 – glucan (Misra *et al.* 2004). However, the effect of this compound on the growth performance and immune response of freshwater crayfish have not been investigated and there is a need to verify the dosage rate and application strategy for freshwater crayfish for the most effective application of β- 1,3 – glucan. In addition to traditional performance indicators of feed additives such as growth and survival, measures of crustacean health can include physiological parameters such as organosomatic indices, moisture content and osmoregulartory capacity (Sang and Fotedar 2004). Moreover, immune– physiological parameters such as total number of haemocytes (THC), proportion of granular cells, bacteraemia and haemolymph clotting time have also been used as indicators of immunological and health status of crustacean (Smith *et al.* 1995; Jussila *et al.* 1999; Fotedar *et al.* 2001; Fotedar *et al.* 2006; Sang *et al.* 2009). The aim of this experiment was to determine the suitable levels of dietary β- 1,3 – glucan by investigating the effects of dietary supplementation of various levels of β- 1,3 – glucan on the survival, growth, physiological and immunological parameters of marron.

7.2 MATERIALS AND METHODS

7.2.1 Test diets

Beta glucan (BG) (Beta-Mune[TM] brand, 100% German long chain Beta -1,3 – D - Glucan) was tested at five supplemented level of 0.08% (D2), 0.1% (D3), 0.2% (D4), 0.4% (D5) and 0.8% (D6) against a control diet (D1). All feed ingredients and supplements used in this trial were supplied by Specialty Feeds Pty Ltd, Western Australia. Proximate compositions of the ingredients and supplements were used as a basis to formulate the all diets using the software FeedLIVE version 1.52 (Table 7.1).

7.2.2 Experiment design

Juvenile marron (0.47 ± 0.02 g total weight and 7.47 ± 0.25 mm carapace length) were supplied by Aquatic Resource Management Pty Ltd, Western Australia and transported to the Curtin Aquatic Research Laboratory (CARL). The marron were placed in the tanks provided with aerated, recirculating filtered freshwater and

acclimated to the culture conditions for 2 weeks. During the acclimation period, the marron were fed with a commercial diet supplied by Enviroplus, Australia (26% protein, 47-50% carbohydrate, 9% fats and 8.9% ash) at the rate of 3% body weight per 2 days.

Table 7.1: Test diet formulations for marron

Ingredients	D1 (%)	D2 (%)	D3 (%)	D4 (%)	D5 (%)	D6 (%)
Fish oil	3.2	3.2	3.2	3.23	3.23	3.23
Soybean meal	10.15	10.15	10.14	10.14	10.14	10.11
Fishmeal	33.78	33.8	33.83	33.83	33.86	34.01
Wheat flour	49.35	49.25	49.21	49.08	48.85	48.33
Ascorbic acid	0.05	0.05	0.05	0.05	0.05	0.05
Betaine	1.2	1.2	1.2	1.2	1.2	1.2
Calcium ascobate	0.02	0.02	0.02	0.02	0.02	0.02
Premix	0.15	0.15	0.15	0.15	0.15	0.15
Cholesterol	0.25	0.25	0.25	0.25	0.25	0.25
Wheat starch	1.85	1.85	1.85	1.85	1.85	1.85
β - 1,3 - D - glucan	0	0.08	0.1	0.2	0.4	0.8
Total	100	100	100	100	100	100

Plastic cylindrical tanks (800 mm diameter, 500 mm high, 250 L capacity) were used for the experiment. Sufficient PVC pipes and oyster net of appropriate sizes were placed in each tank to provide shelters for the marron. Each tank was provided with a biological filtration recirculating water system. The water in the system was filtered through both mechanical and biological filtration at a rate of approximately 3L min^{-1}.

Eighteen culture tanks were used for the trial which lasted for 12 weeks. The marron from the acclimation tanks were randomly distributed among the culture tanks at a density of 18 marron per tank. Each random block of three tanks was supplied one of the above diets so that each diet was represented in three replicates. The formulated diets were provided at the rate of 5 -6 % body weight every 2 days in all tanks. Uneaten food and faeces was siphoned out prior to feeding and sufficient water was added to maintain 200 L in each tank. Water quality parameters including pH, total

ammonium, nitrite and nitrate were monitored weekly using pH meter (WP-80, TPS Pty Ltd, Brisbane) and chemical test kits (Aquarium Pharmaceuticals, Inc). Surviving marron were counted every 2 weeks and the weight of marron was measured to two decimal places using an electronic balance (SHIMADZU AW 220) every 4 weeks.

7.2.3 Data collection

The survival in all tanks was determined using the following formula: survival rate (%): $S = 100 \times (n_t/n_o)$, where: S is the survival rate; n_t is the number of marron at time t and n_o is the number of marron at the commencement. Yield was measured as the total weight of all marron in each tank.

Organosomatic indices analysis: The organosomatic indices of the marron including hepatosomatic index (HSIwet), wet tail muscle index (TMIwet), hepatopancreas moisture content (HM%) and tail muscle moisture content (TM%), dry hepatosomatic index (HSIdry) and dry tail muscle index (TMIdry) were measured by established method (Sang and Fotedar 2004) as described in the chapter 3.

The osmoregulatory capacity of the marron was determined by the method of (Sang and Fotedar 2004) as modified in the chapter 3. Total (THC), granular cells (GCs) and bacteraemia were conducted as per the established procedure for Western rock lobster (Fotedar *et al.* 2001) as described in the chapter 3.

7.2.4 Data analysis

All statistical analysis were performed using SPSS version 15. Results were presented as means ± SE (standard error). ANOVA (analysis of variance) and LSD (least significant difference) post hoc tests were used to determine significant differences between growth, survival, physiological and immunological parameters of the marron fed different diets. Results were judged as significant at $P < 0.05$.

7.3 RESULTS

7.3.1 Growth and survival

After 84 days of culture, survival of marron was significantly different when fed with D2, D3, D4 and D5 diets compared to D1 (Figure 7.1). There was no significant

difference in the final weight of the marron fed different diets. The yield differed in the marron fed D3, D4 and D5 diets when compared to D1 (Figure 7.2).

7.3.2 Physiological parameters

The HSIwet was not significantly different in the marron fed different diets. However, TMIwet was significantly different in the marron fed D3, D4, D5 and D6 compared to D1 and D2. There were no significant differences in the HM%, HSIdry and the osmoregulatory capacity of the marron fed the different diets. The TM% significantly differed in the marron fed D6 to D1 and the TMIdry of marron fed D6 was significantly different to that of marron fed either D1 or D2 (Table 7.2).

7.3.3 Immunological parameters

The THCs were differences (P < 0.05) in the marron fed D2, D3, D4 and D5 compared to D1 whereas the proportion of granular cells was significantly different in all marron fed beta glucan supplemented diets to the marron fed the control diet. The bacterium in the culture medium was not significantly different in all treatments. Bacteraemia showed significant differences when the marron were fed with D3, D4, D5 and D6 compared to the marron fed D1 and D2 (Table 7.3).
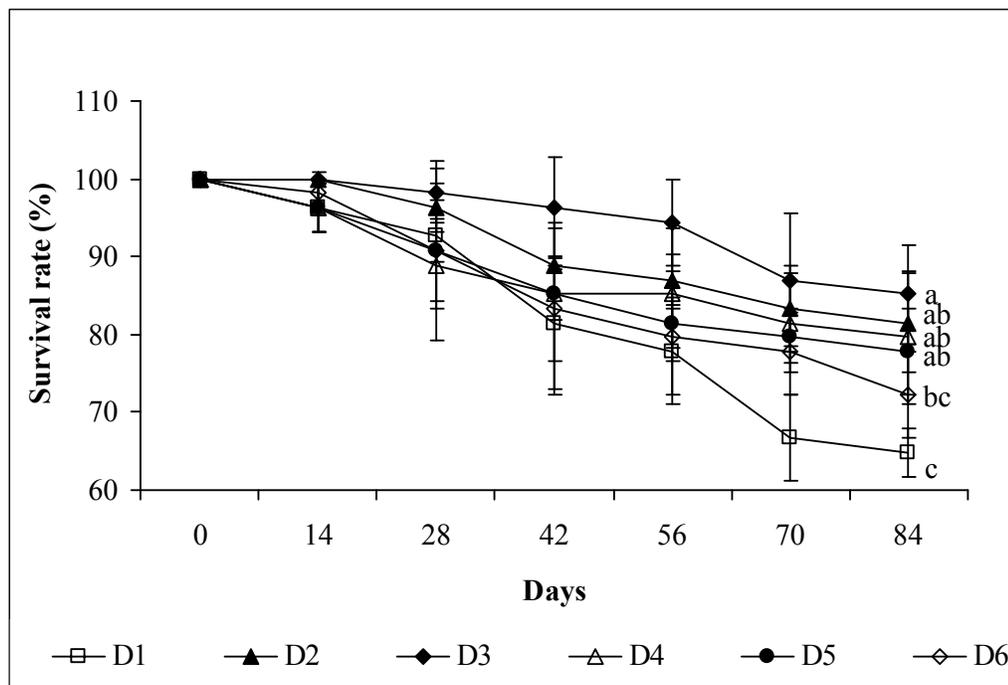
Figure 7.1: Survival of marron fed different beta glucan supplemented diets (mean ± S.E.).

Different letters denote significant differences (P < 0.05) in mean value.
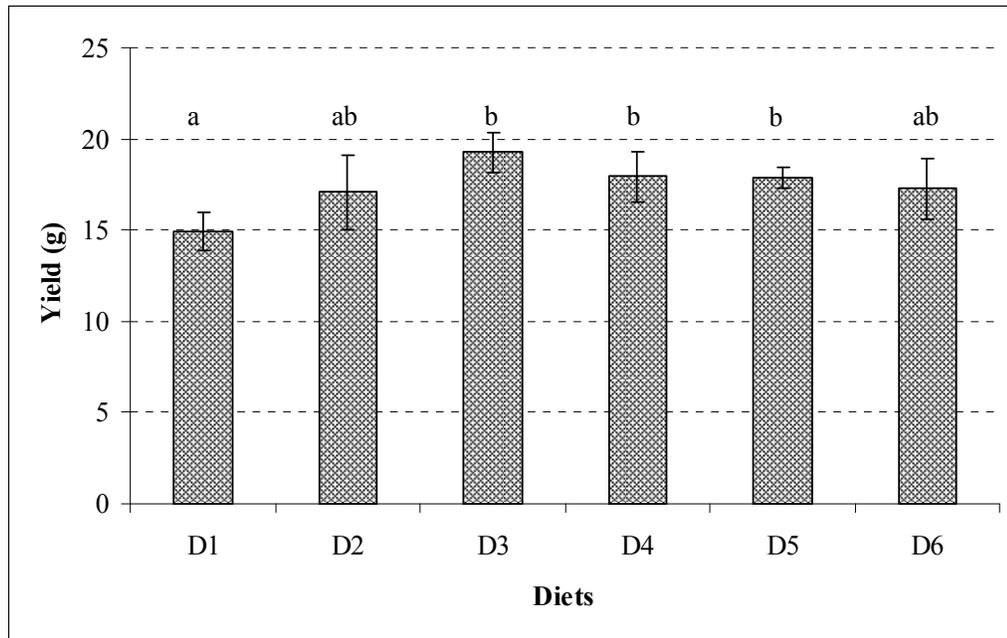
Figure 7.2: Yield of marron fed different beta glucan supplemented diets (mean ± S.E.).

Different letters denote significant differences (P < 0.05) in mean value.

Table 7.2: Physiological respond of marron fed different beta glucan supplemented diets

Parameters	D1	D2	D3	D4	D5	D6
HSIwet	7.17 ± 0.82^a	6.84 ± 0.31^a	6.35 ± 0.65^a	6.46 ± 0.45^a	6.48 ± 0.35^a	7.15 ± 0.43^a
TMIwet	22.91 ± 0.60^a	24.66 ± 1.28^{ab}	26.01 ± 0.56^b	25.75 ± 1.26^b	25.05 ± 0.65^b	27.07 ± 0.55^b
HM%	71.65 ± 1.53^a	73.23 ± 1.58^a	68.14 ± 1.69^a	66.37 ± 4.66^a	71.67 ± 3.95^a	65.66 ± 1.51^a
TM%	80.08 ± 0.89^{ab}	81.36 ± 0.71^a	81.41 ± 0.95^a	76.93 ± 3.95^{ab}	81.03 ± 0.99^{ab}	73.89 ± 3.92^b
HSIdry	2.06 ± 0.33^a	1.83 ± 0.15^a	2.04 ± 0.32^a	2.21 ± 0.44^a	1.86 ± 0.34^a	2.45 ± 0.12^a
TMIdry	4.56 ± 0.22^a	4.61 ± 0.39^a	4.84 ± 0.34^{ab}	6.04 ± 1.33^{ab}	4.75 ± 0.19^{ab}	7.11 ± 1.21^b
OC	578.20 ± 71.17^a	539.57 ± 68.35^a	742.33 ± 71.82^a	597.33 ± 130.90^a	785.03 ± 115.73^a	523.67 ± 88.73^a

Data in the same row having different superscript letters (a, b, c…) are significantly different at P < 0.05.

Table 7.3: Immunological parameters (mean ± S.E.) of marron fed different beta glucan supplemented diets

Parameters	D1	D2	D3	D4	D5	D6
THC (Million cells/mL)	2.87 ± 1.27^a	8.49 ± 1.08^b	7.11 ± 7.76^b	7.02 ± 2.25^b	8.58 ± 2.51^b	5.53 ± 2.83^{ab}
GCs (%)	11.28 ± 0.55^a	16.79 ± 1.60^b	16.80 ± 3.40^b	16.83 ± 1.83^b	17.69 ± 3.73^b	17.07 ± 4.29^b
Bacteraemia rank	10.33 ± 4.61^a	9.33 ± 3.51^a	1.00 ± 0.00^b	3.67 ± 2.30^b	1.00 ± 0.00^b	2.67 ± 2.08^b

Data in the same row having different superscript letters (a, b, c…) are significantly different at P < 0.05.

7.4 DISCUSSION

This study is the first attempt to investigate the effects of beta glucan on the growth, survival, physiological and the immune response of marron, *Cherax tenuimanus*. Results of this study indicate that the inclusion of beta glucan in the diet can enhance the performance and the immune response of marron. The survival, growth and yield are the main parameters for the success of any commercial aquaculture farm. The survivals of the marron in all of the experimental tanks were more than 65% at the initial stocking biomass of about 16.83 gm^{-2}. This rate was much higher when compared with the earthen pond study (Fotedar 2004), which had 13.82 – 34.86% survival and a lower density (4.01 ± 0.28 gm^{-2}). The survival of marron fed the diets without beta glucan supplemented in this experiment was the same and the survival of marron fed the beta glucan supplemented diets was higher than survival of marron reared in an intensive system (Jussila and Evans 1996a). The higher survival of marron fed the beta glucan supplemented diets indicates that beta glucan has improved the survival of marron. Although, beta glucan did not alter the growth performance of marron, some of physiological parameters of marron were positively affected by inclusion of beta glucan in their diets. The higher yield of the marron fed the 0.1% and 0.2% beta glucan supplemented diets was the consequence of higher survival when marron were fed with those diets. These results show that a higher harvest rate of marron is possible at the beta glucan supplementation of 0.1% to 0.2% in the diets.

The significant difference in the immune parameters of marron fed the beta glucan diets compared to the control indicates that the inclusion of beta glucan into the diets has an effect on the immunity of marron. The higher total haemocyte count and the proportion of the granular cells of marron fed the beta glucan supplemented diets compared to the control in the present study indicate that these marron were healthier than the marron fed no beta glucan. In crustaceans, haemocyte numbers or the proportion of different cell types is affected by factors such as sex, growth, life-stage, molt cycle, nutritional status, stressors, and bacteria infection (Fotedar *et al.* 2001). This study shows that the beta glucan can also alter the haemocyte counts and their proportion in marron. The higher number of haemocytes from marron fed with beta glucan supplemented diets compared to the control diet resulted in higher number of semi-granular and granular cells in the haemolymph which strengthens the non-

specific defense system of marron to bacteria and pathogens. This could result in higher survival rate of the marron.

Although the numbers of bacteria in the culture medium of all tanks were not significantly different, the bacteraemia of marron fed the beta glucan supplemented diets at 0.1% were lower than others. This is because of the role of beta glucan in activation of the semi-granular and granular cells functions (Smith *et al.* 2003b). The lower in bacteraemia of marron fed beta glucan supplemented diets suggests that beta glucan have induced the degranulation response to bacteria of the semi-granular cells and granular cells.

In conclusion, the present study suggest that dietary supplementation of beta glucan can result in improved performance of marron in term of survival and yield; increased total haemocyte count and granular cells and reduced bacteraemia levels. The results suggested that 0.1 to 0.2% of beta glucan should be supplemented in the diets of marron.

CHAPTER 8: EFFECTS OF MANNAN OLIGOSACCHARIDE DIETARY SUPPLEMENTATION ON PERFORMANCES OF THE TROPICAL SPINY LOBSTER JUVENILE (*PANULIRUS ORNATUS*, FABRICIUS 1798)

8.1 INTRODUCTION

For the past decades, antibiotics have been practicing at the sub-therapeutic concentrations in the animal feed because of their possible effects on, survival, feed utilization and weight gain (Rosen 1996) which are the most important concerns in aquaculture production. However, the stimulation of antibiotics on the development of resistant bacteria in both animal and humans have been the subjects of controversy (Genc *et al.* 2007a). Restriction or ban of the antibiotic used as additives in fish and crustacean feed has prompted interest in developing the alternative strategies as health promoter and disease control. Thus, in the recent years, there has been increasing research on dietary supplementation in which various health promoting compounds have been studied (Gatlin *et al.* 2006). Those compounds can be classified as immunonutrients and immunostimulants with the differences between the two relates to their mechanisms of action. One group of immunostimulants showing beneficial effects in terrestrial and aquatic animals is referred as prebiotics (Gibson and Roberfroid 1995).

Among the most common prebiotics, mannan oligosaccharide (MOS) has been recently receiving incresing application in aquaculture. Since the first use of MOS in aquaculture, there has been increasing the number of studies demonstrating their ability to increase the survival, growth performance and control of the potential pathogens of fish and crustacean. This prebiotic has also been demonstrated to benefit the gut health by improved absorption and immune modulation in the target species. The effective mechanism of MOS results in higher performance in term of survival, growth and bacterial resistant ability of common carp (*Cyprinus carpio*), rainbow trout (*Salmo gairdneri irideus* G.) (Staykov *et al.* 2006), channel catfish (*Ictalurus punctatus*) (Welker *et al.* 2007), rainbow trout (*Oncorhynchus mykiss*) (Staykov *et al.* 2007). The profitable of MOS inclusion in the diet on performance of crustacean has also been proved for green tiger prawn (*Penaeus semisulcatus*) (Genc *et al.* 2007a) and recently, Sang *et al.* (2009) reported that dietary supplementation at

0.2 to 0.4% of MOS resulted in higher resistant ability to bacterial infection and stressors by improving the immune response of marron (*Cherax tenuimanus*).

The culture of tropical spiny lobster (*Panulirus ornatus*) is becoming an important aquaculture industry in the Asia-Pacific and Caribbean regions (Jeffs and Davis 2003; Tuan and Mao 2004). Annual production of tropical spiny lobster in Vietnam alone worth was US$90 millions at the farm gate in 2005 (Irvin and Williams 2007). The existing farming practices are based on rearing of the wild collected puerulii and early juveniles to the desirable size (approximately 15 g total weight) before the growth out phase. Exclusive feeding of the lobster on fresh fishery bycatch is unsustainable due to competition pressures cause by declining inshore fishery catches, poor food conversions and negative environmental impacts (Jeffs and Hooker 2000; Tuan *et al.* 2000; Jeffs and Davis 2003; Tuan and Mao 2004; Irvin and Williams 2007). High mortality was observed at seed rearing stages (the survival was only 43% for lobsters less than 0.5 g) (Williams 2007b) because of the high vulnerability of young lobster to the environmental stressors. One of the obstacles to sustainable aquaculture of rock lobster is lack of the suitable dietary supplementation that will enable seed to be reared to growth-out phase size with high survival and good health status. While there are considerable studies on nutrient requirement in order to develop suitable pellet as alternative to trash fish as food for commercial farming of rock lobster (Smith *et al.* 2003a; Smith *et al.* 2005; Barclay *et al.* 2006), the use of additives such as immunostimulants to benefit the lobster industry is poorly reported and there is no information available on the use of MOS to the lobster culture. Hence, the objectives of this study were to evaluate the effects of dietary inclusion of MOS on (1) survival and growth performance; (2) physiological status; (3) gut morphology and bacterial count (4) survival and immune response to bacterial infection of early juvenile tropical spiny lobster.

8.2 MATERIAL AND METHODS

8.2.1 Culture system

Twelve fiberglass rectangular blue color tanks (500 x 800 x 1000 mm, 400L capacity) were used as cultural units in the experiment. Sufficient dead corals and bricks with holes were placed in each tank to provide shelters for the lobster. Each tank was supplied with 300 l mechanical filtered seawater. The water in each tank was continuously aerated and independently filtered through a biological seawater filter system at a rate of approximately 5 L min^{-1}.

8.2.2 Experimental animals

Lobster juveniles were supplied by commercial lobster juvenile farm at Nha Trang bay, Vietnam (109°12'53.28"E, 12°17'20.70"N) and shipped to the Institute of Oceanography, Nha Trang Vietnam. 132 lobsters were randomly distributed into 12 culture tanks, so that each tank received 11 animals. The lobsters were then acclimated to the culture conditions for 1 week. During the acclimation period, the lobsters were fed with grinded trash fish twice daily at 0800 and 1700 h till satiation. Uneaten food and feces were siphoned out before next feeding.

8.2.3 Preparation of the test diet

Trash fish (fish and prawn at the ratio 2:1) from the local market was used as the basal food for lobster. Trash fish was washed and thoroughly mixed with 0.4% in weight of mannan oligosaccharide (MOS) (Bio-Mos, Alltech, USA). The mixed ingredient was ground using a grinder to obtain the pasty mix. The trash fish without MOS supplementation was also ground and used as control diet. The pasty mixes were packed and stored frozen. Before feeding, the foods was taken out from the freezer and defrosted in the room temperature for 1 h.

8.2.4 Experimental design

After acclimation, the lobsters (1.28 ± 0.01 g – mean ± S.E.) from different tanks were merged together and then randomly distributed to each tanks at the density of 10 animals per tank. Each experimental diet was randomly assigned to six tanks, giving six replicates per diet. The food was initially provided at the rate of around 30% body weight daily divided into two lots and then fed to lobsters at 0800 and 1700 h. Uneaten food and feces were siphoned before next lot of food was provided.

The amount of water lost during siphoning was added into each tank to retain the water level. The lobsters were reared for 8 weeks. Water quality parameter such as temperature, salinity and pH were monitored daily using thermometer, refractometer and pH meter, respectively. The number of lobster in each tank was checked every two weeks by removing the shelter and counting. The individual weight was measured to two decimal places using a balance SHIMADZU AW 220 (LabComerce Inc, USA) every 2 weeks. Physiological parameters, gut bacteria (total bacteria and *Vibrio* spp.) and morphology and immunological parameters (total haemocyte count, granular cell and bacteraemia) were measured at week 8 of the culture period.

At the end of the culture period, a bacteria challenge trial was conducted to evaluate the response of the lobster to bacteria infection. Stock solution of *Vibrio* spp. (isolated from milky diseased lobster) was obtained from the Research Institute for Aquaculture No.3 at Nha Trang, Vietnam. The concentration of stock solution was approximately 0.50×10^6 CFUmL^{-1}.

Bacterial challenge trial was initiated on day 60 of the feeding trial. Before the challenge trial, 18 lobster of each previous diet fed test were randomly distributed to three culture tank, giving three replicates per diet. The lobster were then injected through the base of the fifth thoracic leg with 20 μL bacteria stock solution. During the challenge trial, the lobster were fed with their original diets. Infected lobsters were monitored for survival, total haemocyte count, granular cell and bacteraemia after 1, 3 and days of injection.

8.2.5 Data collection

Growth and survival

The survival in each tank was measured using the following formula:

Survival (%): S = 100 x (nt/no), where: S is the survival rate; nt is the number of lobster at time t and no is the number of lobster at the commencement.

Growth rate was calculated and expressed as specific growth rates (SGR) and average weekly gain (AWG) according to the following equation: SGR(% day^{-1}) = 100 x (ln We – ln Ws)/d, AWG (g week^{-1}) = (Wf - Wo)/wk where Ws and We are the weights of the lobsters at the start and end of the growth period, respectively, and d and wk are the number of days and weeks, respectively, in the growth period.

Physiological condition parameters

The organosomatic indices of the lobster including wet hepatosomatic index (HSIwet), wet tail muscle index (TMIwet), hepatopancreas moisture content (HM%), tail muscle moisture content (TM%), dry hepatosomatic index (HSIdry) and dry tail muscle index (TMIdry) as the indicator of physiological condition were measured by applying the established methods (Sang and Fotedar 2004).

Immunological parameters

Total haemocyte count (THC), granular cell proportion (GC) and bacteraemia assessment were conducted as per the established procedure for rock lobster (Fotedar *et al.* 2001). The base of the fifth thoracic leg of each lobster from each culture tank was cleaned with 70% alcohol. A 0.2 mL aliquot of haemolymph was withdrawn into a 1-mL sterile syringe containing 0.2 mL of anticoagulant (1% glutaraldehyde in 0.2M sodium cacodylate) and dispensed into an Eppendorf tube kept on ice. The total haemocyte counts for the individual lobster were estimated with a haemocytometer (Neubauer, Germany) under 100-fold magnification, from the anticoagulant/haemolymph mixture. 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). For granular cell count, one drop of haemolymph/anticoagulant mixture was placed on a slide and smeared. The smear was air dried and fixed in 70% methanol for 10 min. Fixed smears were stained with May-Grunwald and Giemsa (10 min in each) (Bancroft and Stevens 1977) and mounted with coverslips. Approximately 200 cells were counted on each slide. The granular cells among the 200 cells were distinguished on the basis of the larger cell size, smaller pale nucleus, and larger number of eosinophillic granules in the cytoplasm, and their proportion was determined.

For bacteraemia assessment, a 0.3 mL aliquot of haemolymph was withdrawn into a sterile syringe. Five drops were quickly placed and smeared on a marine agar (MA) plate; the plate was carefully inverted before being kept in the incubator chamber at temperature of 25°C for 24 h. Colony-forming units (CFU) were counted for each drop and CFUmL^{-1} calculated for each sample on the basis of a total volume of 20 μL for each drop.

Gut bacteria and morphology

At the end of culture period, guts of 6 lobsters from each diet fed treatment were dissected out and weighed to four decimal places (Wgut) and placed in sterile test tube. Five mL of PBS (phosphate buffered saline) was added to the tube and homogenized using glass rod and homogenizer to obtain a solution. Several serial 10-fold (10x, 100x, 1000x, 10000x) dilutions were prepared using PBS solution. Aliquot of each diluted homogenized solution was incubated in MA plate (0.1 mL/plate, replicated in 3 plates for each dilution, for 24 h at 25°C in order to determine the total bacteria. Similarly, diluted solutions were incubated in thiosulfate citrate bile sucrose agar (TCBS) plates for 46 h to determine the total *Vibrio* spp. The plates having 20-200 colonies were counted for the bacterial count determination.

Histological analysis of lobster gut was performed following the method described by Genc et al. (2007a). Gut of the six lobsters from each diet fed treatment at the end of trial were dissected and fixed in 4% buffer formalin for 24 h. After dehydrating by passing the tissue through a series of alcohol solutions of 70, 85 and 98%, the samples were vacuumly embedded in paraffin. The histological sections (4 – 5 μm) were stained for general morphological purposes with hematoxylin and eosin (H&E). The samples were photographically analyzed and documented using the Olympus BX 50 microscope at 40x magnification. The ratio of internal and external perimeter of the gut was used as the indicator of the lobster gut condition (Sweetman and Davies 2007; Dimitroglou *et al.* 2008).

8.2.6 Data analysis

The data were statistically analysed using statistical analysis program SPSS for Windows version 15. Survival percentage data were normalized using an arcsine transformation before analysis. The normality of data were assessed by the Shapiro-Wilk test (Winer 1991) prior to analysis. The homogeneity of variance was assessed by Levene test (Winer 1991) prior to analysis. Normal data were subjected to independent sample T test for data of homogenous variance, and Tamhane's test (Winer 1991) for data of non-homogenous variance. Non-normal data were subjected to non-parametric testing using Kruskal-Wallis H test (Winer 1991). Differences were considered significant at the level of $P < 0.05$.

8.3 RESULTS

8.3.1 Water quality parameters

During the course of rearing period, the water quality parameters were similar in all tanks. Temperatures ranged from 26 to 28.5ºC, the range of the water salinity was 32.5 to 34 gL^{-1} and pH was from 7.01 to 7.94. There was no obvious effect of the MOS on the water quality in treatment group. Total ammonium, nitrate and nitrite were below the detection levels.

8.3.2 Survival and growth

Survival and growth parameters of the lobster fed two different diets are presented in Table 8.1. After one week of acclimation, three lobsters diet out of total 132 lobsters. After 28 day of feeding two different diets, survival was significantly higher (T-test, $P < 0.05$) in lobster fed 0.4% Bio-Mos supplemented diet compared to control diet. This trend remained unchanged to the end of the culture period (56 days rearing). The lobster weights fed the two different diets were different after 14 days of culture (T-test, $P < 0.05$) and the difference in weight of the lobster fed two diets increased till the end of rearing period.

The lobster fed two diets grew from 1.28 g to more than 2.3 g during initial 8 weeks. The total weight of the surviving individuals was higher for lobster fed diet with Bio-Mos supplementation. The significant difference in weight (T – test, $P < 0.05$) of the lobster between two dietary groups was evident from the 14 days of culture. After 8 weeks, the weight of lobster was 21.84 % higher when the lobster were fed with the 0.4% Bio-Mos supplemented diet. The lobster fed 0.4% Bio-Mos supplemented diet also resulted in the higher SGR (34.32%) and AWG (49.79%) than lobsters fed trash fish.

The differences in weight distribution of lobster fed the two different diets are shown in Figure 8.1. While the individual weight of lobster fed only trash fish ranged from 1 to 4 g, the individual weight of lobster fed Bio-Mos supplemented diet ranged from 1.5 to 4.5 g. The proportion of lobster fed diet with 0.4% Bio-Mos supplementation at peak frequencies were higher than that of the lobster fed trash fish alone.

Table 8.1: Survival and growth parameter of the lobster fed the control and 0.4% MOS supplemented diets

Parameters	Days	Test diets	
		Control	0.4% Bio-Mos
Survival (%)	0	100	100
	14	95.24 ± 3.01^a	97.62 ± 2.38^a
	28	71.43 ± 3.69^a	88.10 ± 4.39^b
	42	59.52 ± 2.38^a	71.43 ± 3.69^b
	56	54.76 ± 2.38^a	66.67 ± 4.76^b
Weight (g)	0	1.27 ± 0.01^a	1.27 ± 0.01^a
	14	1.39 ± 0.02^a	1.55 ± 0.03^a
	28	1.60 ± 0.07^a	1.82 ± 0.03^b
	42	1.65 ± 0.07^a	2.03 ± 0.05^b
	56	2.35 ± 0.14^a	2.86 ± 0.07^b
SGR (%)	0 - 56	1.07 ± 0.11^a	1.44 ± 0.04^b
AWG (g/week)	0 - 56	0.13 ± 0.02^a	0.20 ± 0.01^b

Different superscript letters in the same row indicate significantly different means at P < 0.05.

8.3.2 Physiological condition parameters

In general, the lobster fed 0.4% Bio-Mos supplemented diet showed better physiological conditions. TMIwet, HSIwet and TMIdry of the lobster fed Bio-Mos supplemented diet were higher (T-test, $P < 0.05$) than that of the lobster fed the trash fish without MOS supplementation. The HSIdry, TM% and HM% of the lobster were not affected by supplementation of Bio-Mos (Table 8.2).
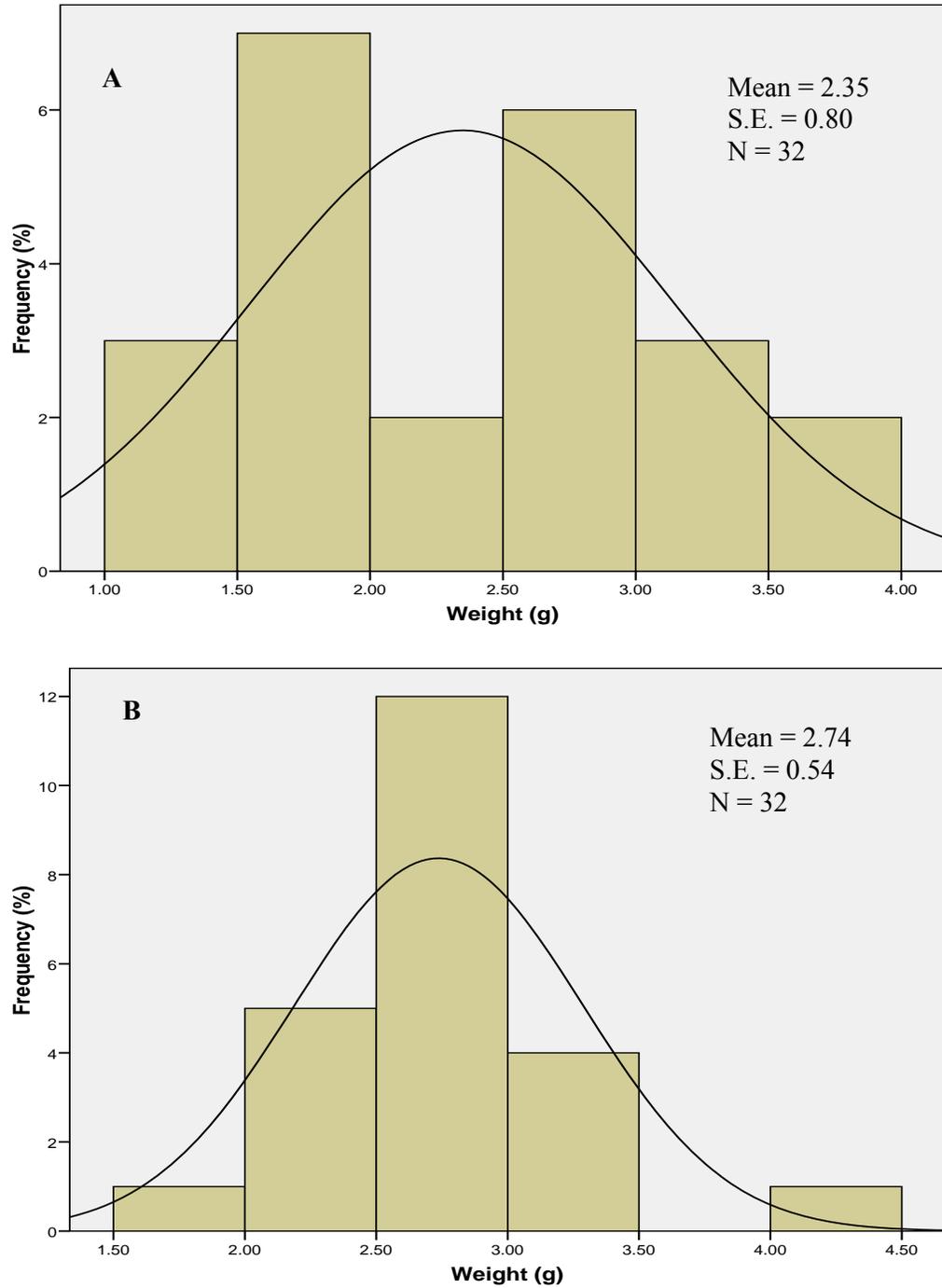
Figure 8.1: Weight distribution of the lobster fed different Bio-Mos supplemented diets.

A: Control, B: 0.4% Bio-Mos supplementation.

Table 8.2: Physiological parameters (mean ± S.E.) of lobster fed the two different diets

Parameters	Control	0.4% Bio-Mos supplementation
TMIwet	11.80 ± 0.44^a	19.40 ± 0.68^b
HSIwet	3.50 ± 0.02^a	4.50 ± 0.42^b
TMIdry	2.14 ± 0.11^a	4.18 ± 0.29^b
HSIdry	0.96 ± 0.14^a	1.24 ± 0.16^a
TM%	81.75 ± 1.18^a	78.44 ± 0.81^a
HM%	72.70 ± 3.90^a	72.57 ± 1.44^a

8.3.3 Gut bacteria and morphology

After 56 days of culture, total aerobic bacteria in the gut of lobster fed 0.4% Bio-Mos supplemented diet was approximate 10 time higher (T-test, $P < 0.05$) than that of the lobster fed the ground trash fish. *Vibrio* spp. was 7 times higher in the gut of lobster fed 0.4% Bio-Mos supplemented diet. However, the total aerobic bacteria/*Vibrio* spp. ratio was not significant different between the lobsters fed diets. The ratio of internal and external perimeter of the gut of lobster fed Bio-Mos supplemented diet was higher (T-test, $P < 0.05$) than that of the lobster fed diet without Bio-Mos supplementation (Table 8.3). Nuclear of epidermis cell of lobster fed Bio-Mos supplemented diet was clearer as shown in Figure 8.2.

Table 8.3: Gut bacteria and morphology of the lobster fed different diets

| Parameter	Control	0.4% Bio-Mos supplementation
Total aerobic bacteria (million CFU/g)	54.00 ± 4.93^a	546.67 ± 53.64^b
Total *Vibrio* spp. (million CFU/g)	28.00 ± 7.09^a	207.66 ± 29.63^b
Total bacteria/*Vibrio* spp. ratio	2.09 ± 0.37^a	2.68 ± 0.14^a
Internal perimeter/External perimeter	2.25 ± 0.10^a	3.76 ± 0.12^b
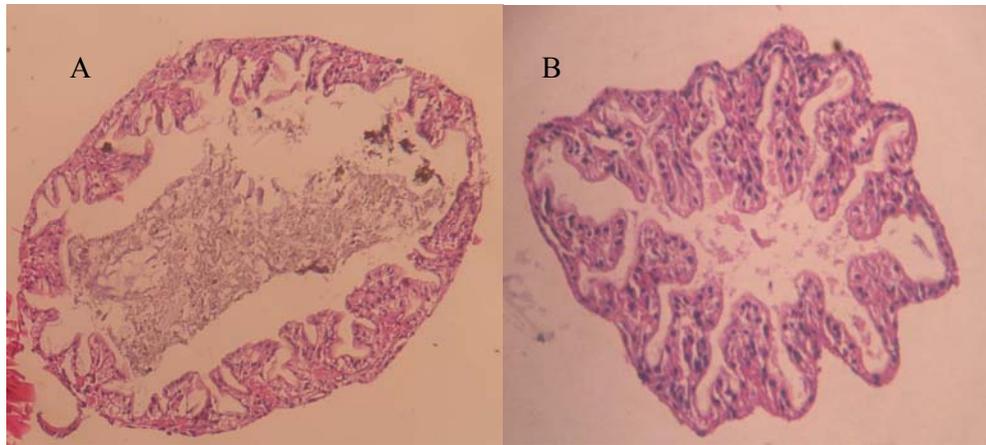
Figure 8.2: Cross section of tropical rock lobster's gut fed different diets.
A: Control, B: 0.4% Bio-Mos supplementation.

8.3.4 Survival and immunological response of the lobster challenged with *Vibrio* spp.

Seven days after *Vibrio* spp. infection, the survival of lobsters fed either the diets significantly lower. However, higher mortality was observed in the lobster fed the control diet giving the survival of 55.56 ± 5.55% compared to 77.78 ± 5.55% in the lobster fed Bio-Mos supplemented diet (Table 8.4).

At the end of culture period, THC and GC of the lobster was higher (T – test, P < 0.05) and bacteraemia was lower (T – test, P < 0.05) when they were fed with 0.4% Bio-Mos supplemented diet. THC, GC and bacteraemia changed when the lobsters were infected with *Vibrio* spp. THC of the lobster fed any diets significantly lower after one day of infection and start increasing till the day 7 of the infection. However, the recovery speed of THC of the lobster fed Bio-Mos supplemented diet was faster than that of the lobster fed trash fish alone. After 1 day of infection, THC was not different in the lobster fed any diets. The difference in THC of lobster fed different diets was observed only after 3 days of infection. THC of lobster fed Bio-Mos supplemented diet after 7 days of infection was similar to that of the lobster before infection while it was still lower in the lobster fed control diet. Bacteraemia increased after 1 day of infection in both lobster groups. The bacteraemia kept on showing increased trend in the lobster fed the control diet while it remained unchanged giving the lower bacteraemia of the lobster fed Bio-Mos supplemented diet. GC increased after 3 days of infection in the lobster fed any diets resulting in

the similar proportion in the both lobster groups and dropped quickly at the day 7 of infection. However, GC of the lobster fed the Bio-Mos supplemented was till higher than that of the lobster fed the control diet (Table 8.4).

Table 8.4: Survival and immunological parameters of the lobster challenged with *Vibrio* spp.

Parameters	Time after infection (day)	Test diets	
		Control	0.4% Bio-Mos
Survival (%)	Initial	$_1100$	$_1100$
	1	$_194.44 \pm 5.55^a$	$_197.22 \pm 0.33^a$
	3	$_277.78 \pm 3.45^a$	$_283.33 \pm 3.55^a$
	7	$_355.56 \pm 5.55^a$	$_277.78 \pm 5.55^b$
THCs (million cells/mL)	Initial	$_12.20 \pm 0.04^a$	$_12.39 \pm 0.04^b$
	1	$_20.74 \pm 0.08^a$	$_20.95 \pm 0.11^a$
	3	$_21.12 \pm 0.12^a$	$_32.04 \pm 0.09^b$
	7	$_21.21 \pm 0.29^a$	$_12.36 \pm 0.05^b$
Granular cells (%)	Initial	$_125.93 \pm 1.48^a$	$_130.33 \pm 0.74^b$
	1	$_126.10 \pm 0.70^a$	$_127.60 \pm 0.21^a$
	3	$_230.80 \pm 0.11^a$	$_229.50 \pm 0.80^a$
	7	$_320.53 \pm 1.38^a$	$_325.46 \pm 0.81^b$
Bacteraemia (CFU/mL)	Initial	$_1623.33 \pm 66.41^a$	$_1283.33 \pm 37.12^b$
	1	$_2826.66 \pm 46.30^a$	$_2386.66 \pm 24.04^b$
	3	$_31026.67 \pm 50.44^a$	$_2380.00 \pm 15.27^b$
	7	$_41306.67 \pm 54.87^a$	$_2373.33 \pm 23.33^b$

Different superscript letters in the same row indicate significantly different means at P < 0.05. Means for each immune parameter in any one column not preceded by the same subscript numbers are significantly different at P < 0.05.

8.4 DISCUSSION

This research is the first attempt demonstrating an enhancement of the growth rate, survival, physiological and immunological condition, digestive tract condition and resistant ability to bacterial infection of the tropical rock lobster, as a result of

supplementing their basal diet with MOS. Similar positive effects of dietary MOS supplementation on growth, survival and immune status of aquatic animal have been reported for Nile Tilapia (*Oreochromis niloticus*) (Nontawith 2008), rainbow trout (*Salmo gairdneri irideus*) and common carp (*Cyprinus carpio*) (Staykov *et al.* 2006), rainbow trout (*Oncorhynchus mykiss*) (Staykov *et al.* 2007), channel catfish, *Ictalurus punctatus* (Welker *et al.* 2007), European lobster (*Homarus gammarus)* (Taylor 2005; Daniels *et al.* 2006), tiger prawn (*Penaeus semisulcatus*) (Genc *et al.* 2007a), European sea bass (*Dicentrarchus labrax*) (Sweetman and Davies 2007; Torrecillas *et al.* 2007), and marron (*Cherax tenuimanus*) (Sang *et al.* 2009). MOS supplemented in the diets has also been reported to improve the health of digestive tract of sea bream (*Diplodus sargus*) (Dimitroglou *et al.* 2005), Sole (*Solea solea*) (Dimitroglou *et al.* 2006), rainbow trout (*Oncorhynchus mykiss*) (Dimitroglou *et al.* 2008) and larval cobia (Salze *et al.* 2008a).

Several attempts have been made to develop a suitable pellet feed for lobster in order to replace or supplement the natural food items such as fresh fish, crustaceans and mollusks (Smith *et al.* 2003a; Smith *et al.* 2005; Williams *et al.* 2005). However, there is still no commercial pellet feed successfully developed for tropical spiny lobster. Thus, the trash fish was the most suitable feed used for the current research. The acceptable range of nitrate, nitrite and total ammonia in the current experiment (Thuy 2004a) demonstrates that feeding the lobster by ground trash fish and the supplementation of MOS in ground trash fish did not alter the water quality. While there was no difference in the water quality and the source of the lobster seed, the differences in performances of the lobster in present study is a function of the MOS dietary supplementation.

In crustacean, assimilated energy is channeled into basal metabolism growth, molting and reproduction (Mootz and Epifanio 1974). Growth may be considered as the energy gained by the individual and stored as body reserves (Lemos and Phan 2001). In the current experiment, lobster fed with MOS results in higher absorption surface of the gut indicated by the internal perimeter/external perimeter (Sweetman and Davies 2007; Dimitroglou *et al.* 2008) of the gut as well as total bacteria in the gut. As a consequence, the lobster fed MOS had faster assimilated process and thus assimilated energy due to higher nutrient uptake. Consequently, higher assimilated

energy was stored in the tail muscle resulting in higher growth rate, TMIwet and TMIdry indices. The same effects of MOS on gut's absorption surface has been reported for sea bream (*Diplodus sargus*) (Dimitroglou *et al.* 2005), sole (*Solea solea*) (Dimitroglou *et al.* 2006), rainbow trout (*Oncorhynchus mykiss*) (Dimitroglou *et al.* 2008) and larval cobia (Salze *et al.* 2008a). Those reports shown that the application of MOS in diets has promoted the health of epithelium as well as the micro villi of the gut indicated by a more complicated architectural gut structure with longer villi, and hence a large surface area for nutrient absorption. In addition, total aerobic microbiota in the gut has also been demonstrated to increase in the gut of marron (Sang and Fotedar 2010a) when MOS is supplemented in the diet. The gut bacteria could be the sum total of natural occurring bacteria (opportunistic bacteria) and beneficial bacteria which are as a direct consequence of prebiotics (MOS). The greater number of those aerobic microbiota in the gut may increase the host survival under suboptimal dietary condition and they may improve digestion efficiency and provide digestive enzymes or vitamin (Dillon and Dillon 2004). Thus, the digestive, absorptive and assimilated processes of the food intake could have been improved by inclusion of MOS in the diet resulting in the higher growth rate. However, the change in digestive enzyme profile of crustacean when manipulation with MOS supplemented diets has not been studied providing the need for further research on the mechanism in which MOS effects on the digestive function of the gut.

Hepatosomatic index, tail muscle index, moisture contents of hepatopancreas and tail muscles have successfully been used as indicators of crustacean condition (Jussila 1997b). A large hepatopancreas size, especially when it related to low hepatopancreas moisture content, can be taken as an indicator of healthy physiological condition in freshwater crayfish (Mannonen and Henttonen 1995; McClain 1995b; Jussila 1997b). The changes in the histology of the midgut gland were observed earlier than growth depression of the crustacean (Storch *et al.* 1984; Vogt *et al.* 1985; Catacutan and De La Cruz 1989). Numerous lipid vacuoles were observed in R-cells of the midgut gland cells of juveniles *P. monodon* when fed a diet deficient in either folic acid, riboflavin and ascorbic acid (Catacutan and De La Cruz 1989). While molt stages are one of the factors influencing the crustacean condition (Jussila 1997a), the lobster used for sample analysis in the current experimental trial were in the same molt stage. Higher HSIwet, TMIwet and TMIdry

of the lobster in the current experiment suggest that the application of MOS improved the physiological condition by stimulating storage of energy in the hepatopancreas and in tail muscle (Fotedar *et al.* 1999). Moreover, the high HSIwet of lobster fed MOS diet indicates the high ability to synthesis and secret digestive enzymes, absorption of digested dietary products, maintenance of mineral reserves and organic substances, lipid and carbohydrate metabolism, distribution of stored reserves during the intermolt cycle and catabolism of some organic compounds (Dall and Moriarty 1983; Shiau and Huang 2001).

The immunological parameters of lobster were not able to be determined at the beginning of the experiment because the specmens were too small for haemolymph extraction. Differences in immune indicator of lobsters fed two diets at the end of the current experiment also suggests that inclusion of MOS in the diets has improved the immune status of the lobsters indicated by the higher THC, higher proportion of granular cells and lower number of bacteria in the haemolymph of the lobster MOS before and after they were challenged with the bacteria. The results are consistent with findings of previous studies on land-based animals (Savage *et al.* 1996) and aquaculture species. Staykov *et al.* (2006) who found higher levels of bactericidal activity, lysozyme activity, antibody levels and alternative complement pathway activity in rainbow trout and common carp fed MOS. In addition, the same effects has also been reported when 0.4% of MOS included in the diet of sea bass (Torrecillas *et al.* 2007). Thus MOS could activate and facilitate antigen processing and serve to stimulate the initial stages of the immune response (Moran 2004). Recently, Sang *et al.* (2009) reported the significant improvement in several immunological indicators such as THC, DHC, bacteraemia in marron fed a diet supplemented with MOS. The improved immune status and bacterial infection resistant ability in a challenge test with the pathogen *Vibrio* spp. were also reported for MOS fed marron (Sang *et al.* 2009). Nevertheless, MOS effects on other immune indicators such as leucocyte count, phagocytic ratio, phagocytic index, lysozyme activity, complement activity, serum bactericidal activity has not been reported for crustaceans. In addition, MOS effect on immune response could be related with other factors (Torrecillas *et al.* 2007). Thus, further studies need to be conducted to understand the role of MOS in crustacean immunomodulation.

In the present study, all lobsters tested were in the intermolt stage, thus considered to have similar immunological status. A primary test on lobsters indicated that the injection of 20 µL (PBS) which was used for preparing bacteria stock solution did not alter the survival and THC, granular cell and bacteraemia of the lobsters. The water quality in all culture tanks during the course of bacterial challenge was at the acceptable level and was same as before the challenge commenced. The changes in THC, granular cell and bacteraemia imply that bacteria infection could influence the circulating haemocyte counts of lobster. The decline in the THC in both lobster group after the injection of bacteria *Vibrio* spp. may be related to haemolymph locomotion to the injection site and haemocyte lysis as a result of defense activities (Omori *et al.* 1989; van de Braak *et al.* 2002). However, the better immune status of lobster fed MOS in the current experiment is indicated by the fact that THC of the lobster have recovered after 3 and 7 days of infection in the lobster fed MOS while it was still low in the control fed lobster. The higher recovery rate of THC results in the lower bacteraemia of those lobsters. In contract, the immune system of control lobsters was not able to cope up with the increased number of bacteria caused by the multiplication of the bacteria in the haemolymph, thus resulting in increased bacteraemia. The same effects have been observed in black tiger prawn (*Penaeus monodon*) (van de Braak *et al.* 2002) and Chinese shrimp (*Fenneropenaeus chinensis*) (Yao *et al.* 2008) when they are injected with *Vibrio anguillarum*. *Penaeus japonicus* haemocytes have the capacity to proliferate, and the proliferation rate can be increased three times when the shrimp are injected with an immunostimulant, lipopolysaccharide (Sequeira *et al.* 1996). The higher THCs of lobster fed MOS diet before and after bacteria infection in the current experiment indicates the role of MOS in proliferation of haemocytes, and the stimulation and increased the proliferation rate of lobster haemocytes to compensate for the loss of haemocytes due to the *Vibrio* spp. infection.

In conclusion, MOS inclusion in the diet have shown that MOS improve growth performance, survival, gut health, physiological condition and immune response of the tropical spiny lobster to bacterial infection. While this work is the first attempt to apply MOS on lobster, further researches need to be conducted to understand the mechanism of MOS action(s) in the performance improvement of lobster and an

applied schedule of MOS in the diet is need to be clarified to optimize the economical benefit of the spiny lobster aquaculture.

CHAPTER 9: EFFECTS OF DIETARY MANNAN OLIGOSACCHARIDE ON THE SURVIVAL, GROWTH, IMMUNITY AND DIGESTIVE ENZYME ACTIVITY OF FRESHWATER CRAYFISH, *CHERAX DESTRUCTOR* CLARK (1936)

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9.1 INTRODUCTION

Restriction or ban on the use of the antibiotic as fish and crustacean feed additives has prompted research direction towards developing alternative products which can be used for health management and disease control. Thus, in the recent years, there has been an heightening research on the dietary supplementation in which various health promoting compound have been studied (Gatlin *et al.* 2006). Those compounds can be classified as immunonutrients and immunostimulants with the differences between the two relates to their mechanisms of action. One group of immunostimulant showing beneficial effects in terrestrial and aquatic animals is referred to as prebiotics (Gibson and Roberfroid 1995). Prebiotics are classified as non-digestible food ingredients that beneficially affect the host by stimulating growth and/or activity of a limited number of beneficial bacteria such as *Lactobacillus* and *Bijidobacter* spp. in the gastrointestinal tract while limiting potentially pathogenic bacteria such as *Salmonella, Listeria* and *Escherichia coli* (Manning and Gibson 2004). The gut microbiota that are affected by prebiotics play an integral role in numerous processes including growth, digestion and immunity (Burr *et al.* 2005). These roles have been demonstrated in poultry (Patterson and Burkholder 2003), other terrestrial livestock and companion animals (Flickinger *et al.* 2003), as well as in humans (Gibson and Roberfroid 1995). In aquaculture, the application of prebiotics is till in its infancy compared with the progress that has been made in the development of prebiotics for poultry, but holds considerable potential (Burr *et al.* 2005). Among the established prebiotics such as fructooligosaccharide (FOS), transgalactooligosaccharide (TOS), inulin and mannan oligosaccharide (MOS), MOS is the most commonly used as the dietary supplementation for fish and crustacean species. The immuno-stimulating and growth promoting roles of MOS have been demonstrated in sea bream (*Diplodus sargus*) (Dimitroglou *et al.* 2005), sole (*Solea senegalensis*) (Sweetman and Davies 2007), rainbow trout (*Oncorhynchus mykiss*)

(Dimitroglou *et al.* 2008) tiger shrimp, *Penaeus semisulcatus* (Genc *et al.* 2007a) and marron (*Cherax tenuimanus*) (Sang *et al.* 2009).

Freshwater crayfish, *Cherax destructor*, called yabby, are widely distributed in temperate and sub-tropical south-eastern and central Australia. They have also been introduced into farm dams in southwestern Australia for recreational fishing and aquaculture (Austin 1985). However, relatively little published information is available on the health management of yabby culture and there is no information available on the role of immunostimulants in yabbies. In addition to traditionally used performance indicators of the feed additives such as growth and survival, measures of crustacean health can also be included in immune–physiological parameters such as total number of haemocyte (THC), differential haemocyte counts (DHC), bacteraemia (Smith *et al.* 1995; Jussila *et al.* 1999; Fotedar *et al.* 2001; Fotedar *et al.* 2006; Sang *et al.* 2009) and digestive enzyme activities (Pedroza-Islas *et al.* 2004; Wang 2007). The aim of the present study was to investigate the role of a prebiotic - MOS on the growth, survival, immunity and digestive enzyme activity of the yabbies under the laboratory conditions.

9.2 MATERIALS AND METHODS

9.2.1 Preparation of the diets

Two isonitrogenous and isocaloric diets were designed to contain 0% (control) and 0.4% Bio-Mos (Alltech, USA) replacing standard carbohydrates. All feed ingredients except for Bio-Mos were supplied by Specialty Feeds Pty Ltd, Western Australia. Bio-Mos were supplied by Alltech, USA. The main ingredients of Bio-Mos are brewers dried yeast and dried *Saccharomyces cerevisiae* fermentation soluble containing 72.6 g kg^{-1} moisture, 311 g kg^{-1} crude protein, 40.4 g kg^{-1} crude fat, 3.6 g kg^{-1} sulfur, 10.2 g kg^{-1} phosphorus, 9.7 g kg^{-1} potassium, 2 g kg^{-1} magnesium, 3.5 g kg^{-1} calcium, 0.9 g kg^{-1} sodium, 44 mg L^{-1} manganese, 57 mg L^{-1} copper, 167 mg L^{-1} zinc and 250 g kg^{-1} glucomannoprotein-complexes (MOS). The proximate compositions of the ingredients and Bio-Mos were used as a basis to form the required formulas using the software FeedLIVE version 1.52 (Table 9.1).

Table 9.1: Formulation and proximate nutrient components of the experimental diets

Ingredient and nutrient components (g kg^{-1})	Control	0.4% Bio-Mos
Fish oil[1]	32.0	32.3
Soybean meal	101.5	101.4
Fish meal[2]	337.8	338.6
Wheat flour	493.5	488.5
Ascorbic acid[3]	0.5	0.5
Betaine[4]	12.0	12.0
Calcium ascobate	0.2	0.2
Premix[5]	1.5	1.5
Cholesterol	2.5	2.5
Wheat starch	18.5	18.5
Bio-Mos	0.0	4.0
Crude protein (per diet n = 6)	346.40 ± 0.30	348.10 ± 1.10
Dry matter (per diet n = 6)	945.60 ± 1.20	941.30 ± 1.40
Moisture content (per diet n = 6)	54.40 ± 2.60	58.70± 1.10
Ash (per diet n= 6)	61.60 ± 1.70	60.40 ± 0.80
Gross energy (MJ kg^{-1}) (per diet n = 6)	18.80 ± 0.06	18.79 ± 0.07

[1] Cod liver oil, Melrose Laboratories (Box Hill, Victoria, Australia).

[2] Peruvian fishmeal, 68% CP (supplied by Specialty Feeds Pty Ltd, Western Australia).

[3] Stay – C (supplied by Specialty Feeds Pty Ltd, Western Australia).

[4] Betaine Anhydrous 97% (BEC Feed Solutions, supplied by Specialty Feeds Pty Ltd, Western Australia).

[5] Commercial vitamin and mineral premix for trout (Specialty Feeds Pty Ltd, Western Australia).

All of the dry feed ingredients and Bio-Mos were passed through a 100-µm mesh sieve in order to obtain a uniform particle size and then thoroughly mixed with a predetermined amount of freshwater (200 mL kg^{-1}) to form a soft dough. The soft dough was then passed through a mince mixer to obtain pellets of 2 mm diameter. The pellets were dried in the direct sunlight for 6 h and then were allowed to cool at room temperature for 30 min followed by storing in plastic containers.

Analysis of moisture content, dry matter, crude protein and ash of the diets were performed according to the standard methods of the Association of Official Analytical Chemists (AOAC 1995) in six replicates for each diet. Gross energies of the diets were determined using a bomb calorimeter (Calorimeter C2000, Crown Scientific, Germany).

9.2.2 Experimental animal and design

The study was conducted at Curtin Aquatic Research Laboratory (CARL), Curtin University, Australia. The experiment was conducted in 6 blue plastic cylindrical tanks (80 cm in diameter and 50 cm water depth). Each tank was provided with aeration and an independent recirculating freshwater system with a bio-filter (Aquastop system Fluval 204, Askoll Italy). Sufficient number of PVC pipes and oyster net were placed in the tank as shelter for the yabbies. The recycling rate of water in each tank was maintained at 0.5 L min^{-1} throughout the experiment. Water temperature in all experimental tanks was kept at approximately 20°C by putting a heater (MasterPet, 200w) in each tank. Yabbies (35.14 ± 0.48 g, initial weight) were purchased from Cambinata Yabbies Ltd, Kukerin, Western Australia and transported to CARL. Eight yabbies were stocked into each tank and acclimated to the experimental condition for 1 week before the feeding. The six tanks were randomly assigned to two dietary treatments. The food was provided daily at the rate of 5% body weight which is considered to be above the satiation feeding level for yabbies. Food was given once at 0900 h everyday after siphoning uneaten food and faeces from the previous day. The amount of water lost during siphoning was added into each tank to maintain the water level. Amount of diets were adjusted according to the total biomass calculated after each sampling period (14 days). Yabbies were culture for 56 days. Water quality parameters such as pH, nitrite and NH_4^+ were monitored inside each tank every 3 days using pH meter WP-80, TPS Company and chemical test kits (Aquarium Pharmaceuticals, INC). Dissolved oxygen was measured by CyberScan DO300 Waterproof Dissolved Oxygen Meter.

9.2.3 Data collection

The survival (S) in each tank was measured using the following formula:

S = 100 x (n_t/n_o), where: S is the survival rate; n_t is the number of yabbies at time t and n_o is the number of yabbies at the commencement.

Specific growth rates (SGR) and average weekly gain (AWG) of yabbies in each tank were calculated as follows: SGR(% day-1) = 100 x (lnWe – lnWs)/d; AWG (g week-1) = (We– Ws)/wk where Ws and We are the mean weights of the yabbies in each tank at the start and end of the growth period, respectively, and d and wk are the number of days and weeks, respectively, in the growth period.

Total haemocyte count (THC), granular cells proportion (GCs), semi-granular cells proportion (SGCs), hyaline cells proportion (HCs) and bacteraemia assessment were conducted as per the established procedure for rock lobster and marron (Fotedar *et al.* 2001; Sang *et al.* 2009). The base of the fifth thoracic leg of three yabbies from each culture tank was cleaned with 70% alcohol. A 0.2 mL aliquot of haemolymph was withdrawn into a 1-mL sterile syringe containing a solution which contained 0.2 mL of 1% glutaraldehyde (fixative) in 0.2M sodium cacodylate (buffer) and then dispensed into an Eppendorf tube kept on ice. The THC for the individual yabby was estimated with a haemocytometer (Neubauer, Germany) under 100-fold magnification from the haemolymph solution. The total haemocyte count was calculated as THC = (cells counted x dilution factor x 1000)/volume of grid (0.1 mm^3). For differential haemocyte counts, one drop of haemolymph solution was placed on a slide and smeared. The smear was air dried and fixed in 70% methanol for 10 min. Fixed smears were stained with May-Grunwald and Giemsa for 10 min in each solution (Bancroft and Stevens 1977). Approximately 200 cells were counted on each slide. The granular cells were distinguished on the basis of the cell size, presence of a smaller pale nucleus, and larger number of eosinophillic granules in the cytoplasm, and their proportion to overall haemocytes was calculated. Hyaline cells had round shape, big nucleus and little or no cytoplasm; while semi-granular cells had longer shape, big nucleus and little or no cytoplasm. For bacteraemia assessment, a 0.1 mL aliquot of haemolymph was withdrawn into a sterile syringe and quickly placed and smeared on a marine agar (MA) plate. The plate was carefully inverted before placing in the incubator chamber at 25°C for 24 h. Colony-forming units (CFU) were counted and CFU mL^{-1} was calculated for each sample on the basis of a total volume of 0.1 mL for each plate.

At the end of the culture period, enzymatic activity of protease and amylase in the digestive tract of the basal diet fed and MOS fed-yabbies were analysed. The hindgut and hepatopancreas of three yabbies from each tank were removed and homogenised in ice-cold (0°C) 0.1M citrate phosphate buffer at pH 5.5. Each homogenate comprised the tissue of each yabby in 5 mL of buffer. The crude homogenates were centrifuged for 5 min using a clinical centrifuge (Eppendorf Centrifuge 5804R, Germany) and the supernates were used for the enzyme assay. Enzyme activity was reported as specific activity expressed by the amount of product formed per mg protein per hour. Protein was determined using the method described by (Hartree 1972). Bovine serum albumin (BSA) was used as the standard for protein determination.

Protease activity was assayed at 37°C using Azocoll at 10 mg mL^{-1} in 0.1 M citrate phosphate buffer at pH 5.5 following the methods described by Biesiot and Capuzzo (1990). Azocoll (2.8 mL) in 0.1 M citrate phosphate buffer was pre-incubated for 10 min at 37°C in shaking water bath before 0.2 mL of digestive tract supernate was added. After 60 min the reaction tubes were placed on ice for 10 to 20 min in order to stop the reaction. The tubes were centrifuged for 2 min and the supernates removed. Absorbance of the supernates was read at 520 nm on a spectrophotometer. A blank for each sample was prepared by adding 0.2 mL of digestive tract supernate to 2.8 mL of buffer. The absorbance at 520 nm was also recorded. The protease activity was reported as the increase in absorbance unit (A_{520}) per mg protein per hour.

Amylase activity was assayed according to the methods described by Biesiot and Capuzzo (1990) which was modified from the method of Bernfeld (1955). The assay was conducted at 37°C using soluble starch at 15 mg mL^{-1} in 0.1 M phosphate buffer at pH 6.5 with 0.05 M NaCl. Amylase activity was reported as micro-equivalents of maltose liberated per mg protein per hour.

9.2.4 Statistical analysis

The data were statistically analysed using statistical analysis program SPSS for Windows version 15. Each tank was used as an experimental unit for water quality parameters, survival, growth parameters. Number of death yabbies was subjected to

nominal regression analysis to determine the significantly different in survival between the two treatments. Water quality and growth parameters were subjected to independent sample T-test for significant difference determination. For the immune and enzyme parameters, the individual crayfish was used as an experimental unit and independent sample T-test was used for significant difference determination. Differences were considered significant at the level of $P < 0.05$.

9.3 RESULTS

9.3.1 Test diet and water quality parameters

Analysed gross energy, crude protein, moisture, dry matter and ash content were similar for both diets (Table 9.1). During the course of trial, water quality parameters were not significantly different between the two treatments. Temperature ranged between 19.7 and 20.9°C, pH was between 7.58 and 8.26, dissolve oxygen was ≥ 6 ppm, NO_2^- and NH_4^+ were below detectable levels.

9.3.2 Survival and growth

No mortalities of yabbies occurred during the acclimation period. Bio-Mos supplemented in the diet did not result in improved survival of yabbies after 56 days of feeding. Weight of yabbies fed 0.4% Bio-Mos were higher ($P < 0.05$) than the yabbies fed the control diet resulting in higher SGR and AWG of Bio-Mos fed yabbies (Table 9.2).

Table 9.2: Survival and growth of yabbies (mean ± S.E.; per diet n = 3)

Parameters	Control	0.4% Bio-Mos
Survival (%)	66.67 ± 4.17[a]	83.33 ± 4.17[a]
Weight (g)	42.10 ± 0.74[a]	45.71 ± 0.73[b]
SGR	0.32 ± 0.02[a]	0.47 ± 0.07[b]
AWG (g week^{-1})	0.87 ± 0.07[a]	1.32 ± 0.06[b]

Different superscript letters in the same row indicate significantly different means at P < 0.05.

9.3.3 Immunological parameters

The yabbies fed 0.4% Bio-Mos diet had higher THC, GCs and SGCs proportions than yabbies fed the control diet. HCs were lower in Bio-Mos fed yabbies while bacteraemia did not show any significant difference ($P > 0.05$) between the two yabby groups (Table 9.3).

Table 9.3: Immunological parameter of yabbies (mean ± S.E.; per diet n = 9)

Parameters	Control	0.4% Bio-Mos
THC[*]	2.35 ± 0.03^{a}	2.66 ± 0.06^{b}
GCs	6.08 ± 0.41^{a}	8.25 ± 0.57^{b}
SGCs	3.49 ± 0.32^{a}	4.92 ± 0.35^{b}
HCs	90.44 ± 0.65^{a}	86.83 ± 0.36^{b}
Bacteraemia	7.17 ± 0.60^{a}	6.50 ± 0.58^{a}

Different superscript letters in the same row indicate significantly different means at P < 0.05.

9.3.4 Digestive enzyme activity

Amylase activity in the gut of yabbies fed Bio-Mos supplemented diet was higher ($P < 0.01$) than the yabbies fed the control diet. Activity of amylase in the hepatopancreas was not significantly different between the two yabby groups (Figure 9.1). Protease activity of Bio-Mos fed yabbies was higher ($P < 0.01$) in the hepatopancreas compared to yabbies fed the control diet (Figure 9.2).

9.4 DISCUSSION

Previous studies have stated that the Bio-Mos contains approximately 250 g kg^{-1} glucomannoprotein-complexes as a source of MOS in the diet for aquaculture species (Dimitroglou *et al.* 2007; Genc *et al.* 2007a; Staykov *et al.* 2007; Dimitroglou *et al.* 2008; Salze *et al.* 2008a). Results from current experiment suggest that MOS through dietary supplementation of Bio-Mos improves the growth and certain immune parameters of yabbies. The results of the current study are consistent with previous findings on the role of MOS in improving performances of several fish species (Bogut *et al.* 2006; Culjak *et al.* 2006; Staykov *et al.* 2006; Torrecillas *et al.* 2007; Welker *et al.* 2007; Nontawith 2008) and crustacean species (Taylor 2005; Daniels *et al.* 2006; Sang *et al.* 2009; Sang and Fotedar 2010c). Higher growth of yabbies fed

MOS diet could be contributed by increase in apparent energy digestibility of MOS supplemented diet which has been demonstrated in Atlantic salmon (*Salmo salar*) (Grisdale-Helland *et al.* 2008). Higher growth may also be contributed by increase in the absorption surface of the gut as well as total bacteria in the gut which were reported for MOS-fed lobster (Sang and Fotedar 2010c) and some fish species (Dimitroglou *et al.* 2005; Dimitroglou *et al.* 2008; Salze *et al.* 2008a). As yabbies in the current experiment were fed to satiation, there is a possibility of higher amount of feed intake by yabbies fed MOS supplemented diet could have contributed to the higher growth rates than those fed the control diet.
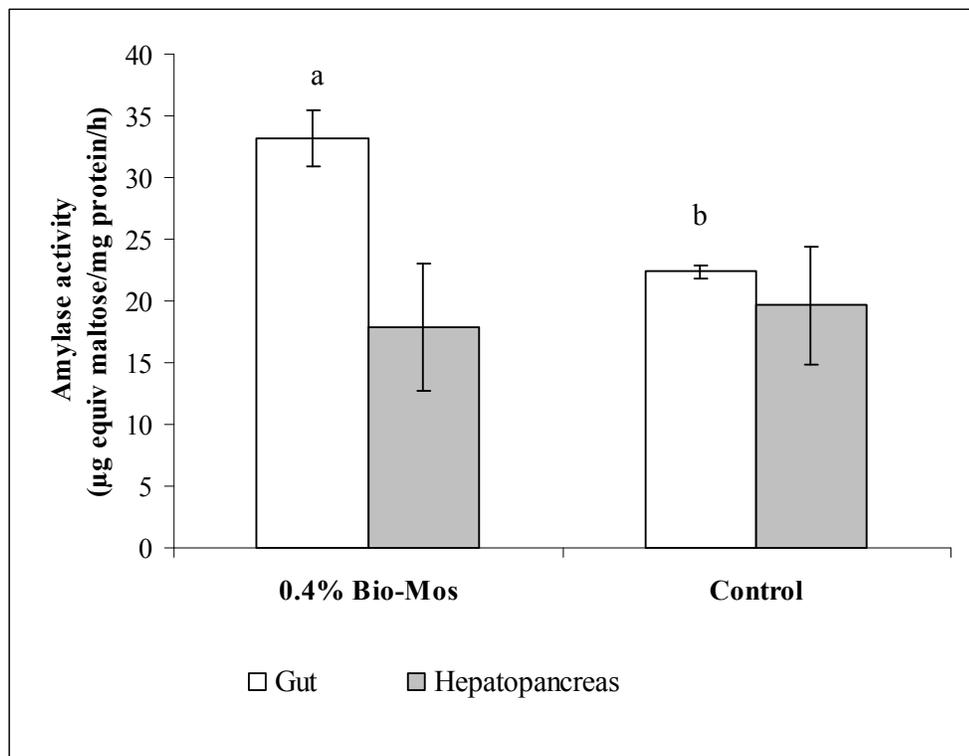

Figure 9.1: Amylase activity in the digestive system of the yabbies (mean ± S.E., per diet n = 9).

*Different letter showing the significantly different in mean value at P < 0.01.*
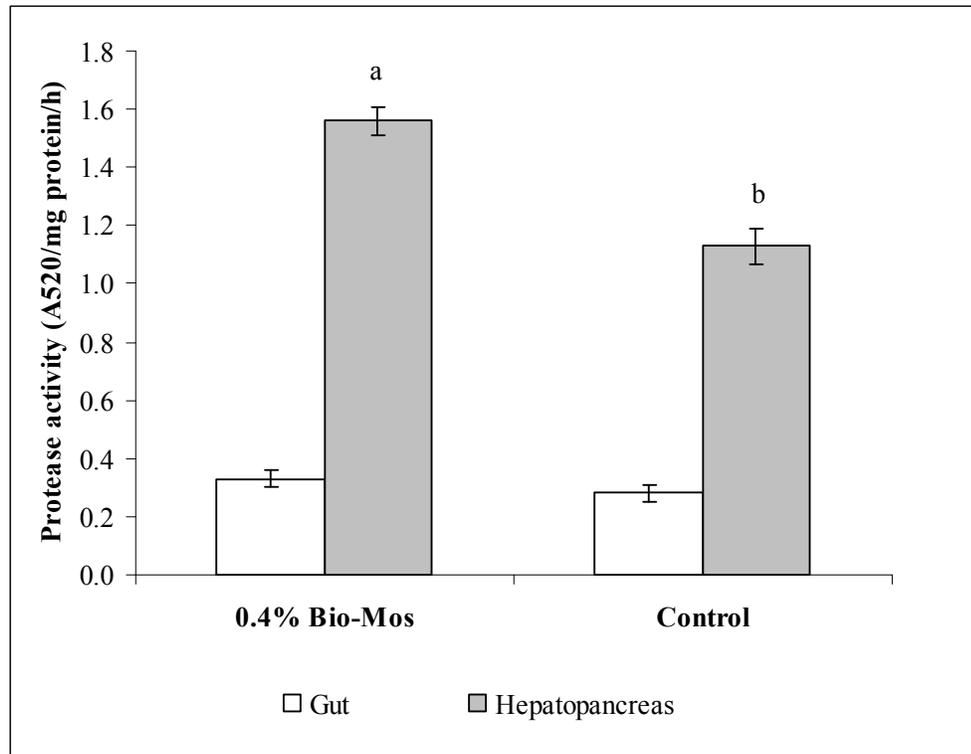
Figure 9.2: Protease activity in the digestive system of the yabbies (mean ± S.E, per diet n = 9).

Different letter showing the significantly different in mean value at P < 0.05.

In crustaceans the innate immune system (non-specific) is the fundamental defense mechanism against infectious microorganisms in which haemocytes play key function (Lee and Söderhäll 2002). The current experiment suggests that inclusion of MOS in the diets improves the immune system of yabbies at cellular level. The mechanism in which prebiotic MOS alter the haemocyte profile of yabbies has not been investigates but it can be postulated that MOS stimulate the benefit gut' bacteria that results in change in immune system at cellular level (Gulliana *et al.* 2004). In the haemolymph of crayfish, the hyaline cells are chiefly involved in phagocytosis, the semi-granular cells are the cells active in encapsulation, while the granular cells participate in storage and release of the prophenoloxidase (proPO) system and create cytotoxicity (Johansson *et al.* 2000). The semi-granular cells are responsible for recognising and responding to foreign molecules and particles by degranulation and subsequently attaching and spreading on the foreign surface (Johansson and Söderhäll 1985). The higher THCs, GCs and SGCs of MOS fed yabbies in the

present experiment indicate the higher ability of these yabbies's haemocyte in degranulation process to defeat against the foreign substances. The participation of MOS in the binding/recognition step of melanisation process has not been investigated, but the role of lypopolysaccharide and beta-glucan in this process has been reported from crayfish species (Lee and Söderhäll 2002). Thus, there is a great need to clarify the involvement of MOS in the initiation of innate immunity in yabbies.

The growth of crustacean species is limited by the capacity of the digestive system to break down and assimilate specific nutrients (Houlihan *et al.* 1988). Profile and activity of digestive enzymes largely determine the capacity of an animal to obtain nutrients from a particular food source (Furne *et al.* 2005). Results of the current study also suggest that MOS has improved digestive enzyme activity in midgut and hepatopancreas of yabbies. The higher digestive enzyme activity contributes to more efficient digestion which can result in higher growth (Houlihan *et al.* 1988). The mechanism in which MOS stimulate the activity of digestive enzyme has not been investigated in crustacean. However, Wang (2007) reported an increase in digestive enzyme activity, especially activity of protease and amylase in whiteleg shrimp (*Penaeus vannamei*) when they fed with the probiotic (photosynthetic bacteria and *Bacillus* spp.) supplemented diets. Thus, MOS as a prebiotic, may have increased the beneficial bacteria in the digestive system and the enhance of the digestive enzymes activity resulting in higher growth rate of MOS fed yabbies.

In conclusion, the results have shown that MOS can be used as an effective dietary supplementation for improving the growth performance of yabbies. Better immune status suggested the role of MOS in improving disease resistance ability of yabbies. Further research is needed to understand the mechanism by which MOS stimulate the immune response.

CHAPTER 10: GENERAL DISCUSSION

Part of the chapter: 'The applications of mannan oligosaccharide in aquaculture'. In:
Oligosaccharides: Sources, Properties and Applications, NOVA publisher (In press)

10.1 INTRODUCTION

Bacterial and viral diseases are known to be the major constraints in aquaculture industry in recent years (Scholz *et al.* 1999) and are impeding the development and sustainability of the industry throughout the world (Bondad-Reantaso *et al.* 2005). Thus, the need to control the loss due to disease outbreaks in aquaculture is becoming increasingly important (Sang *et al.* 2009). For a long time, the most common method for dealing with the occurrence of bacterial infections in aquaculture, was the administration of antibiotics (Yousefian and Amiri 2009). Such practices have led to promote the spread of antibiotic-resistant pathogens in both cultured species and in the environment (Gatlin 2002; Kesarcodi *et al.* 2008). Furthermore, antibiotic use drive the aquaculture industry facing serious problems due to various adverse such as accumulation of antibiotic in the tissue, immunosuppression, and destruction of environmental microbial flora (Yousefian and Amiri 2009) In addition, the application of such practices is expensive and undesirable due to having harmful effects to the environment and human health (Capone *et al.* 1996).

Increased concern about the side effects on the environment and human health of antibiotic used as growth promoter and protector of culture species from bacterial infection has prompted the search for alternative products (Genc *et al.* 2007a). Recently, immunostimulants such as probiotics, prebiotics and beta glucan have been proved promised preventive and environmentally friendly alternative to antibiotic used in aquaculture (Campa-Córdova *et al.* 2002; Bachere 2003; Soltanian *et al.* 2007). Although many obvious benefits gained from the use of probiotics as growth promoter and disease prevention in aquaculture, development and application of probiotics for a specific culture species has to follow strict procedures. The selection of probiotics for specific culture species has to be considered carefully in order to gain the species specific desired outcomes (Wang *et al.* 2008). Moreover, for the best effectiveness, research needs to be undertaken on the particular culture conditions of the selected species in order to understand the rationale, preparation and hazards

from inappropriate species/strains of bacteria selection and/or usage of inadequate population densities of probiotics (Moriarty *et al.* 2005; Balcázar *et al.* 2006).

On the other side, prebiotics defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Gibson *et al.* 2004) effect the host indirectly due to it selectively feeds one or a limited number of microorganisms thus causing a selective modification of the host's intestinal microbiota (Teitelbaum and Walker 2002). The main advantage of prebiotics over probiotics is that they are natural feed ingredients. Their incorporation in the diet does not require particular precautions and their authorization as feed additives may be more easily obtained, in spite of some concerns about their safety and efficacy (Hai and Fotedar 2009; Yousefian and Amiri 2009). Furthermore, prebiotics can be applied in wide range of organisms in husbandry, poultry and aquaculture industry. In different studies since 1999, many substances have been investigated as prebiotic for aquaculture such as inulin, fructooligosaccharide (FOS) and transgalactooligosaccharides (TOS). Recently, mannan oligosaccharides (MOS) which derived from cell wall of yeast *Saccharomyces cerevisiae* has been receiving heightening application as a prebiotic and as an immunostimulant in aquaculture. Another immunostimulant, beta glucan (BG), has successfully been used to enhance the resistance of fishes and crustacean against bacterial or viral infections (Liao *et al.* 1996; Chang *et al.* 1999; Sakai 1999; Chang *et al.* 2000; Chang *et al.* 2003; Misra *et al.* 2004; Marques *et al.* 2006; Katzenback *et al.* 2008). Since the first use of MOS and BG in aquaculture, there has been an increasing number of studies demonstrating their ability to improve the survival, growth performance and control of the potential pathogens in fish and crustacean. The roles of MOS and BG on culture of crustaceans are discussed in this chapter.

10.2 APPLICATION METHOD AND DOSAGE

Application methods for immunostimulants need to be considered carefully in order to obtain the desired results. The selected applied methods depend on various factors such as culture species, life stage of species and cultivate form. In aquaculture industry, MOS is included in the diets as an non-digestive ingredient (Dimitroglou *et al.* 2006; Staykov *et al.* 2006; Genc *et al.* 2007a; Dimitroglou *et al.* 2008; Sang *et al.*

2009) and/or through live feed enrichment (Daniels *et al.* 2006; Salze *et al.* 2008a). On the other hand, BG is applied though a immersion or injection (Sung *et al.* 1994), live feed enrichment (Marques *et al.* 2006) and/or directly added to the diet (Chang *et al.* 2003). In the culture of freshwater crayfishes, marron (*Cherax tenuimanus*) and yabby (*Cherax destructor*), and tropical rock lobster (*Panulirus ornatus*), MOS and BG was supplemented with diets (Sang *et al.* 2009; Sang and Fotedar 2010c, 2010b). To date, literature on the effectiveness of different administration methods of MOS and BG in culture of crayfish is not available. During the early larvae development stage of European lobster (*Homarus gammarus)*, MOS was applied to the animal through enrichment of live feed artemia (Daniels *et al.* 2006). Inclusion of MOS and BG in the diets of marron did not affect nutrient value of the diets as well as the water quality of culture media (Sang and Fotedar 2010c, 2010b).

Dosage of MOS and BG in aquaculture diets is another important criterion to get the optimum economical returns. The dosage below minimum threshold levels may result in null effect on the animal while dosage above the maximum requirement can result in the unnecessary high feeding costs and even negative effects on the performance the animal. In marron, the appropriate inclusion dietary level is 0.2 to 0.4% of MOS (Sang *et al.* 2010a) and 0.1% to 0.2% of BG (Sang and Fotedar 2010b). The dosages less than 0.2% of MOS and 0.1% of BG and higher than 0.4% of MOS and 0.2% BG did not improve the performance of the species (Sang and Fotedar 2010b; Sang *et al.* 2010a). There are no reports available on the adverse side effects of higher dietary inclusion of MOS and BG however, the cost of MOS and BG seem to be the drivers for the inclusion levels in crayfish culture. Chang *et al.* (2003) concluded that further increasing the level of MOS above the optimum level in grass prawn (*Penaeus monodon*) did not result in any higher performance of the prawn. In tropical rock lobster and yabby, MOS was applied at 0.4% in the diets resulting in positive performance (Sang and Fotedar 2010c; Sang *et al.* 2010b). Although the optimum dosage of MOS for these species is not known , 0.4% dietary inclusion levels is the most common practice in many of the crustacean (Genc *et al.* 2007a) and fish species (D'Abramo and Robinson 1989; Dimitroglou *et al.* 2006; Dimitroglou *et al.* 2008). Therefore, further research is required to determine the optimum inclusion levels of MOS in freshwater crayfish and lobsters.

10.3 EFFECTS OF MOS AND BETA GLUCAN ON GROWTH, SURVIVAL AND PHYSIOLOGICAL CONDITION OF CRAYFISH

Dietary MOS and BG, resulted in improved survival of marron (Sang and Fotedar 2010b; Sang *et al.* 2010a). Similar survival improvement has been reported by MOS for tropical rock lobster (Sang and Fotedar 2010c), yabbies and European lobster (Daniels *et al.* 2006) under the optimum culture conditions and even when these species were infected with bacteria (Sang *et al.* 2009; Sang and Fotedar 2010c) (Table 10.1). Dietary inclusion of MOS increased the survival by 38.71% for marron after 112 days of rearing; 21.74% for tropical rock lobster after 56 days of rearing and 24.98% for yabbies after 56 day of rearing. 0.2% BG supplemented in the diet also increased survival of marron by 31.42% after 84 day of rearing. The higher survival in turn, increased the total biomass production of the crayfish when MOS and BG were supplemented in the diet. The improved growth rates was another desired outcome in most of the investigated crayfish species, except marron when dietary MOS and BG was used. The growth of yabbies and tropical rock lobster was improved by 8.7% and 21.7% respectively after 56 days of rearing with MOS supplemented diets (Sang and Fotedar 2010b; Sang *et al.* 2010b). On the contrary, MOS and BG did not improve the growth of marron may be due to the fact that MOS and BG were only applied for a short duration compared to long culture life of marron.

Some physiological parameters reflecting health status of crayfish have also been improved when MOS and BG were included in the diets (Table 10.1). Changes in tail muscle index (wet and dry) and tail moisture content of marron indicated the improved health status after MOS and BG supplementations (Sang and Fotedar 2010b; Sang *et al.* 2010a). Similar improvements, except tail moisture content were also positive indicators of health improvements in tropical rock lobster when MOS was applied (Sang and Fotedar 2010c). Osmoregulatory capacity of marron also improved by MOS application (Sang *et al.* 2010a). The reports indicated that MOS and BG enhance the health condition of these crayfish through stimulating the energy storage capacity in the tail muscles and thus indirectly reducing the impact of stressors (Fotedar *et al.* 1999). However, MOS and BG failed to alter hepatosomatic index, moisture content of hepatopancreas and moisture content of tail muscle of marron and tropical rock lobster (Sang and Fotedar 2010c, 2010b; Sang *et al.* 2010a).

Longer term research is required when these species are used to assess the effectiveness of the MOS and BG on their physiological conditions.

The mechanisms in which prebiotics directly effect the physiological condition of crayfish have not been yet investigated though reports have shown that MOS increase digestive enzyme activities in yabbies. For example, protease activity was higher in hepatopancreas and amylase activity was higher in the gut of yabbies fed MOS diet (Sang *et al.* 2010b). Higher activity of digestive enzyme increases capacity of animals to obtain nutrients from food (Furne *et al.* 2005), and helps in the breakdown and assimilation of specific nutrients (Houlihan *et al.* 1988) thus, improves physiological condition and finally growth (Houlihan *et al.* 1988). The evidence of MOS influencing the enzyme activity comes from the reports showing that MOS have stimulated the beneficially bacterial community in the intestine which directly stimulated the activity of digestive enzyme (Wang 2007).

Table 10.1: Performance of some crayfish fed by MOS inclusion diet

Species - prebiotic	Growth	Survival	Biomass increment	HSIwet	HSIdry	TMIwet	TMIdry	TM%	HM%	OC
Cherax tenuimanus – BG [1]	-	+	+	-	-	+	+	+	-	N/A
Cherax tenuimanus – MOS [2]	-	+	+	-	-	+	+	-	-	+
Panulirus ornatus – MOS[3]	+	+	+	+	-	+	+	-	-	N/A
Cherax destructor [4]	+	+	+	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Homarus gammarus[5]	+	+	+	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Source: [1] (Sang and Fotedar 2010b); [2] (Sang *et al.* 2010a); [3] (Sang and Fotedar 2010c); (Sang *et al.* 2010b); [5] (Daniels *et al.* 2006).

HSIwet: Wet hepatosomatic index; HSIdry: dry hepatosomatic index; TMIwet: wet tail muscle index; TMIdry: dry tail muscle index, TM%: moisture content of tail muscle; HM%: moisture content of hepatopancreas; OC: Osmoregulatory capacity; - : none effect; +: positive effect; N/A: data not available.

Table 10.2: Effect of MOS on immunological indicators of crayfish

Species - prebiotic	THC	GCs	SGCs	HCs	Haemolymph clotting time	Bacteraemia	Resistance to bacterial infection
Cherax tenuimanus – BG [1]	+	+	N/A	N/A	+	+	N/A
Cherax tenuimanus – MOS [2]	+	+	+	+	+	+	+
Panulirus ornatus – MOS[3]	+	+	N/A	N/A	+	+	+
Cherax destructor - *MOS*[4]	+	+	+	+	N/A	+	N/A

Source: [1] (Sang and Fotedar 2010b); [2] (Sang *et al.* 2010a); [3] (Sang and Fotedar 2010c); [4](Sang *et al.* 2010b).

THC: Total haemocyte count; GCs: Granular cells; SGCs: Semi-granular cells; HCs: Hyaline cells; - : none effect; +: positive effect; N/A: data not available.

10.4 EFFECTS OF MOS ON DIGESTIVE TRACT CONDITION OF

CRAYFISH

Health digestive tract of tropical rock lobster and marron was improved by dietary MOS inclusion (Sang and Fotedar 2010c, 2010a). Sang and Fotedar (2010a) reported the alteration of intestine microbial population of marron when 0.2 to 0.4% MOS was supplemented in their diet with the increase in total bacteria and bacteria/Vibrio ratio approximately by ten times. Similar positive effect was observed in tropical rock lobster when the diet was supplemented with 0.4% MOS (Sang and Fotedar 2010c). However, there is a lack of the information on the intestine bacterial strain/species which are stimulated/eliminated by MOS inclusion in the diets of crayfish, unlike shown in some fish species (Zhou and Li 2004; Dimitroglou *et al.* 2008). In Jian carp (*Cyprinus carpio*) culture, dietary MOS has altered the intestinal microbial populations by reducing the population of *Escherichia coli* and increasing *Bifidobacterium* and *Lactobacillus* (Zhou and Li 2004). MOS inclusion has also reduced the bacterial load in the gut of both rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Diplodus sargus*) by reducing the total aerobically cultivated bacteria. In rainbow trout, MOS supplementation has reduced the numbers of *Micrococcus* spp., *Staphylococcus* spp., *Aeromonas/Vibrio* spp. and other unidentified gram + bacteria and increased *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacter* spp. (Dimitroglou *et al.* 2008). Thus, identification of beneficially bacterial strain/species in the gut of crayfish benefited by dietary MOS should be priority in the further research.

Prebiotics in many organisms have also increased the efficiency of the digestive tract by increasing the regularity, height and integrity of the gut villi (Hooge 2004). In crustaceans, such effects of MOS were observed in tropical rock lobster and marron (Sang and Fotedar 2010c, 2010a). Dietary MOS improved the gut morphology of marron by increasing the number of villi/group and density of villi (Sang and Fotedar 2010a). This in turn resulted in improved ability to irrigate the gut, to protect the cuticle layer and/or in smooth movement of faces and to intake environmental water (Chisaka *et al.* 1999). Higher density of gut epidermis cells of marron was also consequence MOS dietary (Sang and Fotedar 2010a) indicating the improved ability in the transport, assimilation, storage of nutrients (Ferna´ndez *et al.* 2002) and transport of ions and water (Mykles 1979) through the digestive tract. In tropical

rock lobster, dietary MOS increased the absorption surface of the gut indicated by the higher internal perimeter/external perimeter ratio (Sang and Fotedar 2010c). Higher absorption area enhances the nutrient and energy assimilated process due to higher nutrient uptake, consequently, higher assimilated energy is stored in the tail muscle in the form of dry tail muscle biomass. The same effect of MOS on gut's absorption surface has been reported for sea bream (*Diplodus sargus*) (Dimitroglou *et al.* 2005), sole (*Solea solea*) (Dimitroglou *et al.* 2006), rainbow trout (*Oncorhynchus mykiss*) (Dimitroglou *et al.* 2008) and larval cobia (Salze *et al.* 2008b). Similarly, the digestive, absorptive and assimilated efficiency could be improved by the inclusion of MOS in the diets of crayfish. However, evidences of nutrient uptake and transportation, assimilation and digestive efficiency enhanced by MOS in crayfish are still unavailable in the literature. In addition, the changes in digestive enzyme profile of crayfish when manipulation with dietary MOS has not been studied. The influence of MOS on digestibility of main diet ingredients is not available for crayfish as reported for rainbow trout (Glencross *et al.* 2003). Those limitations provide the great opportunities for further researches on the mechanism in which MOS affects the digestive function of the gastrointestinal track of crayfish.

10.5 EFFECT OF MOS AND BETA GLUCAN ON IMMUNE SYSTEM OF CRAYFISH

In the haemolymph of crayfish, the hyaline cells are chiefly involved in phagocytosis. The semi-granular cells are the cells active in encapsulation which recognize and respond to foreign molecules and particles by degranulation and subsequently attaching and spreading on the foreign surface (Johansson and Söderhäll 1985). Together with granular cells, semi-granular cells also participate in cytotoxicity and storage and release of prophenoloxidase (proPO) activating system (Jackson 1994; Johansson *et al.* 2000).

MOS and BG applications have positive effects on the immune system of crayfishes as indicated by their haemocyte profiles (Table 10.2). MOS increased total haemocyte count, the proportion of granular and semi-granular cell and reduced proportion of hyaline cells of marron, yabbies and tropical rock lobster (Sang and Fotedar 2010c; Sang *et al.* 2010a, 2010b). Similar effects on total haemocyte count and granular cells were reported by BG on marron (Sang and Fotedar 2010b).

Although the proportion of hyaline cells was reduced, the total number of hyaline cells increased because of increase in total haemocyte counts when MOS and BG were applied (Sang *et al.* 2010a, 2010b). Haemolymph clotting time of marron and tropical rock lobster also decreased when MOS and BG were supplemented (Sang and Fotedar 2010c, 2010b; Sang *et al.* 2010a). In addition, MOS and BG reduced the bacteria loaded in the haemolymph of crayfish (Sang and Fotedar 2010c, 2010b; Sang *et al.* 2010a, 2010b). Therefore, this research has clearly shown that the applications of MOS and BG enhance the immune capacity of crayfish at cellular level.

The direct involvements of BG in activating the immune reaction of crayfish have been established. Söderhäll (1983) reported that serine protease, which involves in activation of proPO and a coagulation process can be specifically activated by the glucans. Moreover, proPO activating system of freshwater crayfish, *Pacifastacus leniusculus*, which consists of several proteins involved in the immune defence leading to melanin production, cell adhesion, encapsulation and phagocytosis, can also be activated by BG application (Söderhäll 1983). If previously reacted with glucan, the granular cells can be triggered to undergo exocytosis and a subsequent release of the proPO system from the granules by two endogenous proteins that are associated with the proPO-system, namely the 76 kD factor and the beta-1,3-glucan binding protein. The BG then contributes to activate the proPO to form phenoloxidase enzyme (Söderhäll and Cerenius 1992). In addition, BG improves the phagocytosis activity through increasing the activity of a series of microbicidal substances including phenoloxidase, superoxide anions, hydrogen peroxide, hydroxide ions, singlet oxygen, myeloperoxidase catalysed hypochlorites and various lysosomal enzymes (Ma *et al.* 1999; Misra *et al.* 2004). By contrast, the direct role of MOS in immune reaction of crayfish is still unknown and only reported for a few fish species such as rainbow trout (Staykov *et al.* 2007), tilapia (Abdel-Tawwab *et al.* 2008). Thus, the role of MOS on biological functions of haemocyte cells in immune reaction such as melanisation, encapsulation, cytotoxicity and phagocytosis are most obvious suggestions for future research.

MOS improved the higher ability of marron and lobster in defending bacterial infection and environmental stress. Accumulative mortality of MOS-fed marron was

reduced by 121% after 96 h of infection with *Vibrio mimicus* (Sang *et al.* 2009). Similarly, survival of MOS-fed tropical rock lobster (77.78 ± 5.55%) was higher comparing to non MOS-fed lobster (55.56 ± 5.55%) after 7 days infected with *Vibrio* spp. (Sang and Fotedar 2010c). Other immune indicators such as higher total haemocyte count, granular cells and neutral red retention time of lysosome were also observed in MOS fed marron and tropical rock lobster after challenged with *Vibrio* spp. (Sang *et al.* 2009; Sang and Fotedar 2010c). Sang *et al.* (2009) also reported the improved ability of MOS-fed marron to resist stressors such as NH_3 and air exposure. Total haemocyte count and granular cells were also higher in MOS fed lobster during the infected period (Sang and Fotedar 2010c). Higher performances of crayfish to bacterial infection are as a consequence of higher capacity of immune system generated by MOS administration. Unfortunately, very little information is available on the bacteria-infected crustaceans when fed MOS. On the contrary, in fish culture, improvement in the bacterial resistance was achieved by dietary MOS and was species specific and bacterial strain. Dietary inclusion of MOS did not reveal the improvement in resistance ability of channel catfish, *Ictalurus punctatus,* to *Edwardsiella ictaluri* infection (Welker *et al.* 2007). By contrast, the improvement in the resistant ability was observed in MOS fed tilapia, *Oreochromis niloticus,* infected with *Aeromonas hydrophila* (Abdel-Tawwab *et al.* 2008) and MOS fed sea bass (*Dicentrarchus labrax*) infected with *Vibrio alginolyticus* (Torrecillas *et al.* 2007). Therefore, difference bacterial strains/species infected on the MOS treated crayfish is highly recommended in the future research.

10.6 SUGGESTIONS FOR FURTHER RESEARCH

The dietary supplementation of immunostimulants, MOS and BG, resulted in numerous benefits to marron, tropical lobsters and yabbies and can be used as alternative for antibiotic in crayfish culture. However, research into the direct roles of MOS and BG is currently limited and, of the available literature, only some species of crayfish have been investigated for the short term application of those immunostimulants. To further maximize the productivity of crayfish and obtain the most desired outcomes of immunostimulant application, a number of key research priorities are suggested based on the information reviewed previously.

The period to reach typical marketable size for marron is about three years; it is more than one and a haft year for tropical rock lobster and about 1 year for yabbies. However, the effects of MOS and BG have only been only examined in those crayfishes for a short time at juvenile stages. Thus there is a need to validate the effects of MOS and BG in the whole culture cycle of crayfishes at commercial farm. In addition, apparent digestibility of diet ingredient when MOS are supplemented needs to be determined. This would assist in the usage of cheaper protein sources instead of expensive and unreliable fishmeal. In addition, the economical and environmental benefits of MOS applications in crayfish culture need to be quantified in future research.

To date, the understanding on benefits of MOS on the digestive tract of crayfishes within the literature is rather limited, only morphological and total bacterial dynamic in the gut is available. Thus, future researches need to focus on validation of gastrointestinal track microbial community of crayfishes. Furthermore, roles of MOS in the nutrient transportation, nutrient uptake digestive enzyme activity profile in the gastrointestinal track of crayfishes need to be investigated in order to obtain more fully gastrointestinal characterizes manipulated by MOS.

Although MOS have been reported to improve the immune capacity of crayfish, whether the MOS stimulate the immune reactions such as prophenoxidase activating system, phagocytosis, cytotoxicity is not clear. Therefore, further research to understand this is warranted.

CHAPTER 11: CONCLUSIONS AND RECOMMENDATIONS

11.1 CONCLUSIONS

The hypothesis (H_o) is "that there is no significant difference in the performance, physiological condition and immunological capacity in terms of growth, survival, total haemocyte count, different haemocyte count, clotting time, bacteraemia, gut health status as well as tolerant capacity to stressor of decapod crustaceans fed with and without immunostimulant". Based on the results obtained from the experiments conducted for the project, H_o is rejected. The following summary indicates that the objectives of the research have been achieved:

1. Physiological indicators of marron such as TMIdry, TMIwet and TM% changed with size class while HSIwet, HSIdry and HM% were size-class independent (Objective 1).

2. Immunological parameters of marron were size dependent except for DHC (Objective 1).

3. Micro villi density is depended on marron size. The micro villi group distribute in the hindgut of marron is 4 - 8 villi/group (Objective 1).

4. MOS inclusion levels of 0.2 and 0.4% in the diet or BG inclusion levels of 0.1 and 0.2% in the diet are the most appropriate concentrations for marron culture (Objective 2).

5. Dietary MOS or BG enhanced survival and total production yield of marron, however, the growth of marron is not improved when MOS or BG is applied (Objective 2).

6. Dietary MOS or BG improves the physiological condition by increasing TMIwet and TMIdry of marron (Objective 2).

7. MOS supplemented diet results in higher density of micro villi and epidermis cells in the gut of marron (Objective 2).

8. Higher total bacteria and bacteria/vibrio ratio is observed in the gut of marron fed MOS supplemented diets (Objective 2).

9. Higher growth and survival are attained for yabbies fed MOS supplemented diet (Objective 3).

10. Dietary MOS changes the digestive enzyme activity by increasing amylase activity in the gut and protease activity in the hepatopancreas of yabbies (Objective 3).

11. Higher survival and better growth rates are obtained when MOS is supplemented in the diet of tropical rock lobster juvenile (Objective 4).

12. MOS inclusion diet results in a better health status of lobster, indicated by higher TMIwet, TMIdry and HSIwet (Objective 4).

13. Dietary MOS increases total bacteria and bacteria/vibrio ratio in the gut of tropical rock lobster (Objective 4).

14. Increased nutrient absorption surface area, indicated by higher internal/external perimeter ratio of the gut, is attained in MOS fed lobster (Objective 4).

15. Dietary administration of BG reduces bacteria load in haemolymph while increases total haemocyte count and proportion of granular cells in marron (Objective 5).

16. Dietary MOS enhances total haemocyte count, proportion of granular cells whereas decreases bacteria load in haemolymph and shortens haemolymph clotting time of marron (Objective 5).

17. Dietary MOS increases total haemocyte count and proportions of granular and semi-granular cells while decreases the proportion of hyaline cells of yabbies (Objective 6).

18. Supplementation of MOS in the diet increases total haemocyte count and proportion of granular cells and reduces the bacteria load in the haemolymph of tropical rock lobster (Objective 6).

19. Mortality of MOS fed is lower than non MOS fed marron and lobster when they are infected with *Vibrio* spp. (Objective 7).

20. Dietary MOS also improves survival of marron subjected to NH_3 stress (Objective 7).

21. Immunological indicators such as total haemocyte count, differentiate haemocyte count, bacteraemia, neutral red retention time of lysosome of MOS fed marron are better than non MOS-fed marron when they are challenged with bacteria, subjected to NH_3 or air exposures (Objective 7).

22. Better immunological condition is attained in tropical rock lobster fed MOS diet compared to the lobster fed non MOS supplemented diet when they are infected with *Vibrio* spp. (Objective 7).

11.2 RECOMMENDATIONS

Based on the outcomes from the current research, further research should focus on following aspects (Objective 8):

1. Performance in terms of growth, survival, physiology and immunology of decapod crayfish and lobster should be evaluated when MOS is applied throughout the whole culture cycle.

2. Other immunological indicators such as synthesise cells, agglutinating, phenoloxidase, haemocyte phagocyte and antibacterial activities, haemolymph protein profile, cell adhesion, superoxide anion production, levels of glucose and lactate in the plasma are important to evaluate the immune response of crustacean. Thus, future study should incorporate all these parameters to evaluate the MOS efficiency in crayfish and lobster.

3. Study involving the roles of MOS in stimulation/elimination of specific bacteria species/strains in the gut of crayfish and lobster is warranted.

4. The role of MOS in nutrient uptake and transportation needs to be investigated.

5. Study into the effects of dietary MOS on gene expression of crayfish and lobster is also recommended.

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