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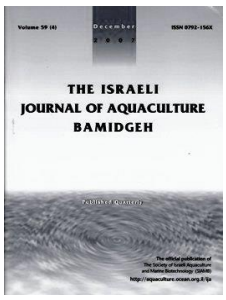

Effects of Mannan Oligosaccharide (MOS) on the Survival, Physiological, and Immunological Response of the Black Tiger Prawn (*Penaeus monodon* Fabricius, 1798) when Challenged with two Different Stressors

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Key words: black tiger prawns, mannan oligosaccharide, *Penaeus monodon*, immune response, physiological response

Abstract

Two trials were conducted to determine the effects of mannan oligosaccharide (MOS) on the resistance of the black tiger prawn (*Penaeus monodon*) to two different stressors, bacterial infection by *Vibrio alginolyticus*, and the environmental pollutant ammonia (NH_3). Prawns were fed two different diets, 0% (control diet) and 0.15% MOS, for 8 weeks prior to exposure to the stressors. They were then tested for survival, physiological, and immunological parameters, as indicators of health status. When the two groups were exposed to NH_3 and bacterial infection, survival of prawns fed the MOS diet was significantly higher ($P<0.05$) than prawns fed the control diet. Similarly, the wet hepatosomatic index (Hiw), dry hepatosomatic index (Hid), hepatopancreatic moisture content (HM), total hemocyte count (THC), and granular cell percentage (GC%), of the MOS fed prawns was significantly higher ($P<0.05$) than in prawns fed the control diet. Bacteremia of the MOS fed prawns was lower ($P<0.05$) than the control diet-fed prawns after bacterial infection. Findings demonstrated the potential of MOS to improve the survival, health status, and immunity of black tiger prawns when challenged with bacterial infection and NH_3 exposure.

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Introduction

The Black tiger prawn culture industry expanded significantly during the 1980s, becoming a multi-billion dollar industry, thus one of the most lucrative industries due to high prices and demand for black tiger prawns in the international market (Callinan and Robertson, 2006).

To meet the ever-increasing demand for animal protein, the shrimp aquaculture industry continuously seeks new techniques to increase production. However, along with updated aquaculture practices, cultured shrimp are continuously exposed to stress-inducing environmental conditions and subsequent outbreaks of diseases. Intensively cultured shrimp are often challenged with stressors such as rapid changes in environmental parameters (temperature, pH, salinity, insufficient O_2), bio-chemical toxins (algal bloom, un-ionized ammonia, hydrogen sulfide), diseases (parasites, bacteria), and handling. Environmental stress greatly affects mortality and disease susceptibility of cultured shrimps (Callinan and Robertson, 2006). Disease outbreaks are one of the major problems related to the sustainable development of the shrimp culture industry (Flegel, 2009). Thus, prevention and minimization of factors that directly and indirectly cause stress and disease are important components for successful shrimp culture.

In shrimp culture ponds, stressors are controlled mechanically, by water renewal and aeration, chemically, with the addition of quick lime to lessen algal blooms, and with the use of critical nutritional diets and selective immunostimulants which include both prebiotics and probiotics (Ganguly et al., 2010). Among the established prebiotics such as fructooligosaccharides, trans-galactooligosaccharide, inulin and mannan oligosaccharide (MOS), MOS is most commonly used as a dietary supplement for fish and crustacean species (Ringo et al, 2010). MOS has improved growth performance, immune response, and health status in many cultured fish and various crustacean species, such as marron, *Cherax tenuimanus* (Sang et al., 2010a), tropical rock lobster, *Panulirus ornatus* (Sang and Fotedar, 2010), yabby, *Cherax destructor* (Sang et al., 2010b), tiger prawns, *Penaeus semisulcatus* (Genc et al., 2007), and white leg shrimp, *Liptopeaneus vannamei* (Sangeetha et al., 2005). Black tiger prawn (*Penaeus monodon*) is an Indo-West-Pacific species, found from the eastern coast of Africa and the Arabian Peninsula, to Southeast Asia, the Sea of Japan, and northern Australia. It is one of the most popular species for aquaculture. In order to maximize production, extensive research into disease management and stress reduction of prawns has been carried out. MOS can be supplemented in food for aquaculture organisms such as *P. monodon*. There is only one published study showing the effectiveness of dietary MOS on improved survival, growth, physiological, and immunological conditions of the black tiger prawn (Sang et al., 2013). Information on the role of MOS in stress resistance of the prawn to bacterial infection and NH_3 is scarce. Since limited research has been carried out in this field, examination of several parameters is needed (Mangkurat, 2008). The objective of this study is to evaluate the effectiveness of dietary inclusion of MOS in enhancing the survival, physiology, and immunity of black tiger prawns when exposed to bacteria and NH_3.

Materials and Methods

A basal shrimp diet containing 40% crude protein, 4% crude fat, 11% moisture, 13% ash and 3% fiber (Uni-President, Taiwan), was supplemented with 0.15% MOS (Bio-Mos, Alltech Inc, USA) and compared to the basal control diet (0% MOS). The concentration of MOS at 0.15% has been shown to be the most effective on performance of the black tiger prawn (Sang et al., 2013). Pellets were ground and MOS was added at 0 g/kg and 1.5 g/kg, to produce diets with, 0% and 0.15% MOS inclusion, respectively. The mixed ingredients were then minced with water to obtain pellets of 1.5 mm diameter. The pellets were dried in direct sunlight for 6 h, allowed to cool at room temperature for half an hour, and then packed in plastic containers.

Juvenile black tiger prawns (2-3 cm) were purchased from a commercial hatchery (VINA, Ltd, Ninh Thuan, Vietnam) and shipped to the Institute of Oceanography, Nha Trang Vietnam, then stocked in six fiberglass rectangular blue culture tanks (2000 x 2000

x 1000 mm, 4000 l (capacity) at a density of 300 individuals per tank. Water in each tank was aerated and contained an independent recirculating seawater system with a biofilter. The recycling rate of the water in each tank was maintained at approximately 20 l/min throughout the culture period. Each of the two test diets was randomly assigned to three tanks, giving three replicates per diet. The prawns were fed twice daily at 8:00h and 16:00h at an initial feeding rate of 5% of their body weight. This was adjusted for each tank after every feeding. Uneaten food and feces were removed before every feeding. The prawns were reared for 8 weeks before being challenged with bacteria and NH_3. The survival of prawns in each culture tank was about 90% after the 8 week period.

The bacterial challenge: 60 prawns from each culture tank were randomly selected and distributed into two test tanks 30 prawns/tank (aerated fiberglass rectangular blue tanks, 500 x 700 x 1000 mm, 350 l capacity). There were six challenge test tanks for MOS fed prawns, and six tanks for the MOS-free (control) prawns. A stock suspension (approximately 25 x 10^5 cfu/ml) of *V.aginolyticus* (isolated from the gut of black tiger prawn) was obtained from the Department of Environmental Ecology, Institute of Oceanography, Vietnam. All prawns in each challenged test tank were injected in the base of the fifth thoracic leg with 20 µl bacterial stock suspension. The prawns in one tank from the group of two challenged test tanks were monitored for survival, and the prawns from other tank were monitored for immunological conditions, 12, 24 and 96 h after being injected; physiological parameters were measured 96h after being injected.

The NH_3 challenge: the same procedure as that used for testing the bacterial challenge was used for testing the stressor, NH_3. Each test tank of prawns was exposed to NH_3 by adding NH_4Cl into the culture media to obtain NH_4Cl concentration of 78 ppm (NH_3 concentration of 1,96 ppm at 29°C and pH of 8.1). Survival was monitored 12, 24, 48 and 96 h after exposure and physiological parameters were measured 96 h after exposure.

Prawn survival rate in each experiment was measured using the following calculation: $S = 100 \times (n_t/n_o)$; where S = survival rate; n_t = number of prawns at time t and n_o = number of prawns at the commencement. The physiological indices of the prawns, wet hepatosomatic index (Hiw), wet tail muscle index (Tw/B), hepatopancreas moisture content (HM), tail muscle moisture content (TM), dry hepatosomatic index (Hid) and dry tail muscle index (Td/B), were measured according to the established methods for the western king prawn, *Penaeus latisulcatus* (Sang and Fotedar, 2004).

For immunological evaluation, total hemocyte count (THC), granular cell (GC), and bacteremia, were measured following the established procedure for rock lobsters with some modification. Seventy percent alcohol was used to clean the base of the fifth thoracic leg of 3 prawns from each tank. A 0.1 ml aliquot of hemolymph was withdrawn into a 1 ml sterile syringe containing 0.3 ml of saline solution (1.8%) and dispensed into an Eppendorf tube kept on ice. The total hemocyte count for individual prawns was estimated with a hemocytometer (Neubauer, Germany) under 100X magnification, from the anticoagulant and hemolymph mixture. To differentiate hemocyte counts, one drop of the anticoagulant and hemolymph mixture was placed on a slide and smeared. The smear was air dried and fixed in 70% methanol for 10 min. Fixed smears were stained with May-Grunwald and Giemsa (10 min. in each) and mounted with coverslips. Approximately 200 cells were counted on each slide. The granular cells were distinguished on the basis of larger cell size, smaller pale nucleus, and larger number of eosinophillic granules in the cytoplasm. The proportion of each hemocyte type to overall hemocytes was calculated. Bacteremia assessment was calculated by withdrawing 0.05 ml aliquot of hemolymph into a sterile syringe; small drops were quickly placed on a nutrient agar (NA) plate, and smeared; the plate was carefully inverted and kept in the incubator chamber at 25°C for 24 h. Colony-forming units (CFU) were counted and CFU/ml calculated for each sample on the basis of total volume of 0.05 ml/plate. The CFU/ml were ranked 1(1-250 CFU/ml) to 12(2751-3000 CFU/ml), and a final rank of 13 was assigned to those samples in which the colonies were too numerous for an accurate count (Fotedar et al., 2001).

Statistical analyses were performed using SPSS 15.0 for Windows. Survival percentage data were normalized using an arcsine transformation before analysis. The normality of data was assessed by the Shapiro-Wilk test prior to analysis. Levene's test was used to

assess the homogeneity of variance prior to analysis. Data were subjected to an independent sample T-test for data of homogenous variance, and Tamhane's test for non-homogenous variance. Non-normal data was subjected to non-parametric testing using Kruskal-Wallis H test (Winer, 1991). Differences were considered significant at the level of $P<0.05$

Results

Survival of the prawns decreased ($P<0.05$) 48h after NH_3 and bacterial infection exposure. Survival of the prawns fed MOS diet was higher ($P<0.05$) than the prawns fed the control diet 96 h after the two groups of prawns were exposed to NH_3 (Fig. 1), and 48 h to 96 h after exposure to bacterial infection, compared to the prawns fed the control diet (Fig. 2).

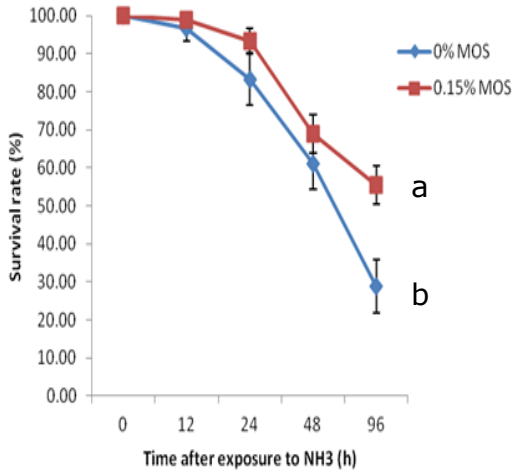

Fig. 1. Survival of the prawn when exposed to NH_3 (Different letters show significant difference at *P<0.05*)

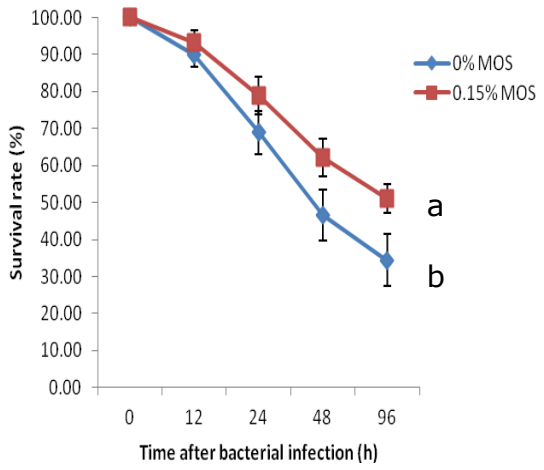

Fig. 2. Survival of the prawn when exposed to bacterial infection (Diff.letters show significant difference at *P<0.05*)

The Hiw and Hid of MOS fed prawns were higher ($P<0.05$) 96h after exposure to NH_3 than of the prawns fed the control diet. There was no significant difference in the HM, Tw/B, Td/B and TM between the two prawn groups 96h after NH_3 exposure. Hiw, Hid and HM were higher ($P<0.05$) in the MOS fed prawns compared to the prawns fed the control diet 96h after bacterial infection (Table 1).

Table 1. Physiological parameters of prawns fed different diets 96 h after exposure to NH_3 and bacterial infection

	NH3 Exposure		*Bacterial infection*	
Parameters	*0% MOS*	*0.15% MOS*	*0% MOS*	*0.15% MOS*
Hiw (%)	2.81 ± 0.34^a	3.59 ± 0.31^b	3.11 ± 0.22^a	3.79 ± 0.11^b
Hid (%)	0.64 ± 0.11^a	0.78 ± 0.14^b	0.73 ± 0.19^a	0.81 ± 0.16^b
HM (%)	78.35 ± 3.34^a	77.23 ± 1.64^a	80.35 ± 1.24^a	74.13 ± 0.64^b
Tw/B (%)	40.90 ± 1.14^a	44.64 ± 2.49^a	43.10 ± 1.54^a	44.64 ± 2.49^a
Td/B (%)	11.38 ± 0.61^a	11.99 ± 0.88^a	11.18 ± 0.31^a	11.39 ± 0.48^a
TM (%)	72.18 ± 0.74^a	73.16 ± 0.59^a	74.28 ± 0.14^a	73.96 ± 0.59^a

Different superscript letters in the same row indicate significantly different means at P<0.05.
Hiw:wet hepatosomatic index,
Hid:dry hepatosomatic index,
HM:hepatopancreas moisture content,
Tw/B:wet tail muscle index,
Td/B:dry tail muscle index
TM: tail muscle moisture content.

THC of the MOS fed prawns was higher ($P<0.05$) 12, 24 and 96h after bacterial infection, than the prawns fed the control diet. In both sets of prawns, THC was lower ($P<0.05$) 12 h after infection compared to the THC of the prawns prior to infection. The THC of MOS fed prawns 96h after infection was not significantly different ($P>0.05$) to that of the MOS fed prawns before infection while the difference was observed in the prawns fed the control diet. Before infection there was no difference ($P>0.05$) in GCs between the two prawn groups, however, 12, 24 and 96h after infection, the GCs of the MOS fed prawns were higher ($P<0.05$) than GCs of the prawns fed the control diet. Bacteremia in

the both groups increased significantly (P<0.05) 12h after bacterial infection. Bacteremia of the MOS fed prawns were lower (P<0.05) than the control diet fed prawns after infection. Bacteremia of prawns fed MOS diet were not different (P>0.05) 96h after infection, than the bacteremia of the MOS fed prawns before infection. In the control diet group, bacteremia were higher (P<0.05) 96h after infection compared to bacteremia of the prawns before infection (Table 2).

Table 2. Immunological parameters of the prawns challenged with *Vibrio alginoliticus*

		Diets	
Parameters	*Time after infection (h)*	*0% MOS*	*0.15% MOS*
THCs (million cells/mL)	Initial	$_{1}28.26 \pm 0.47^{a}$	$_{1}32.66 \pm 0.04^{b}$
	12	$_{2}20.73 \pm 0.04^{a}$	$_{2}22.56 \pm 0.44^{b}$
	24	$_{2}21.85 \pm 0.62^{a}$	$_{2}26.70 \pm 1.08^{b}$
	96	$_{2}23.30 \pm 0.54^{a}$	$_{1}29.91 \pm 0.41^{b}$
GCs (%)	Initial	$_{1}6.40 \pm 0.57^{a}$	$_{1}6.21 \pm 0.81^{a}$
	12	$_{1}3.86 \pm 0.33^{a}$	$_{2}5.31 \pm 3.32^{b}$
	24	$_{2}2.86 \pm 0.60^{a}$	$_{2}4.78 \pm 0.69^{b}$
	96	$_{3}3.73 \pm 1.0^{a}$	$_{1}5.18 \pm 2.54^{b}$
Bacteremia	Initial	$_{1}1.00 \pm 0.00^{a}$	$_{1}1.00 \pm 0.00^{a}$
	12	$_{2}7.33 \pm 0.33^{a}$	$_{2}4.33 \pm 0.33^{b}$
	24	$_{3}5.00 \pm 0.00^{a}$	$_{2}3.00 \pm 0.00^{b}$
	96	$_{4}3.33 \pm 0.33^{a}$	$_{1}2.33 \pm 0.33^{b}$

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

Discussion

The current study has shown that dietary supplementation of MOS can significantly improve the resistance of black tiger prawns to stressors such as bacterial infection and NH_3 exposure, increase survival rates and improve physiological and immunological parameters. Research with other MOS fed crustaceans such as marron (Sang et al., 2009), tropical rock lobster (Sang and Fotedar, 2010) and fish such as sea bass (Torrecillas et al., 2007) and cobia larvae (Salze et al., 2008) have yielded similar results. Physiological, immunological, and gut health conditions of black tiger prawns improved significantly when given 0.1-0.2% MOS supplemented diets (Sang et al., 2013). The increased resistance to stressors in prawns fed a MOS supplemented diet may be a direct consequence of improved overall health as seen in many other aquaculture species such as common carp, *C. carpio,* (Staykov et al., 2005b), rainbow trout, *S. gairdneri irideus* G., (Staykov et al., 2005a), channel catfish, *I. punctatus,* (Welker et al., 2007), and rainbow trout, *O. mykiss*, (Staykov et al., 2007).

Physiological parameters such as hepatosomatic index, tail muscle index, moisture contents of hepatopancreas, and tail muscle, have successfully been used as crustacean health indicators (Fotedar et al., 2006; Jussila and Mannonen, 1997; Jussila et al., 2001). A large hepatopancreas, especially when related to low hepatopancreas moisture content, may be an indicator of healthy physiology in freshwater crayfish (Jussila and Mannonen, 1997). While molt stages are important in assessing the crustacean condition (Jussila and Mannonen, 1997), the prawns used for sample analysis in the current experimental trial were in the same molt stage. In the current experiment, higher Hiw and Hid of the MOS fed prawns, suggests that MOS supplementation increases resistance to environmental pollutants such as NH_3, and bacterial infection, and increases the ability of the prawns to synthesize and secrete digestive enzymes, to absorb digested dietary products, enhances maintenance of mineral reserves and organic substances, lipid and carbohydrate metabolism, distribution of stored reserves during the intermolt cycle and catabolism of some organic compounds (Dall and Moriarty, 1983).

Immunological indicators such as total number of hemocytes, proportion of hemocyte types, bacteremia in decapod crustaceans, life cycle, food intake, disease outbreaks,

pollutants, and environmental stress affect the quantity and quality of circulating hemocyte count (Le Moullac and Haffner, 2000). In the present study, all prawns tested were in the intermolt stage, thus considered to have similar physiological and/or immunological status. Changes in physiological condition and the THCs and GCs of the prawns after exposure to stressors in the current trials imply that bacterial infection and NH_3 exposure could influence the health of the prawns. After being injected with *V. alginoliticus*, THCs and proportion of granular cells of prawns fed the control diet dropped significantly. The decline in THC after the bacterial injection could be associated with hemolymph locomotion to the injection site and hemocyte lysis as a result of defense activities (van de Braak et al., 2002). However, THC of prawns fed the MOS diet recovered 96h after infection indicating that MOS stimulated the hemocyte proliferation process. *Penaeus japonicus* hemocytes are able to proliferate, and the proliferation rate can be increased three times when the shrimp are injected with an immunostimulant, lipopolysaccharide (Sequeira et al., 1995). In the present study, the incorporation of MOS in diets may stimulate and increase the proliferation rate of black tiger prawn hemocytes to compensate for the loss of hemocytes due to the bacterial infection, resulting in recovered THC and GCs proportions 96 h after infection.

Our results have shown that dietary MOS supplementation improves the survival of the black tiger prawns, improves physiological responses, health, and enhances immunity by increasing resistance to bacterial infection and NH_3 stress. Further research is needed to clarify the mechanisms of MOS-action(s) in health and enhanced immunity in black tiger prawns.

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