Immunological Responses of Customised Probiotics-Fed Marron,*Cherax tenuimanus*, (Smith 1912) When Challenged with *Vibrio mimicus*

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Abstract

A two-phased experiment was conducted to investigate the effects of dietary supplementation of customised probiotics on marron physiology. During the first phase marron were fed probiotic supplemented feed for 70 days, while in phase two the same marron were challenged with *Vibrio mimicus* and their physiological responses were investigated for 4 days post-challenged.

The experiment was carried out in a purpose-built room, designed for aquaculture research, using 18 of 250 L cylindrical plastic tanks. Five species of isolated probiotic bacteria from commercial probiotic products and marron's intestine were tested in this experiment. The probiotic bacteria were (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); and PM4 (*Bacillus* sp.), which were added to the formulated basal marron diet (34% crude protein, 8% crude lipid, 6% ash) at a concentration of 10^8 cfu/g of feed. Immune responses of marron fed probiotics were evaluated by investigating organosomatic indices, growth rate, survival, intermoult period, total haemocytes counts (THC), proportion of granular cells (GC), bacteraemia, bacteria load in the intestine and water quality. The results showed that dietary supplementation of probiotics in marron had no significant impact on growth, intermoult period and survival of the marron. However, their supplementation improved the physiological condition of marron in terms of significantly higher tail muscle indices, THC and proportion of granular cells (GC) and reduced bacterial load in the haemolymph. The addition of probiotics in marron diets also increased the bacteria load in the marron intestine.

In addition, dietary supplementation of the customised probiotics was effective in improving the resistance of marron against *V. mimicus* as they had higher THC, higher proportion of GC and lower presence of bacteria in their haemolymph, after marron were challenged with *V. mimicus*. The results also showed that probiotic *Bacillus mycoides* (A10) and PM4 are the most beneficial dietary probiotics for marron health.

Key words: probiotics, physiological condition, immunological responses, marron

1. Introduction

Since the abuse of antibiotics and other chemicals for disease management in many aquaculture facilities were uncovered, safety in seafood products has received public attention. The research has shown that mismanagement of antibiotics use can lead to the emergence of bacterial resistance species [1]. Consequently, the research now is more focused in finding an alternative to antibiotics for disease management. Recently, probiotics have emerged as an alternative to antibiotics for disease management [2, 3]. The increasing demand for environment-friendly aquaculture has also led to probiotics becoming more popular as prophylactic agents and providers of improved nutrition [4, 5].

In recent years, studies in shrimp aquaculture have demonstrated that probiotics are beneficial for enhancing growth [6-9] and the immune system [10-15], by combating the pathogens through competitive exclusion mechanism [16-20]. The use of probiotics has also resulted in improving water and sediment quality [6, 10, 21, 22] and thus, leading to higher survival rates of healthier animals [1, 23-25]. Some species of probiotics have the ability to protect against viruses, although the mechanism of combating the virus is not fully understood [6, 26-28]. In aquaculture, probiotics can be applied either as a food supplement or as a water additive [21].

Marron, *Cherax tenuimanus* (Smith 1912) is one of the important freshwater crayfish species native to Western Australia [29]. Marron have attracted global interest as a potential aquaculture species due to their positive attributes, such as large harvest-size (up to 2 kg), higher price, non-burrowing behaviour, simple life cycle and ease of live transport [29-32].

Though fungus and parasites are dominant disease causing agents in marron aquaculture, yet prevalence of infection and incidence of mortality are relatively low [33, 34]. *Epistylis* and *Temnocephala* are two epibionts which are commonly found in marron, caused by poor water quality, particularly in unaerated ponds containing excessive organic matter [29]. These epibionts can decrease growth rates and reduce consumer appeal [31]. Although there is no current report on the losses in marron aquaculture caused by bacterial infection, the threat of marron getting bacterial infection are realistic as the industry grows and expands. *Vibrio mimicus* has emerged as a dominant bacterial pathogen of freshwater crayfish in aquaculture [35, 36].

The marron health can ultimately be assessed by growth and accepted survival rates, however, other physiological indicators, such as organosomatic indices, moisture content and osmoregulatory capacity, total haemocyte counts (THC), proportion of granular cells, bacteraemia and haemolymph clotting time [37], can also be used to understand the underlying mechanism for marron health. There has been no study on the effect of supplementation of probiotics in marron diet, therefore it is important to evaluate the effectiveness of dietary probiotics for the benefit of marron health under aquaculture environment.

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

2. Materials and Methods

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

2.1. Experimental system

The experimental system was setup in a purpose-built laboratory designed for aquaculture research in the indoor aquarium facility of the Curtin Aquatic Research Laboratory (CARL), Curtin University, Perth, Western Australia. The experimental system

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

2.2 Experimental animals

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

2.3. Test diets

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

Five species of probiotic bacteria isolated from the various sources were selected for their growth inhibition capabilities against *Vibrio mimicus* and *V. cholera* non-01 and then tested for pathogenicity by feeding to marron in a tank trial (unpublished results). *Bacillus mycoides* (A10) and *Shewanella* sp. (A12) were selected from a number of healthy farmed marron intestines. ; *Bacillus* sp. (AQ2) was selected a commercial product from Aquasonic Pty. Ltd. and finally, *Bacillus subtilis* (PM3) and *Bacillus* sp. (PM4) were selected from another commercial probiotic product supplied by Enviroplus Pty Ltd., Perth Australia.: All probiotics were identified by the Bacteriology Laboratory, Animal Health Laboratories, Department of Agriculture and Food, Western Australia. These selected probiotics have not been reported as known pathogens of marron [38]. A basal diet without any probiotic supplementation was used as a control diet.

The probiotics were supplemented to the basal diet using a described procedure [28], with some modifications. The isolated probiotics from stock culture were re-grown onto a new blood agar plate. After overnight incubation at 25^{o}C, an appropriate inoculum of each probiotic species was diluted into 20 mL of sterilized normal saline. Before being sprayed onto the basal diet, all feeds were coated with fish oil blend (Bait mate®, Western Australia) at 20 mL per kg basal diet. A concentration of 10^{8} cfu/g of feed was selected following the previous studies [39-42]. The probiotic species were sprayed onto 1 kg of basal diet (10^{8} cfu/g feed) and then immediately covered with aluminium foil and stored in a refrigerator at 4^{o}C to avoid bacterial growth. The concentration in cfu/mL, of each probiotic bacterium sprayed onto the feed was determined using an established method [43] where optical density (Spectrophotometer, BOECO S-20, Hamburg, Germany) correlates to the bacterial concentration. The concentration sprayed onto the feed was confirmed by performing a total bacterial count using blood agar plates and an overnight incubation at 25^{o}C.

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

Eighteen 250 L cylindrical plastic tanks were used to culture marron in a laboratory scale experiment. Each tank was stocked with 7 healthy juvenile marron which were cultured

for 70 days, a time considered optimal for studying the marron growth and effects of probiotics. The marron were fed with a basal (control) diet and probiotic-supplemented diets at a rate of 1.5% / body weight every alternate day during the experimental period. Each treatment was set up in triplicate. The effect of feeding treatments were measured in terms of growth, survival, intermoult period, physiological response (organosomatic indices), immune responses (total haemocyte count, differential haemocyte count and bacteraemia), bacteria load in the intestine and water quality parameters.

2.5. Challenge test with Vibrio mimicus – phase 2

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

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

2.6. Data collection

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

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

Marron survival rate, specific growth rate (SGR) and biomass increment were determined after every 14 days.

The presence of moults in tanks were checked daily and removed immediately. intermoult period was determined as per established equation [44, 45].

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

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

The wet hepatosomatic index (H_{iW}), tail muscle to wet body ratio (T/B_W), the percentage moisture of the hepatopancreas (HM%) and tail muscle (TM%), wet hepatopancreas index (Hiw), dry hepatopancreas index (Hid), wet tail muscle to wet body weight ratios (T/Bw) and dry tail muscle to wet body weight ratios (T/Bd) were calculated as per established equations [46, 47]. Moisture content and organosomatic indices assessments were conducted at day 0, 35 and 70.

The immune parameters of marron, such as total haemocyte count (THC) and differential haemocyte counts (DHC) were determined following the established protocol [46, 48]. Bacterial loads in the haemolymph (bacteraemia) were assessed by using the established method [28]. All of the immune parameters were determined at commencement of the experiment (0 day), in the middle of the experiment (35^{th} day) and at the end of the experiment (70^{th} day).

The bacterial load in the haemolymph (bacteraemia) was assessed using total bacterial counts on blood agar plates. Individual drops of the haemolymph aliquot were placed onto

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

The bacterial load in the intestine (IBL) was determined following the protocol [4], with some modification, on the initial (0 day), middle (35^{th} day) and at the end of the experiment (70^{th} day). The marron samples were selected and rinsed in distilled water, quickly washed with 70% alcohol and then rinsed again in sterilised distilled water to remove the external bacteria. The intestinal tract of each marron was removed and weighed. A sterilised mortar was used to homogenise the intestine, then diluted serially with sterilised normal saline water, and was lawn inoculated to blood agar plates. The plates were incubated for 24 hours at 25 °C. The bacterial loads were ranked from1 (1 – 250 cfu/mL) to 10 (2501-3000 cfu/mL) and the rank 11 was used for "too numerous for an accurate count".

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

2.6.2. Challenge test with Vibrio mimicus – phase 2

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

2.7. Data analysis

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

3. Results

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

3.1.1. Organosomatic indices

There were no significant differences ($P > 0.05$) in HM%, TM%, H_{iw}, H_{id}, T_{iw} and T_{id} among any marron fed probiotic supplemented diets over any sampling periods (Table1). However, marron fed AQ2 showed a significantly higher ($P<0.05$) in T_{iw} and T_{id} at day 35 than day 0.

3.1.2. Immune responses

The mean THC, the mean proportion of granular cell (GC) and the mean haemolymph bacteria ranks (bacteraemia) of the marron fed different probiotic diets were not significantly different (P>0.05) until day 35 of feeding (Table 2). However, at the end of phase-1, the mean THC in all marron fed probiotic diets was significantly higher (P<0.05) than the control diet. The highest mean THC was for the A10 diet and the lowest was for the marron fed the control diet, while other treatments had similar levels of THC. At the end of the day 70, the mean GC was significantly higher (P < 0.05) in the marron fed AQ2, A10 and PM4 supplemented diets. The highest proportion of GC was associated with the PM4 supplemented diet (35.54± 4.79 %) and the lowest one was with the control diet (10.64±0.60). At the end of phase-1, the mean bacteraemia was significantly lower (P<0.05) in the marron fed probiotic supplemented, than in the marron fed the control diet. The marron fed PM3 and PM4 diets had the lowest bacteraemia, followed by marron fed A10 and AQ2 respectively. The bacteraemia of PM3-treated marron decreased gradually over the culture period, while other parameters fluctuated during phase 1 of the trial.

3.1.3. Intestinal bacteria load (IBL)

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

3.1.4. Survival rate, growth and intermoult period

There was no significant difference ($P > 0.05$) in the mean survival rates, mean biomass increment (BI %) and specific growth rate (SGR) in marron fed any diets. Intermoult period (days) ranged between 25.25 ± 8.50 to 43.00 ± 1.00 and there was no significant difference ($P > 0.05$) between marron fed different diet.

3.2. Challenge test with Vibrio mimicus – phase 2

3.2.1. Total haemocyte count (THC)

The mean THC of marron were not significantly different among any dietary treatments before the *V. mimicus* challenge (Table 3). After *V. mimicus* injection, there were significant decreases ($P <0.05$) in the mean THC in all treatments at 24 hours, 48 hours and 96 hours. At 24 hours post injection, the THC of marron fed probiotic-supplemented diets were significantly higher (P<0.05) than those fed the control diet. The marron fed the diet supplemented with PM3 showed the highest THC (1.53±0.35 $\cdot$ x 10^6 cells/mL), whereas the marron fed the control diet had the lowest THC (0.36±0.07 x 10^6 cells/mL). After 48 hours into the challenge, the THC of marron fed probiotic-supplemented diets were significantly higher (P<0.05) in marron fed A10, A12 and PM4, whereas the other dietary treatments showed similar values as the control. After 96 hours of *V. mimicus* injection, A10, PM3 and PM4 treatments showed significant differences ($P <0.05$) than the control. Whereas, other probiotic fed marron had similar mean THC to those of the control.

3.2.2. Differential Haemocyte Count (DHC)

Before the *V. mimicus* was injected, the mean proportions of granular cells (GC) of marron fed the probiotic-supplemented diets were significantly different ($P < 0.05$) than the control. The PM4 treatment produced the highest GC proportion, followed by the A10 treatment (Table 3). Other treatments had a similar proportion of GC as the control. After *V. mimicus* injection, marron exhibited a declining trend ($P <0.05$) in the mean GC proportions in all treatments. At 24 hours post-injection, there was a significant difference (P<0.05) among treatments, with the PM3 producing the highest GC proportion and the control

showing the lowest. At 48 hours into the challenge, there was a significantly higher (P < 0.05) GC proportion for A12 and PM4 supplemented diets than others. At 96 hours, the GC proportion of marron fed probiotic-supplemented diets were significantly higher (P<0.05) than the marron fed control diet.

3.2.3. Bacteraemia

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

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

3.2.4. Survival Rate

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

4. Discussion

Hepatopancreas and tail muscle indices have been widely used as tools to monitor the effects of different culture environments and diets on penaeid [49-53] and non-penaeid shrimps [54, 55]. The present study shows that probiotic supplementation of *Bacillus sp* on marron diet did improve the physiological condition of marron, as shown by the changes in dry tail index (T_{id}). The significantly higher T_{id} observed in marron fed a diet with *Bacillus* sp. indicates that this probiotic bacteria helped the marron to absorb nutrients more efficiently and thus, they could store more energy in their tail muscle [56]. Similarly, probiotic bacteria in the formulated shrimp diet, act as a facilitator to digest all of the protein components, which in turn synthesises required enzymes responsible for increasing the shrimp's protease activity and food digestibility [57].

Haemocytes perform a key role in the host-defence mechanism of crustaceans by destroying micro-organic invaders [58, 59]. Environmental conditions, such as the presence of stressors and disease, have a relationship with the number of circulating haemocytes in crayfish [60]. A poor health condition in crayfish can be reflected by a low number of total haemocyte counts in their haemolymph [55]. The results from this study showed that marron fed probiotic diets had higher THC counts than those fed the control diet; this indicates that marron are healthier when they are fed a probiotic supplemented diet. The THC of the western king prawn (*Penaeus latisulcatus*) also increased gradually when they were treated with two selected probiotics (*Pseudomonas synxantha* and *Pseudomonas aeruginosa*) [61]. Administration of *Bacillus licheniformis* in the *Litopenaeus vannamei* diet enabled an increase in the THC [62]. In the present study, *Bacillus mycoides* produced the highest THC, demonstrating its higher effectiveness than other probiotics.

After being injected with *V. mimicus,* the THC decreased sharply. Similar results has been reported in Chinese shrimp (*Fenneropenaeus chinensis*) injected with *Vibrio anguillarum* [63]. Another study also found the THC of *V. mimicus* injected marron declined [64]. Environmental stressors and diseases can influence the number of circulating haemocytes [55, 60, 65]. The decrease in THC after *V. mimicus* injection, could be associated with an inflammatory response of the haemocytes leaving the circulation and migrating to the site of the injection [66, 67]. However, the THC of marron fed probiotic diets remained higher than those fed the probiotic-free diet during the challenge test. Similar results has been reported in white shrimp (*Litopenaeus vannamei*) when challenged with *V.*

harveyi, after administration of probiotic bacteria (*Lactobacillus plantarum*) [68]. The higher THC of the marron fed a probiotic-supplemented diet in the present study indicates the greater ability of the marron haemocytes in the degranulation process to fight against the foreign substance. Among the probiotics diet, PM4 (*Bacillus* sp.) resulted in the highest THC. *Bacillus* sp. is known as an effective probiotic bacteria in shrimp aquaculture and has been studied for its effectiveness by many researchers, under both laboratory and commercial trials [6, 12, 24, 69, 70].

In the present study, the marron diets with probiotic supplementation of AQ2 (*Bacillus* sp.), A10 (*Bacillus mycoides*) and PM4 (*Bacillus* sp.) produced significantly higher proportions of granular cell in marron haemolymph than the control diet (Table 2). Therefore, indicating a positive effect of the probiotic, *Bacillus spp* in enhancing the health condition of these marron [55, 71]. After *V. mimicus* injection, the proportions of granular cells in marron were decreased in all treatments. Similar results were also reported where an injection of 20 uL of 0.53 ± 10^6 cfu/mL *V. mimicus* reduced the proportion of GC [64]. In other species the reduction of GC proportion have been reported in black tiger shrimp (*Penaeus monodon*) [67] and Chinese shrimp (*Fenneropenaeus chinensis*) [63] when they were injected with *Vibrio anguillarum.* A decreasing GC proportion suggests that GC degranulates at first, followed by lysis of the lysosomes [63]. Nevertheless, the proportion of GC of marron fed with probiotic diets remained higher than GC of marron fed probiotic-free diet (control) during the challenge test. Similar results have been reported for western king prawn (*Penaeus latisulcatus*) when challenged with *Vibrio harveyi* at 10^3, 10^5, and 10^7 CFU/mL [72]. The marron fed the diets supplemented with genus *Bacillus* (AQ2, PM3 and PM4) and then challenged with high dosage of *V. mimicus* had higher GC proportions than other treatments. Similar to shrimps, *Bacillus* surface antigens, or their metabolites, act as immunogens by stimulating phagocytic activity of granulocytes [73].

The bacteraemia of marron fed probiotic supplementation in their diets was lower than the marron fed control diet. The probiotics act as immunostimulants to stimulate the non-specific immune system against bacterial infection [6, 13, 74]. PM3 (*Bacillus subtilis*) and PM4 (*Bacillus* sp) supplementations had the lowest haemolymph bacteraemia. Probiotic bacteria *Bacillus,* not only competes for nutrients and thus inhibits other bacteria from growing rapidly [5], they naturally are also able to produce many different antibiotic compounds [21]. At 24 and 48 h post injection with *V. mimicus,* the bacteraemia values increased sharply in all treatments. Similar results has been reported wherein bacteraemia of marron fed the control diets (without immunostimulant Bio-Mos^T) increased significantly 24 and 48 h post *V. mimicus* injection [64]. However, the bacteraemia of marron fed probiotic-supplemented diets had lower levels of bacteraemia than the control diet after 96 hours. This indicates that the probiotics were effective in reducing the bacterial load in the marron haemolymph. In western king prawn, administration of two combined probiotics, *Pseudomonas synxantha* and *Pseudomonas aeruginosa* (10^5 cfu/mL), at 20 mL/kg feed, reduced the number of bacteria in the haemolymph [28, 61]. The increase bacteraemia levels in haemolymph indicates that the immune capacity of the animal has declined and thus, possibly can result in increased susceptibility to infections [71].

The average survival rate of the marron in the feeding trial (phase 1) was 46.03% of the initial stocking biomass of approximately 103.82 g/m^2. There was no evidence to prove that supplementation of the probiotics in marron diets improved marron survival rate. However, this rate is still higher than marron cultured in the earthen ponds, where the survival of marron ranges from 13.82% to 34.66% with a lower stocking density (4.01 ± 0.28 g/m^2) [53, 75]. When beta 1,3 β glucan is used as an immunostimulant, more than 65% of the initial stocking biomass (16.83 g/m^2) of marron can be achieved [37]. In an intensive battery culture system the survival rate can also be higher (71%) than the present trial, due to absence of cannibalism [76]. In the present study, the average survival was relatively low as marron mortality was mainly caused by cannibalism. Cannibalism frequently occurs when a population of crayfish is at high density and it is often triggered by moulting individuals [77].

In shrimp aquaculture, the application of probiotics, either through mixing into the water or supplementing with feed, has been reported to increase the survival rate [3, 6-8, 12,

28, 61, 70]. The injection of *V. mimicus* did not affect the survival of marron (59.04 ± 1.64 g initial mean weight). However, it took a few days for marron to recover and resume its response to the feed given. Different results have been reported where 20 uL of 0.53 x 10^6 cfu/mL of *V. mimicus* stock solution injected into marron (10.44±0.20 g initial mean weight) resulted in 100% mortalities in marron [64]. The different results in the present experiment could be attributed to the low dosage of injected *V. mimicus* per unit weight of the marron, as the mean weight of the marron was five times higher than those used by other authors [64].

Injection of *V. alginolyticus* into white shrimp (*Litopenaeus vannamei*) at dosages of 10^5 and 10^6 cfu/shrimp could produce survival rate of 40 – 50% [78, 79]. *P. monodon* mean weight of 18g survived for 6 days after challenged by bath exposure to 10^7 cfu/mL of *V. harveyi* 1526 in aquarium water [80], whereas, when challenged with 40 – 200 x 10^6 cfu of *V. anguillarum* at body weight of 2 – 4 g, *P. monodon* survived for a week [67]. Furthermore, the isolates of *V. mimicus a*t a dosage of 10^5 cfu, can cause high mortalities in *Cherax albidus* [81]. This comparison shows that the survival of injected animals is dependent on species of the pathogenic bacteria, the mean weight of the host species and the virulent effects of the pathogen, rather than numbers of bacteria. Hence, in considering the resistance of marron against *V. mimicus*, the virulent effect and the size of marron need to be considered to explain the effects of a probiotic on marron survival.

Marron fed probiotic-supplemented diet in the present study did not show any growth improvement or biomass increment, although supplementation has been reported to increase the growth in other cultured shrimps [7, 8, 82, 83]. The average SGR in the present experiment ranged from 0.13 to 0.66, with moult interval ranging between 25.5 to 43 days. The average SGR of marron was different in various culture conditions; pond culture studies range from 0.5 – 2.1 and intensive crayfish culture system studies range from 0.4 – 1.1 [84]. The intermoult period in juvenile marron ranged between 15 to 45 days [55, 85]. The presence of probiotic bacteria in shrimp intestine can increase the health status of the host by providing competitive exclusion in the shrimp's gut [86, 87]. Moreover, probiotics may synthesise the vitamins that can lead to increased digestive activity or improve enzymatic activities; therefore, this could be responsible for weight increase, improved digestion and/or nutrient absorption [9].

The present study showed that marron fed probiotics had higher intestinal bacteria ranks than those fed the control diet, especially diet supplemented with *Bacillus mycoides*. Though our studies could not confirm whether higher bacterial load in the intestine of marron are due to increased densities of probiotic species, other studies have reported that the administration of probiotic *Bacillus licheniformis* can improve the white shrimp's intestinal micro-flora and its immunity by colonizing and replacing pathogen bacteria in their intestine [12, 69].

In a shrimp's digestive tract, *Bacillus* enhance the specific activity of lipase, protease and amylase [8]. In the current study, most species of probiotic bacteria for the trial came from the genus *Bacillus,* as the enzymes of *Bacillus* are very efficiently in digesting a large variety of carbohydrates, lipids and proteins into smaller units [5]. In shrimp culture within ponds, *Bacillus* species also degrade organic materials [1, 12], thus improving the water quality.

In conclusion, using the matrix (Table 4) results from phase 1 and 2 showed that A10 can achieved the highest score, followed by PM4, AQ2, PM3, and A12. Furthermore, A10 (*Bacillus mycoides*) and PM4 (*Bacillus sp*.) are most effective probiotic supplements in the marron diets. In the present study, marron fed probiotic supplemented diets produced beneficial outcomes in terms of physiological condition (organosomatic indices), immune parameters (THC, DHC, GC proportion and bacteria load in haemolymph) and level of the bacteria load in the intestine than control diet. However, further research is required to investigate the effects of supplementing two or three different species in marron diets under a commercial farming environment.

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References

1. Verschuere, L., et al., *Probiotic Bacteria as Biological Control Agents in Aquaculture.* Microbiol. Mol. Biol. Rev., 2000. **64**(4): p. 655-671.
2. Farzanfar, A., *The use of probiotics in shrimp aquaculture.* FEMS Immunology & Medical Microbiology, 2006. **48**(2): p. 149-158.
3. Zhou, X.-x., Y.-b. Wang, and W.-f. Li, *Effect of probiotic on larvae shrimp (Penaeus vannamei) based on water quality, survival rate and digestive enzyme activities.* Aquaculture, 2009. **287**(3-4): p. 349-353.
4. Li, J., B. Tan, and K. Mai, *Dietary probiotic Bacillus OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (Litopenaeus vannamei).* Aquaculture, 2009. **291**(1-2): p. 35-40.
5. Ninawe, A.S. and J. Selvin, *Probiotics in shrimp aquaculture: Avenues and challenges.* Critical Reviews in Microbiology, 2009. **35**(1): p. 43-66.
6. Balcázar, J.L., et al., *The role of probiotics in aquaculture.* Veterinary Microbiology, 2006. **114**(3-4): p. 173-186.
7. Gomez, R., D. Geovanny, and M.A. Shen, *Influence of Probiotics on the Growth and Digestive Enzyme Activity of White Pacific Shrimp (Litopenaeus vannamei).* Ocean Univiversity of China., 2008. **7**(2): p. 215-218.
8. Ziaei-Nejad, S., et al., *The effect of Bacillus spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp Fenneropenaeus indicus.* Aquaculture, 2006. **252**(2-4): p. 516-524.
9. Gullian, M., F. Thompson, and J. Rodriguez, *Selection of probiotic bacteria and study of their immunostimulatory effect in Penaeus vannamei. .* Aquaculture, 2004. **233**((1-4)): p. 1-14.
10. Li, J., et al., *Comparative study between probiotic bacterium Arthrobacter XE-7 and chloramphenicol on protection of Penaeus chinensis post-larvae from pathogenic vibrios.* Aquaculture, 2006. **253**(1-4): p. 140-147.
11. Perdigon, G. and S. Alvarez, *Probiotic and the immune state*, R. Fuiller, Editor 1992, Chapman and Hall. p. 144-180.
12. Rengpipat, S., et al., *Effects of a probiotic bacterium on black tiger shrimp Penaeus monodon survival and growth.* Aquaculture, 1998. **167**(3-4): p. 301-313.
13. Rengpipat, S., et al., *Immunity enhancement in black tiger shrimp (Penaeus monodon) by a probiont bacterium (Bacillus S11).* Aquaculture, 2000. **191**(4): p. 271-288.
14. Rengpipat, S., et al., *Enhanced growth and resistance to Vibrio challenge in pond-reared black tiger shrimp Penaeus monodon fed a Bacillus probiotic* Diseases Of Aquatic Organisms, 2003. **55**: p. 169-173.
15. Sahu, M., et al., *Probiotics in aquaculture: importance and future perspectives.* Indian Journal of Microbiology, 2008. **48**(3): p. 299-308.
16. Thompson, J., et al., *An in vitro and in vivo assessment of the potential of Vibrio spp. as probiotics for the Pacific White shrimp, Litopenaeus vannamei.* Journal of Applied Microbiology, 2010: p. no-no.
17. Castex, M., et al., *Effect of probiotic Pediococcus acidilactici on antioxidant defences and oxidative stress of Litopenaeus stylirostris under Vibrio nigripulchritudo challenge.* Fish & Shellfish Immunology, 2010. **28**(4): p. 622-631.

18. Hai, N.V., N. Buller, and R. Fotedar, *Effects of probiotics (Pseudomonas synxantha and Pseudomonas aeruginosa) on the growth, survival and immune parameters of juvenile western king prawns (Penaeus latisulcatus Kishinouye, 1896).* Aquaculture Research, 2009. **40**(5): p. 590-602.
19. Purivirojkul, W., M. Maketon, and N. Areechon, *Probiotic Properties of Bacillus pumilus, Bacillus sphaericus and Bacillus subtilis in Black Tiger Shrimp (Penaeus monodon Fabricius) Culture.* Kasetsart Journal Natural Science 2005. **39**: p. 262 - 273.
20. Sun, Y.-Z., et al., *Probiotic applications of two dominant gut Bacillus strains with antagonistic activity improved the growth performance and immune responses of grouper Epinephelus coioides.* Fish & Shellfish Immunology, 2010. **29**(5): p. 803-809.
21. Moriarty, D.J.W., *Control of luminous Vibrio species in penaeid aquaculture ponds.* Aquaculture, 1998. **164**(1-4): p. 351-358.
22. Wang, Y.B., Z.R. Xu, and M.S. Xia, *The effectiveness of commercial probiotics in northern white shrimp Penaeus vannamei ponds.* Fisheries Science, 2005. **71**(5): p. 1036-1041.
23. Far, H.Z., et al., *Effect of Bacillus subtilis on the growth and survival rate of shrimp (Litopenaeus vannamei).* African Journal of Biotechnology, 2009. **8**: p. 3369-3376.
24. Gomez-Gil, B., A. Roque, and J.F. Turnbull, *The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms.* Aquaculture, 2000. **191**(1-3): p. 259-270.
25. Irianto, A. and B. Austin, *Probiotics in aquaculture.* Journal of Fish Diseases, 2002. **25**(11): p. 633-642.
26. Direkbusarakom, S., et al., *The dominant flora in shrimp hatchery against some fish pathogenic viruses.* Journal of Marine Biotechnology, 1998. **6**(4): p. 266-267.
27. Girones, R., J.T. Jofre, and A. Bosch, *Isolation of marine bacteria with antiviral properties.* Can. J. Microbiol, 1989. **35**: p. 1015-1021.
28. Hai, V.N. and R. Fotedar, *Comparison of the effects of the prebiotics (Bio-Mos® and [beta]-1,3-D-glucan) and the customised probiotics (Pseudomonas synxantha and P. aeruginosa) on the culture of juvenile western king prawns (Penaeus latisulcatus Kishinouye, 1896).* Aquaculture, 2009. **289**(3-4): p. 310-316.
29. Lawrence, C. and C. Jones, *Cherax*, in *Biology of Freshwater Crayfish*, D.M. Holdich, Editor 2002, Blackwell Science Ltd. p. 377-438.
30. Morrissy, N.M., *Experimental pond production of marron, Cherax tenuimanus (Smith) (Decapoda: Parastacidae).* Aquaculture, 1979. **16**: p. 319-344.
31. Morrissy, N.M., L. Evans, and J. V. Huner, *Australian freshwater crayfish: Aquaculture species. World Aquaculture* 1990. **21**: p. 113-122.
32. Rouse, D.B. and I. Kartamulia, *Influence of salinity and temperature on molting and survival of the Australian freshwater crayfish (Cherax tenuimanus).* Aquaculture, 1992. **105**(1): p. 47-52.
33. Herbert, B., *Notes on diseases and epibionts of Cherax quadricarinatus and C. tenuimanus (Decapoda: Parastacidae).* Aquaculture, 1987. **64**(3): p. 165-173.
34. Langdon, J.S., *Microsporidiosis due to a pleistophorid in marron, Cherax tenuimanus (Smith), (Decapoda: Parastacidae).* Journal of Fish Diseases, 1991. **14**(1): p. 33-44.
35. Wong, F., K. Fowler, and P.M. Desmarchelier, *Vibriosis due to Vibrio mimicus in Australian freshwater crayfish. .* Journal of Aquatic Animal Health, 1995. **7**: p. 284-291.
36. Evans, I.H. and B.F. Edgerton, *Pathogens, Parasites and Commensals, in Biology of Freshwater Crayfish, D.M. Holdich, Editor 2002, Blackwell Science Ltd. p. 439-464.*
37. Sang, H.M. and R. Fotedar, *Effects of dietary β – 1,3 – glucan on the growth, survival, physiological and immune response of marron, Cherax tenuimanus (smith, 1912).* Fish & Shellfish Immunology, 2010. **28**(5-6): p. 957-960.
38. Buller, N., *Bacteria from Fish and Other Aquatic Animals: A Practical Identification Manual, .* CABI Publishing, Oxford shire, UK, 2004.

579 39. Keysami, M.A., *Effect of Bacillus subtilis on growth development and survival of larvae*
580 *Macrobrachium rosenbergii (de Man).(Author abstract).* Aquaculture nutrition, 2007. **13**(2):
581 p. 131.
582 40. Keysami, M., *Probiotic activity of Bacillus subtilis in juvenile freshwater prawn,*
583 *Macrobrachium rosenbergii (de Man) at different methods of administration to the feed.*
584 Aquaculture international, 2012. **20**(3): p. 499-511.
585 41. Liu, C.-H., *Dietary administration of the probiotic, Bacillus subtilis E20, enhances the growth,*
586 *innate immune responses, and disease resistance of the grouper, Epinephelus coioides.* Fish
587 & shellfish immunology, 2012. **33**(4): p. 699.
588 42. Zhang, Q., *Dietary administration of Bacillus (B. licheniformis and B. subtilis) and*
589 *isomaltooligosaccharide influences the intestinal microflora, immunological parameters and*
590 *resistance against Vibrio alginolyticus in shrimp, Penaeus japonicus (Decapoda: Penaeidae).*
591 Aquaculture research, 2011. **42**(7): p. 943.
592 43. Van Hai, N., R. Fotedar, and N. Buller, *Selection of probiotics by various inhibition test*
593 *methods for use in the culture of western king prawns, Penaeus latisulcatus (Kishinouye).*
594 Aquaculture, 2007. **272**(1-4): p. 231-239.
595 44. Reynolds, J.D., *Growth and Reproduction in Biology of Freshwater Crayfish, D.M. Holdich,*
596 *Editor. Blackwell Science Ltd.* 2002: p. 152-191.
597 45. Jussila, J. and L.H. Evans, *On the factors affecting marron (Cherax tenuimanus) growrth in*
598 *intensive culture.* Freshwater Crayfish 1996. **11**: p. 428 - 440.
599 46. Jussila, J., *Physiological responses of Astacid and Parastacid crayfishes (Crustacea:*
600 *Decapoda) to conditions of intensive culture. Kuopio University: Finland.* 1997.
601 47. Fotedar, R., *Nutrition of marron, Cherax tenuimannus (Smith) under different culture*
602 *environments—a comparative study* 1998: p. 174
603 48. Hai, N.V. and R. Fotedar, *Comparison of the effects of the prebiotics (Bio-Mos® and β-1,3-D-*
604 *glucan) and the customised probiotics (Pseudomonas synxantha and P. aeruginosa) on the*
605 *culture of juvenile western king prawns (Penaeus latisulcatus Kishinouye, 1896).* Aquaculture,
606 2009. **289** p. 310–316.
607 49. Prangnell, D.I. and R. Fotedar, *Effect of sudden salinity change on Penaeus latisulcatus*
608 *Kishinouye osmoregulation, ionoregulation and condition in inland saline water and*
609 *potassium-fortified inland saline water. .* Comparative Biochemistry and Physiology- Part A:
610 Molecular & Integrative Physiology, 2006a. **145**: p. 449-457.
611 50. Prangnell, D.I. and R. Fotedar, *The growth and survival of western king prawns, Penaeus*
612 *latisulcatus Kishinouye, in potassium-fortified inland saline water. .* Aquaculture, 2006. **259**:
613 p. 234-242.
614 51. Tantulo, U. and R. Fotedar, *Comparison of growth, osmoregulatory capacity, ionic regulation*
615 *and organosomatic indices of black tiger prawn (Penaeus monodon Fabricius, 1798) juveniles*
616 *reared in potassium fortified inland saline water and ocean water at different salinities.*
617 Aquaculture, 2006. **258**: p. 594-605.
618 52. Tantulo, U. and R. Fotedar, *Osmo and ionic regulation of black tiger prawn (Penaeus*
619 *monodon Fabricius 1798) juveniles exposed to K+ deficient inland saline water at different*
620 *salinities. .* Comparative Biochemistry and Physiology - Part A: Molecular & Integrative
621 Physiology, 2007. **146**: p. 208-214.
622 53. Sang, H.M. and R. Fotedar, *Growth, survival, haemolymph osmolality and organosomatic*
623 *indices of the western king prawn (Penaeus latisulcatus Kishinouye, 1896) reared at different*
624 *salinities.* Aquaculture, 2004. **234**(1-4): p. 601-614.
625 54. Fotedar, R., *Nutrition of marron, Cherax tenuimannus (Smith) under different culture*
626 *environments—a comparative study (Unpublished) PhD thesis.*, 1998, Curtin University of
627 Technology. pp.174: Perth, Western Australia.
628 55. Jussila, J., *Physiological responses of Astacid and Parastacid crayfishes (Crustacea:*
629 *Decapoda) to conditions of intensive culture.*, 1997, Kuopio University: Finland.

56. Gullian, M., F. Thompson, and J. Rodriguez, *Selection of probiotic bacteria and study of their immunostimulatory effect in Penaeus vannamei.* Aquaculture, 2004. **233**(1-4): p. 1-14.
57. Leonel Ochoa-Solano, J. and J. Olmos-Soto, *The functional property of Bacillus for shrimp feeds.* Food Microbiology, 2006. **23**(6): p. 519-525.
58. Langdon, J.S., *Description of Vavraia parastacida sp. nov. (Microspora: Pleistophoridae) from marron, Cherax tenuimanus (Smith), (Decapoda: Parastacidae).* Journal of Fish Diseases, 1991. **14**(6): p. 619-629.
59. Soderhall, I. and K. Soderhal, *Immunhe Reaction*, in *Biology of Freshwater Crayfish*, D.M. Holdich, Editor 2002, Blackwell Science Ltd. p. 439-464.
60. Soderhall, K., M.W. Johansson, and V.J. Smith, *Internal Defense Mechanism*, in *Freshwater crayfish : biology, management, and exploitation*, D.M. Holdich and R.S. Lowery, Editors. 1988, Croom Helm, London.
61. Hai, V.N., N. Buller, and R. Fotedar, *The use of customised probiotics in the cultivation of western king prawns (Penaeus latisulcatus Kishinouye, 1896).* Fish & Shellfish Immunology, 2009. **27**(2): p. 100-104.
62. Li, K., et al, *Beneficial effects of Bacillus licheniformis on the intestinal microflora and immunity of the white shrimp, Litopenaeus vannamei.* Biotechnology Letters, 2007. **29**(4): p. 525-530.
63. Yao, C.-L., et al., *The lysosome and lysozyme response in Chinese shrimp Fenneropenaeus chinensis to Vibrio anguillarum and laminarin stimulation.* Journal of Experimental Marine Biology and Ecology, 2008. **363**(1-2): p. 124-129.
64. Sang, H.M., L.T. Ky, and R. Fotedar, *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, 2009. **27**(2): p. 341-348.
65. Le Moullac, G. and P. Haffner, *Environmental factors affecting immune responses in Crustacea.* Aquaculture, 2000. **191**(1-3): p. 121-131.
66. Omori, S.A., G.G. Martin, and J.E. Hose, *Morphology of hemocyte lysis and clotting in the ridgeback prawn, <i>Sicyonia ingentis</i&gt.* Cell and Tissue Research, 1989. **255**(1): p. 117-123.
67. van de Braak, C.B.T., et al., *The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (Penaeus monodon).* Fish & Shellfish Immunology, 2002. **12**(3): p. 253-272.
68. Vieira, F.d.N., et al., *Time-related action of Lactobacillus plantarum in the bacterial microbiota of shrimp digestive tract and its action as immunostimulant.* Pesquisa Agropecuária Brasileira, 2008. **43**: p. 763-769.
69. Li, K., et al., *Beneficial effects of <i>Bacillus licheniformis</i> on the intestinal microflora and immunity of the white shrimp, <i>Litopenaeus vannamei</i&gt.* Biotechnology Letters, 2007. **29**(4): p. 525-530.
70. Rahiman, K.M.M., et al., *Probiotic effect of Bacillus NL110 and Vibrio NE17 on the survival, growth performance and immune response of Macrobrachium rosenbergii (de Man).* Aquaculture Research, , , , 2010. **41**: p. 120-134.
71. Fotedar, S., E. Tsvetnenko, and L. Evans, *Effect of air exposure on the immune system of the rock lobster Panulirus cygnus. Marine and Freshwater Research.* 2001. **52**: p. 1351 - 1355.
72. Hai, N.V., N. Buller, and R. Fotedar, *Effect of Customized Probiotics on the Physiological and Immunological Responses of Juvenile Western King Prawns (Penaeus latisulcatus Kishinouye, 1896) Challenged with Vibrio harveyi.* Journal of Applied Aquaculture, 2010. **22**(4): p. 321-336.
73. Itami, T., et al., *Enhancement of disease resistance of kuruma shrimp, Penaeus japonicus, after oral administration of peptidoglycan derived from Bifidobacterium thermophilum.* Aquaculture, 1998. **164**(1-4): p. 277-288.

74. Kumar, R., et al., *Enhanced innate immune parameters in Labeo rohita (Ham.) following oral administration of Bacillus subtilis.* Fish & Shellfish Immunology, 2008. **24**(2): p. 168-172.
75. Minh Sang, H. and R. Fotedar, *Growth, survival, haemolymph osmolality and organosomatic indices of the western king prawn (Penaeus latisulcatus Kishinouye, 1896) reared at different salinities.* Aquaculture, 2004. **234**(1-4): p. 601-614.
76. Jussila, J. and L.H. Evans, *Impact of Sump Tank Size on Growth, Survival, and Production of Marron, Cherax tenuimanus, in an Intensive System.* Journal of Applied Aquaculture, 1996. **6**(3): p. 23-31.
77. Nystrom, P., *Ecology*, in *Biology of Freshwater Crayfish*, D.M. Holdich, Editor 2002, Blackwell Science Ltd. p. 192-235.
78. Liu, C.-H., et al., *The immune response of the white shrimp Litopenaeus vannamei and its susceptibility to Vibrio infection in relation with the moult cycle.* Fish & Shellfish Immunology, 2004. **16**(2): p. 151-161.
79. Hsieh, S.-L., et al., *Immune and physiological responses in Pacific white shrimp (Penaeus vannamei) to Vibrio alginolyticus.* Aquaculture, 2008. **275**(1-4): p. 335-341.
80. Rengpipat, S., et al, *Enhanced growth and resistance to Vibrio challenge in pond-reared black tiger shrimp Penaeus monodon fed a Bacillus probiotic.* Diseases Of Aquatic Organisms, 2003. **55**: p. 169-173.
81. Wong, F.Y.K., K. Fowler, and P.M. Desmarchelier, *Vibriosis Due to Vibrio mimicus in Australian Freshwater Crayfish.* Journal of Aquatic Animal Health, 1995. **7**(4): p. 284-291.
82. Balcázar, J.L., T. Rojas-Luna, and D.P. Cunningham, *Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (Litopenaeus vannamei) following immersion challenge with Vibrio parahaemolyticus.* Journal of Invertebrate Pathology, 2007. **96**(2): p. 147-150.
83. Wang, Y.-B., *Effect of probiotics on growth performance and digestive enzyme activity of the shrimp Penaeus vannamei.* Aquaculture, 2007. **269**(1-4): p. 259-264.
84. Evans, L.H. and J. Jussila, *Freshwater Crayfish Growth Under Culture Conditions: Proposition for a Standard Reporting Approach.* Journal of the World Aquaculture Society, 1997. **28**(1): p. 11-19.
85. M., M.N., *Assessment of artificial feeds for battery culture of a freshwater crayfish, marron (Cherax tenuimanus) (Decapoda: Parastacidae).* , 1984, Department of Fisheries and Wildlife. Western Australia, Report No. 63.
86. RENGPIPAT, S., et al., *Immunity enhancement in black tiger shrimp (Penaeus monodon) by a probiont bacterium (Bacillus S11).* . Aquaculture, 2000. **191**: p. 271-288.
87. RENGPIPAT, S., et al., *Effect of probiotic bacterium on black tiger shrimp (Penaeus monodon) survival and growth.* Aquaculture, 1998. **167**: p. 301-313.

Table 1. Hepatopancreas moisture content (HM%), tail muscle moisture content (TM%), wet hepatosomatic index (H_{iw}), dry hepatosomatic index (H_{id}), wet tail muscle indices (T_{iw}) and dry tail muscle indices (T_{id}) of marron with different species of probiotic bacteria supplementation

Index	Day	Treatment					
		AQ2	A10	A12	PM3	PM4	Control
HM%	0	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16	$_{1}$61.97±1.16
	35	$_{1}$62.53±1.46^{a}	$_{1}$62.05±5.38^{a}	$_{1}$65.33±6.48^{a}	$_{1}$62.76±5.61^{a}	$_{1}$68.42±5.54^{a}	$_{1}$75.16±7.07^{a}
	70	$_{1}$58.44±1.30^{a}	$_{1}$63.16±5.40^{a}	$_{1}$64.17±3.28^{a}	$_{1}$65.11±3.92^{a}	$_{1}$61.44±1.38^{a}	$_{1}$70.79±6.26^{a}
TM%	0	$_{2}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30	$_{1}$80.52±0.30
	35	$_{1}$79.13±0.26^{a}	$_{1}$82.00±2.193^{a}	$_{1}$79.55±0.88^{a}	$_{1}$79.47±0.90^{a}	$_{1}$80.57±0.727^{a}	$_{1}$80.82±0.41^{a}
	70	$_{1}$78.74±0.19^{a}	$_{1}$79.34±0.83^{a}	$_{1}$80.36±1.13^{a}	$_{1}$86.46±6.36^{a}	$_{1}$79.15±0.09^{a}	$_{1}$80.67±0.94^{a}
Hiw	0	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{1}$6.06±1.10	$_{12}$6.06±1.10
	35	$_{1}$7.83±0.87^{a}	$_{1}$6.99±0.64^{a}	$_{1}$5.87±0.85^{a}	$_{1}$6.73±1.29^{a}	$_{1}$6.70±1.27^{a}	$_{1}$5.50±0.51^{a}
	70	$_{1}$5.86±0.68^{a}	$_{1}$6.30±0.66^{a}	$_{1}$6.17±0.65^{a}	$_{1}$6.72±0.50^{a}	$_{1}$6.44±0.81^{a}	$_{2}$8.15±0.91^{a}
Hid	0	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49	$_{1}$2.33±0.49
	35	$_{1}$2.96±0.43^{a}	$_{1}$2.72±0.63^{a}	$_{1}$1.94±0.223^{a}	$_{1}$2.65±0.81^{a}	$_{1}$2.26±0.84^{a}	$_{1}$1.39±0.48^{a}
	70	$_{1}$2.76±0.17^{a}	$_{1}$2.34±0.46^{a}	$_{1}$2.19±0.19^{a}	$_{1}$2.32±0.13^{a}	$_{1}$2.50±0.29^{a}	$_{1}$2.29±0.88^{a}
Tiw	0	$_{12}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35	$_{1}$26.44±1.35
	35	$_{2}$29.69±0.91^{a}	$_{1}$35.32±6.51^{a}	$_{1}$28.60±0.77^{a}	$_{1}$28.04±0.54^{a}	$_{1}$27.21±1.51^{a}	$_{1}$27.43±1.21^{a}
	70	$_{1}$25.80±1.04^{a}	$_{1}$25.42±1.07^{a}	$_{1}$28.58±2.55^{a}	$_{1}$24.90±3.00^{a}	$_{1}$27.96±0.94^{a}	$_{1}$27.64±1.09^{a}
Tid	0	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}	$_{1}$5.15±0.24^{a}
	35	$_{2}$6.20±0.26^{a}	$_{1}$6.07±0.26^{a}	$_{1}$5.84±0.17^{a}	$_{1}$5.75±0.23^{a}	$_{1}$5.27±0.24^{a}	$_{1}$5.27±0.32^{a}
	70	$_{12}$5.49±0.25^{a}	$_{1}$5.24±0.12^{a}	$_{1}$5.64±0.72^{a}	$_{1}$3.72±1.86^{a}	$_{1}$5.83±0.18^{a}	$_{1}$5.36±0.45^{a}

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

Note: AQ2 (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); PM4 (*Bacillus* sp)

Table 2. Mean ± SE marron immune parameters

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

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

Table 3. Mean ± SE marron immune parameters when challenged with high dosage of *V. mimicus*

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

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

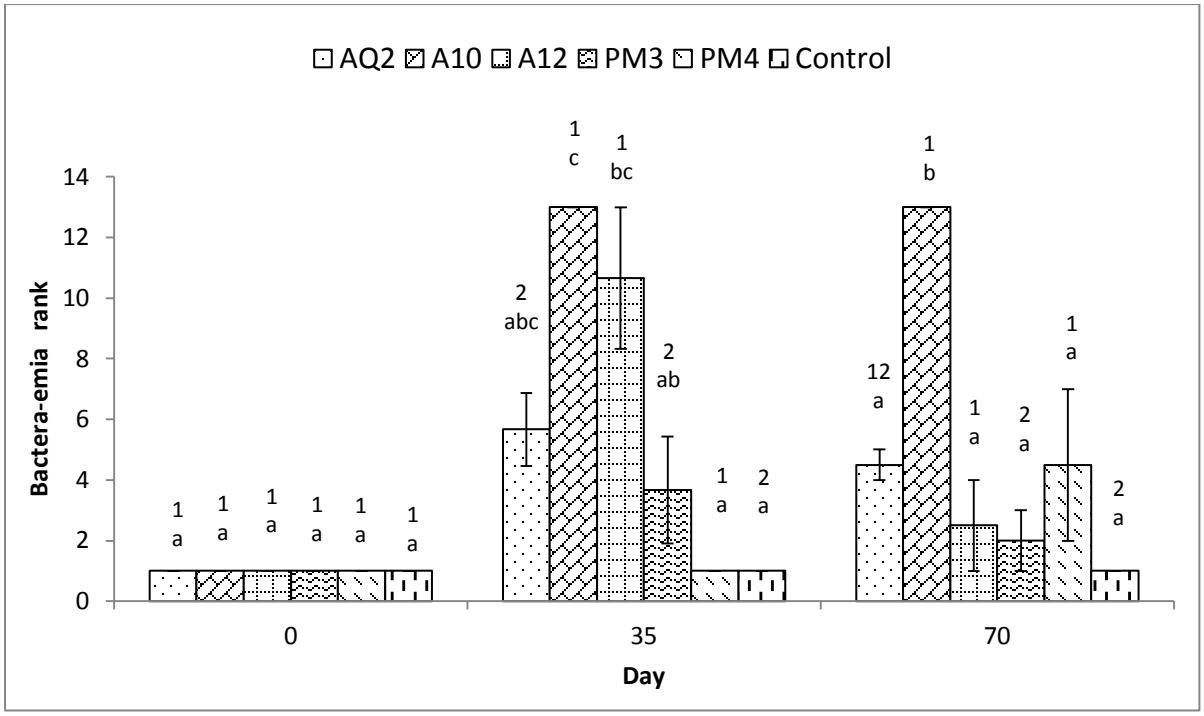

Fig. 1. Mean ± SE of intestine bacteria rank (IBL) of marron fed on different probiotic supplementation diets. Different letters ($_{a, b, c}$) over bars indicate significantly different means for different treatments at P <0.05. Different numbers ($_{1, 2}$) over bars indicating significantly different means at different times at P <0.05

Note: AQ2 (*Bacillus* sp.); A10 (*Bacillus mycoides*); A12 (*Shewanella* sp.); PM3 (*Bacillus subtilis*); PM4 (*Bacillus* sp)

762 Table 4. Matrix of comparison between five probiotic diets using various parameters

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

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