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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.

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.

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

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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 [32]. 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 °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 marron shelters in the form 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 °C until used.

Five species of probiotic bacteria isolated from the various sources were selected for their growth inhibition capabilities against *V. mimicus* and *Vibrio 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. 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 [27], with some modifications. The isolated probiotics from stock culture were re-grown onto a new blood agar plate. After overnight incubation at 25 °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 [38–41]. 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 °C to avoid bacterial growth. The concentration (cfu/mL) of each probiotic bacterium sprayed onto the feed was determined using an established method [42] where optical density (Spectrophotometer, BOECO S-20, Hamburg, Germany) correlates to the bacterial concentration (cfu/mL). The concentration sprayed onto the feed was confirmed by performing a total bacterial count using blood agar plates and an overnight incubation at 25 °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 V. 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