

Survival and Immunity of Marron *Cherax cainii* (Austin, 2002) Fed *Bacillus mycooides* Supplemented Diet under Simulated Transport

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Abstract

The present study examined the effect of simulated transport on marron, *Cherax cainii*, (Austin, 2002) after a 10 week feeding trial using basal diet or customised probiotic, *Bacillus mycooides* supplemented diet by measuring intestinal bacterial population, total haemocyte count (THC), bacteraemia, morbidity, dehydration and mortality.

Packing steps followed the established standard packing method for live transportation of marron. Each treatment group consisted of six polystyrene boxes (65 × 30 × 40 cm³), and each box contained 30 marron from each feeding group. The sealed boxes were placed on a trolley at room temperature to give the effect of transportation. Boxes were opened at 24th and 48th hour post simulated transport and marron from each treatment group were returned to the culture tank. After temperature acclimation, the marron were observed for mortality and samples were collected to assess marron health and immunity.

The results demonstrated that no mortality was observed at 24 h of transport both in basal diet and probiotic diet fed marron, however at 48h of transport the survival of probiotic fed marron was significantly higher (100 ± 0.0%) compared to survival (93.3 ± 2.8%) of basal diet fed marron. The higher survival rate of probiotic fed marron was also sustained by superior health and immune status indicated by higher intestinal bacterial population, higher total haemocyte count and lower haemolymph bacteria (bacteraemia) level. In brief, supplementation with host origin customized probiotic *B. mycooides* significantly improved marron tolerance to a live transport stress test, which resulted in no mortality up to 48h of transport.

Keywords: Simulate transport; Intestinal bacteria population; THC; Bacteraemia; Mortality

Introduction

Practices and methods used for crustacean handling and live trade may lead to serious physiological stress responses in aquatic animals [1-3] including marron [4-6]. Marron, *Cherax cainii* (Austin, 2002), is endemic to Western Australia and recently was introduced into South Africa, Zimbabwe, Japan, USA, China and the Caribbean as a commercial aquaculture species [6-8].

The reasons for live trade of crustaceans include for consumption, grow-out, restocking, and the aquarium trade; hence survival and quality of the animals is extremely important for welfare and economic reasons. The duration of the stressors encountered in the live trade process leads to short and long term changes in immune parameters as stress response shifts from adaptive to maladaptive [2,9]. Beyond this point the physiological stress response may reduce disease resistance and growth reduced quality and eventually death [2,10]. Therefore, improving immunity, stress tolerance and optimising health conditions of crustaceans during storage and live transport is of fundamental importance [3].

The successful culture and stocking of marron relies on better understanding the factors affecting their well-being during transport and their recovery afterwards [5]. Marron are an economically important aquaculture species in Western Australia and they show significant environmental stress tolerance post handling and live transport. Jussila observed no mortality of marron post 24 h handling and simulated transport, whereas detected no mortality of marron up to 36 h under simulated transport [5,6]. Moreover, marron may undergo live transport up to 72 h without mortality, however longer periods of transportation resulted in an average dehydration of 4.5% of body weight [4,5].

Feed additives such as probiotics and prebiotics can improve stress tolerance and immunity of aquatic animals including marron [6,11-16]. Our previous studies indicated that *Bacillus mycooides*, a marron origin customised probiotic, possessed a number of favourable probiotic properties [17], significantly improved gastrointestinal (GIT) health of marron [18] and improved immunity against the pathogenic bacterium *Vibrio mimicus* [19], a dominant bacterial pathogen of freshwater crayfish in aquaculture [20-22].

Cruz et al. suggested that aquatic animals should be treated with probiotics before exposure to transport and environmental stressors [23]. To date, improved stress tolerance and immunity by feeding probiotics have been documented in fish [11,24,25], shrimps [26-29] and molluscs [30,31] however information on probiotic-fed marron under practical transport conditions is not available. The present study evaluated the effect of simulated transport conditions on marron fed the probiotic *B. mycooides* by measuring intestinal bacterial population, total haemocyte count (THC), bacteraemia, morbidity, dehydration and mortality.

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Material and Methods

Acclimation and feeding trial

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

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

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

Experimental diet and set up

The experimental diets used in this feeding trial were (1) basal diet of a marron commercial feed supplied by Specialty Feed Pty Ltd, WA and (2) the basal diet supplemented with customised probiotic *B. mycooides*. Before use, the pelleted diet was homogenised with a blender to obtain a desirable pellet size before supplementation with the *B. mycooides* at 10⁸ cfu/g of feed. The density of *B. mycooides* was based on the density used in other *Bacillus* species studies [32-36] and from results of our previous studies [18,19].

Supplementation of the probiotic followed established methods [37]. In brief, the basal diet was placed on tray covered with sterilised aluminium foil and sprayed with 20 mL of fish oil per kg of feed to improve attachment of probiotic bacteria. The feed was wrapped in individual sterilised aluminium foil packs containing the amount adjusted to marron biomass for each tank, and stored at 4°C until used. The diet was prepared each week and the feeding rate adjusted according to the marron weight.

Simulated live transport

After feeding with the test diet for 10 weeks, the animals were subjected to a simulated live transport following the "Code of Practice for the Harvest and the Post-Harvest Handling of Live Marron for Food" established by Department of Fishery Western Australia [38] and the standard packing of marron commonly used by marron growers. Feeding ceased one day before the commencement of simulated transport trial.

In brief, healthy marron of equal size (12.3 ± 0.5 g) from probiotic fed and a control basal diet were selected and placed in a polystyrene box (60 × 40 × 30 cm) for 24 h and 48 h simulated transport. Each box contained sufficient ice-gel bags covered by a moist foam layer and a temperature data logger (Onset HOBO). Marron from each treatment group were placed in a ventilated container prior to placing in the polystyrene boxes. Placing the marron in the ventilated container not only avoided the marron from mixing with different treatment groups, but also protected the marron from the moist foam layer and

ice-gel bags, and was based on the method used in a previous study [6]. Subsequently, another moist foam layer and ice bag were placed over the marron ventilated containers, before the outer polystyrene box was sealed with a lid. The sealed boxes were placed on a trolley at room temperature and being moved intermittently to give simulated transport effects.

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

Measurements of the parameters

Intestinal bacteria population: Bacterial population of the marron was measured before and during simulated transport at 24h and 48h. In brief, marron from each treatment group were sacrificed by placing them at -20°C for 5 minutes before aseptic removal of the GIT. The marron dorsal shell was cut-off horizontally from tail to head until the intestines were exposed. The intestine from individual animals was collected aseptically and placed in a sterilised pestle, weighed and then homogenised. The homogenates of intestines were serially (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶) diluted using sterile normal saline. Fifty microliters of each serial dilution was inoculated onto a blood agar (BA) plate and incubated overnight in a CO₂ incubator at 25°C. A colony count was performed for each dilution to determine the total number of aerobic bacteria [39].

Total haemocyte count (THC): The total haemocyte count was measured following the established methods used in western rock lobsters *Panulirus cygnus* George [40] and marron [6]. In brief, 0.5 mL of haemolymph withdrawn from the second last ventral segment of marron was inserted into a haemocytometer (The Neubauer Enhanced Line, Munich, Germany) counting chamber and immediately viewed under 100-fold magnification on a camera-equipped microscope and images were taken for THC counts. Cells were counted in both grids, and the mean was used as the haemocyte count. For each treatment group, the procedure was repeated using ten different animals. The total haemocyte count was calculated as THC = (cells counted × dilution factor × 1000)/volume of grid (0.1 mm³).

Haemolymph bacteria (Bacteraemia): Bacteraemia of marron was determined following the established method described by sang et al. [6] with a minor modification. Briefly, the haemolymph was withdrawn into a sterile syringe and placed onto a sterile glass slide to avoid bubbles before a 0.05 mL aliquot was lawn inoculated onto a BA plate. The plates were placed in a sterilised container and incubated overnight at 25°C. The total colony forming units (CFU) for each plate and CFU/mL were calculated on the basis of a total volume of 0.05 mL/plate.

Dehydration: Dehydration of marron was measured using the established method [5]. Ten marron from each treatment group of equal size were weighed prior to the commencement of the simulated transport, then weighed at 24 h and 48 h post transport and the percentage of weight loss was recorded.

Morbidity and survival rate (%)

Morbidity (vigour index) of marron was measured following the established method proposed by Jussila et al. [5]. In this study, morbidity of marron was identified based on the response to stimuli at a time after simulated transport, and the time of recovery was recorded after being returned to the culture tanks.

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

$$SR = (N_t/N_0) \times 100$$

where SR is the survival rate (%); N_t is the number of marron at time t and N_0 is the number of marron at the commencement (0), respectively.

Data analysis

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

Results

Intestinal bacteria population

Intestinal bacterial population of marron declined at 24 and 48 h simulated transport both for basal diet and probiotic fed marron. Reduction of the intestinal bacterial population occurred at 24 h and 48 h of transport; however a significant reduction of more than half the initial population levels were observed at 48 h, both in basal diet and probiotic fed marron (Table 1). This result suggests that the longer the stress disturbance, the greater the reduction of intestinal bacterial population. Nevertheless, at 48 h post-transport, the bacterial population (646 ± 16.4) of probiotic fed marron was comparable to the initial bacterial population (626.7 ± 19.7) of basal diet fed marron.

Total haemocyte count (THC)

Prior to the simulated transport test, the THC of marron fed probiotic and basal diets for 10 weeks was measured. The THC of marron fed probiotic supplemented diet was significantly higher compared to THC of basal diet fed marron at the commencement of simulated transport test (Figure 1). This result indicates that the health status of marron fed probiotic was higher at the initiation of the simulated transport test.

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

Haemolymph bacteria (Bacteraemia)

Haemolymph bacteria were observed in basal diet and probiotic fed marron after feeding with the test diets for ten weeks prior to the simulated transport test. Total bacterial count in the haemolymph of probiotic fed marron was significantly ($P < 0.05$) lower compared to basal fed marron, indicating the customised probiotic of host origin may induce protection from bacteria and other foreign particles in the haemolymph. This result was strongly related to THC of marron in each treatment group, as haemocytes play an important role in removal of bacteria and foreign particles from the haemolymph of crayfish [41,42].

Dehydration

Dehydration occurred in marron fed either the basal diet or the probiotic supplemented diet. During the first 24 h, dehydration in the basal diet fed marron was 3.8 percent, whereas in probiotic fed marron it ranged between 3.0 and 3.7%. After 48 h of transport, the dehydration still occurred in both treatment groups but in the basal diet it was 0.45% while in probiotic fed marron the extra dehydration was 0.55%. There

was no significant different ($P > 0.05$) in the mean dehydration between basal diet and probiotic fed marron after 24h and 48h of transport.

Morbidity and survival rate (%)

Marron showed a very weak response to stimuli after 24 h of transport and this condition was more noticeable after 48 h of transport. Marron started to show response to stimuli after 30 minutes during the temperature acclimation in the boxes. As most marron in the boxes were actively crawling and swimming, they were returned to the tanks for mortality observation. No mortality was observed in marron fed probiotic supplemented diet; while in basal diet fed marron mortality (6.7%) occurred at 48 h of transport. No clinical signs were observed on dead marron shells (Figure 2).

Discussion

A number of immune and physiological parameters are involved in the stress response following handling and live transport of crustaceans and fish such as behaviour, morbidity and vigour, THC, blood glucose, dehydration, oxygen uptake, blood composition, pH, hormones and ion [1,3]. In marron, the common selected parameters for testing following handling and simulated transport include dehydration [4] THC, haemolymph/plasma glucose, serum protein, dehydration [5], proportion of granule cells, clotting time and bacteraemia [6].

The circulating haemocytes of crustaceans are an essential part of the immune system, and perform functions such as phagocytosis, encapsulation, and lysis of foreign cells and much research in the defensive role of haemocyte in crustacean is being conducted [26,40,43]. The results suggest total haemocyte count (THC) is a reliable indicator of stress in crustaceans [2,44] including in marron [5,6].

In the present study, THC was investigated as an indicator for stress tolerance. THC of marron fed probiotic was significantly higher compared to basal diet fed marron both at 24 h and 48 h post simulated transport, indicating that the customised probiotic *B. mycoides* was able to improve marron immunity. Enhancement of THC in probiotic fed marron leads to increased stress tolerance and diseases resistance, which results in a significantly higher survival rate (100%) over 24 h and 48 h post live transport. Higher THC of probiotic fed marron also provides better protection to the gill from parasites and bacteria pathogens which may cover and reduce respiration efficiency. Tinh et al. [45] suggested that probiotic bacteria can also be active on the gills and skin of the host. Inefficient respiration during handling and live transport may contribute to marron mortality in basal diet fed marron. Thus prior to transport, purging is generally essential in freshwater crayfish [3] to evacuate the GIT content and clean the gills and skin.

Handling and live transport creates physiological stresses which reduce THC in many crustacean species such as American lobster *Homarus americanus* [1], crab *Cancer pagurus* [2], western rock lobster *Panulirus cygnus* [2,46] marron [5,6], and a mollusc abalone *Haliotis tuberculata* [47]. Therefore, increasing the THC by feeding probiotic supplemented diets may improve stress tolerance and protect the animals from pathogens. This has been demonstrated in crayfish, *Pacifastacus leniusculus*, where a higher THC corresponded

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

Table 1: Intestinal bacteria population (million CFU \pm SE) of marron fed different diets at 24 h and 48 h post transport.

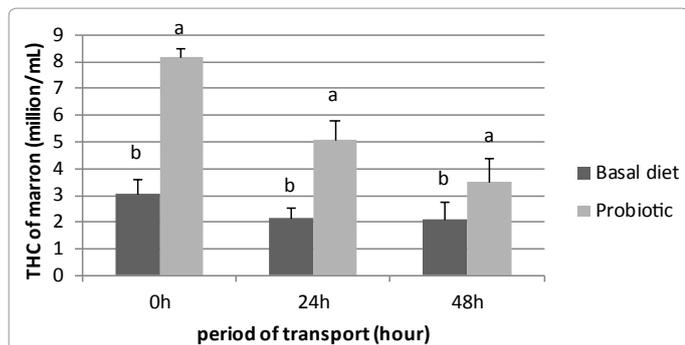


Figure 1: THC of marron fed basal and probiotic diets at 24h and 48h of transport.

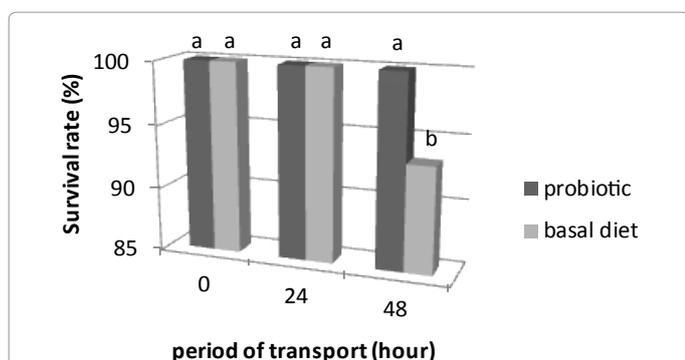


Figure 2: Survival rate (%) of marron fed basal and probiotics diets at 24 h and 48 h of transport.

to improved defence reactions of the animal when infected with the Oomycete fungus *Aphanomyces astaci* [48] and in marron where a higher THC corresponded to reduced bacteraemia and improved immunity against the pathogen *Vibrio mimicus*[6,19].

Bacterial population and diversity in the GIT is an important health component for aquatic animals [49,50] significantly affected by acute stress [51]. Stress due to high stocking density could also affect the performance of probiotics [24]. In the present study, the bacterial population level decreased both in probiotic and basal diet fed marron at 24h and a significant reduction was observed at 48h of transport indicating that prolonged stress significantly reduces intestinal bacteria of the animal. However, the higher bacterial population level of probiotic fed marron prior to transport resulted in the bacterial population remaining higher at 48h of transport compared to the population level of basal diet fed marron at the initiation of the transport test. The reduction of intestinal bacterial population due to handling and transport stress was also determined in other species. In Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss* (Walbaum), adherent bacterial population in the mid-gut and hindgut were significantly reduced following acute handling stress, whereas the level increased in faeces, which suggests that considerable amounts of mucus was lost following stress[51,52]. This significant reduction of the intestinal bacterial population implies decline in the health status of the animal. The microbiota within the GIT can be considered a metabolically active organ, is an essential health component, provides protection against infection and instructs mucosal immunity [50]. Beneficial bacteria such as lactobacilli and bifidobacteria decrease following a stress response [50] and this may provide opportunistic pathogens to become established [2,44,53].

Haemolymph bacteria (bacteraemia) of marron fed probiotic diet was significantly lower compared to basal diet fed marron suggesting that greater THC plays an important role in reducing bacteria and foreign particles in marron haemolymph. Once pathogens or foreign particles enter the haemocoel, the haemocytes initiate phagocytosis [54]. In crayfish, hyaline cells are chiefly involved in phagocytosis, whereas semi-granular cells are active in encapsulation [41]. Crustacean haemocytes contain antibacterial activity [55,56] which can reduce the viable count of bacteria within 4 hours, however the antibacterial potency (per unit protein) varies from species to species [42].

Morbidity and mortality post live transport often occurs as a result of stress [3]. In the present study, morbidity and weakness indicated by no response to stimuli were observed both in basal diet and probiotic fed marron at 24 h and 48 h post simulated transport. However, after returning to the culture tanks the probiotic fed marron fully recovered and swam normally in less than 30 minutes, whereas basal diet fed marron took more time to recover and had several mortalities.

Other than mortality, marron also could be losing weight through dehydration from tissue and gill chambers while out of water during handling and live transport [3-5]. The present study indicated that dehydration of marron was observed in both test diets at 24 h and 48 h post transportation, however the dehydration was not significantly different between the two treatments. The results revealed that the dehydration of marron was comparable to the previous marron handling and live transport studies indicating that dehydration of 4 to 5% of the body weight over 24 to 72 h transportation is a common phenomenon. Jussila et al. observed a minor dehydration of marron during the first 4 hours that remained at 4.0 to 4.5% up to 24 h post handling and transportation, whereas Morrissy et al. observed wet dehydration of 3.9% during the first 24 h, with a further additional loss of 0.4% over the next 24 h [4,5]. Acute stress requires high energy which could reduce the hepatosomatic indices and contribute to the dehydration of the animal [23]. In marron, hepatopancreas significantly reduced after 24 h of transport [5]. Therefore, dehydration should be considered when crayfish are going to be transported for a long distance [5].

Probiotics have improved stress tolerance and immunity of various aquatic animal groups such as fish species including sea bream, *Sparus aurata* [11], grouper *Epinephelus coioides* [57], Nile tilapia *Oreochromis niloticus* [58]; shrimp such as western king prawns, *Penaeus latisulcatus* [59] shrimp, *Litopenaeus vannamei* [60-62], and tiger shrimp, *Penaeus monodon* [63]. Among the bacterial genera evaluated for probiotic use, *Bacillus* species have been successful in improving the stress tolerance in these aquatic animal hosts and more recently in black swordtail, *Xiphophorus helleri* [16]. Our previous work using *B. mycoides* also showed improved marron immunity when experimentally challenged with the pathogen *Vibrio mimicus* [19].

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

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