Int.J.Curr.Microbiol.App.Sci (2018) *7*(4): 569-579

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 04 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.704.067

Enhancement of Growth, Immunity, Resistance and Survival of Freshwater Prawn, *Macrobrachium rosenbergii* against White Muscle Disease (WMD) Due to Dietary Administration of Probiotic and Biogut

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Keywords

Macrobrachium rosenbergii, Biogut, White muscle disease, Growth and survival, Disease resistance, Phenoloxidase activity and Respiratory burst

Article Info

Accepted:
07 March 2018
Available Online:
10 April 2018

A B S T R A C T

The effect of oral administration of probiotic, biogut on growth, survival, and immuno response and disease resistance against white muscle disease was examined in freshwater prawn, *M. rosenbergii*. Biogut was administered to prawn through the diet at 1%, 1.5% and 2.0% body weight/day for a period of 90-day in recirculatary rearing system. The prawns were sampled on 90 day and challenged with white muscle disease virus, which is highly potential disease to prawn through injection route at 50 µl / juvenile. The mean weight gain and survival rate of prawn fed biogut group was significantly higher than the control ($P< 0.01$). The mortality of prawn fed control diet, 1%, 1.5% and 2.0% biogut containing diet was 53.33%, 46.66%, 33.33% and 40% respectively. Phenoloxidase activity and respiratory burst activity of prawn fed control diet, 1.0, 1.5 and 2.0% biogut containing diet was 38.0%, 48.70%, 65.0%, 51.70% and 40.0%, 51.30%, 65.0%, 52.70% respectively. These results indicate that the oral administration of biogut to freshwater prawn enhances growth, survival, disease resistance and immuno response against white muscle disease of prawn and they can be recommended for prawn culture.

Introduction

Macrobrachium rosenbergii is the most important and economically cultured palaemonid in the world and it is now farmed large scale in different parts of the world including India. The global production of the prawn has increased from 130,689 tons in 2000 to 203,211 tons in 2011 (FAO, 2013). The total scampi production from India in 2010–2011 was about 8778 metric tons. The giant freshwater prawn, *Macrobrachium rosenbergii* is a major commercially important candidate species for aquaculture. For high export potential, the giant freshwater prawn enjoys immense potential for culture in India.

M. rosenbergii was regarded as disease resistant when compared to farmed *Penaeid* shrimps. As in *Penaeus monodon* culture, intensification and mismanagement could lead to disease and health problems in *M. rosenbergii*. In India its culture was introduced to compensate the heavy economic losses due to the epidemic white spot syndrome (WSS) in penaeid shrimp farming, hypothesizing the resistance of the giant freshwater prawn to WSS (Sahul Hameed *et al.,* 2000). Since November 2001 prawn hatcheries situated on the South- East coast of India have been facing heavy losses because of newly emerged disease called "white muscle disease" (WMD), this disease also called "whitish disease" or "white tail disease" in some other countries.

The occurrence of this disease in giant freshwater prawn hatchery and farms has caused a major setback in the freshwater prawn aquaculture. In India more than 18 cases of WMD in freshwater prawn hatcheries with post larvae mortalities ranging from 30% to 100% were recorded from November 2001 to December 2002 (Vijayan *et al.,* 2005) The mass mortalities up to 60% in 28 day old post larvae cultured under intensive conditions showing signs of a milky diffuse white body described as idiopathic muscle necrosis (IMN) was reported in *M. rosenbergii* by (Nash *et al.,* 1987) from Thailand and (Arcier *et al.,* 1999) from Taiwan. The association of gram positive cocci, *Lactococcus garviae* and yeasts are the causative agent of a white muscle disease in *M. rosenbergii* (Chen *et al.,* 2001). In present scenario *Macrobrachium rosenbergii* Nodavirus (*Mr*NV) and extra small virus (XSV) have been found to be associated with the WMD. However, the role of *Mr*NV and XSV is not yet clear. In crustaceans circulating haemocytes are involved in the production of melanin via the prophenoloxidase (proPO) system, which plays an important role in the defence reaction (Johansson and Soderhall, 1989) and (Perazzolo and Barracco, 1997). Based upon the recent classification of *M. rosenbergii* haemocytes (Sierra *et al.,* 2001) large ovoid haemocytes and undifferentiated round haemocytes might be carrying out the functions of the proPO system, like semigranular and granular haemocytes in other crustaceans (Johansson and Soderhall, 1989). The activity of phenoloxidase has already been reported in *M. rosenbergii* (Jaya Kumari *et al.,* 2004; Yeh *et al.,* 2005; Smith *et al.,* 1984 and Chang *et al.,* 2000). Therefore, the health of prawn and enhancement of its immunity are of primary concern. The use of antimicrobials is a common practice in shrimp hatcheries, the abuse of antimicrobials can result in the development of resistant strains of bacteria.

There is an increasing interest within the industry in the control or elimination of antimicrobial use. Hence, the use of probiotic bacteria to control potential pathogens is gaining acceptance within the industry. Fuller (1989) defined probiotic as "a live microbial feed supplement which beneficially affects the animal by improving its intestinal microflora. The certain microbes can induce immune responses in crustaceans such as Yeast β-glucan (Sung *et al.,* 1994), yeast zymosan (Sung *et al.,* 1996) and dead bacterial cells (Adams, 1991; Itami *et al.,* 1991; Sung *et al.,* 1991) have stimulated immune responses in *P. monodon. Bacillus* S11 surface antigens, or their metabolites might act as immunogens for shrimp immune defense (Rengpipat *et al.,* 2000). *Bacillus* S11 cell wall peptidoglycan might elicit an immune function in shrimps (Itami *et al.,* 1998) by acting on granulocytes for higher phagocytic activity. Uma *et al.,* (1999) reported that probiotic; Lacto-sace supplement improves the growth, survival and disease resistance of white shrimp, *P. indicus.* (Rengpipat *et al.,* 2000) reported that probiont *Bacillus* S11 provided both cellular and

humoral immune defense responses in *P. monodon*. Disease has been considered as one of the important constraints to limit the production of freshwater prawn worldwide. Generally, *M. rosenbergii* is considered to be moderately disease-resistant in comparison to penaeid shrimp. The present study was aimed at examining the effect of dietary administration of biogut on growth, survival immune response and disease resistance of *M. rosenbergii* against the white muscle in the recirculatary rearing system after three months.

Materials and Methods

Experimental design

Larvae of prawn, *Macrobrachium rosenbergii* produced in the Freshwater Prawn Hatchery of College of Fisheries Mangalore were reared up to post larval stage and used in the study. Prior to start of experiment, the post larvae were acclimatized in the closed circulatory system which consists of 12 circular fiber glass tanks of 120 l capacity and fed with control diet for one week. Uniform sized post larvae with an average initial weight of 0.25 gm were stocked at a rate of 30 numbers/ tank. Post larvae were fed their respective diets at a rate of 10% of their body weight twice daily in three replicate groups for a period of 90 days.

Prawn was weighed biweekly and the daily ration was adjusted accordingly. Tanks received continuous aeration and 10% of the water was exchanged daily to remove uneaten feed and feceal matter to maintain the water quality. During the rearing period, water temperature ranged from 25.20^{o}C to 26.80^{o}C, pH from 6.62 to 7.76, carbon dioxide from 0.1 to 3.6mg/l, dissolved oxygen concentration from 6.46 to 8.10 mg/l and ammonia nitrogen from 0.02 to 5.37µg at N/l. Growth and survival of prawn was calculated at the end of the experiment

Diet preparation

Four diets containing different levels of biogut were prepared (Table 1), by using the square method (Hardy, 1980). Proximate analysis of basal diet was 34.29% crude protein, 5.60% crude lipid, 8.50% ash and 9.80% moisture. The probiotic, biogut is obtained from Varsha Group, a Division of Aquapro, Bangalore. It contains combination of prebiotics, probiotics such as *Lactobacillus sporogens*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Saccharomyces cervisiae* and enzymes such as amylase, protease, lipase cellulase, phytase and beta-galctosidase. It was added to the test diets at levels of 1.0, 1.5 and 2.0 g /kg diet with corresponding decrease in the amount of rice bran. Experimental diets were prepared by mixing the dry ingredients with water until stiff dough resulted. The dough obtained was cooked under steam in a pressure cooker at 105^{O}C for 30 minutes. The cooked feed was cooled to room temperature rapidly by spreading in an enamel tray and required dose of biogut and pre-weighed vitamin and mineral premix was added and blended. The dough was thoroughly mixed again and extruded through a pelletizer having 2mm die. Pellets were dried in a hot air oven at 60^{O}C till the moisture content was reduced to less than 10%. After drying, the finished pellets were then stored in plastic bins at −4°C until use.

Preparation of white muscle disease viral inoculum

Collection of sample

The samples used for the studies comprised of a batch of naturally infected moribund post larvae and juveniles of *M. rosenbergii* having abdomens of milky white appearance were collected from one of the diseases out break hatchery and farms at Nellore region of Andra Pradesh during January 2006. For virus

purification, prawn samples were brought to laboratory on ice and stored at -20^{0}C until it is used for infection and susceptibility studies.

Preparation of inoculum

The inoculums prepared by the filtration of 2g homogenized post larvae of *M. rosenbergii* having abdomens of milky white appearance in 1:10 (w/v) of TNE buffer (0.1-M Tris-Hcl, 0.4M Nacl 0.02M EDTA-Na_2 and pH 7.4). In order to prepare the viral extract the homogenate was centrifuged at 20,000 rpm for 15 minutes at 4^{0}c and resultant supernatant was filtered through 0.25µm syringe filter. The filtrate obtained was collected in to 2 ml aliquots and stored at -40^{o}C until use.

Resistance of *M. rosenbergii* to white muscle disease

After three month of rearing period 45 prawns (Fig. 1) from each treatment and control group (15 prawns from each replication) were randomly sampled and transferred to 60l fiber glass aquaria (15 no/ aquarium) and fed with control diet at the rate of 10% of body weight twice daily for a week. Only prawns in the intermoult stage were used. The moult stage was determined by examination of uropoda in which partial retraction of the epidermis could be distinguished (Robertson *et al.,* 1987). The resistance test was conducted in triplicate by the injection of 50 µl of white muscle viral inoculum into the ventral sinus of the cephalothorax. The prawn that received saline (20 µl) with no injection served as the saline group. There were total of three treatments. Each treatment was conducted with 45 prawns. Water was renewed daily, and the experiment lasted 8 days. During resistance study clinical signs and mortality of prawn was observed. The relative percentage of survival (RPS) and mortality of prawn was calculated at the end of the study.

Immune parameters of *M. rosenbergii*

At the end of the susceptibility study all the survived prawns from treatments and control group were sampled for PPO and NBT assay to study the immuno response of *M. rosenbergii* against the white muscle disease.

Prophenoloxidase assay (PPO)

L-dihydroxyphenylalanine (L-DOPA) used as substrate as per the procedure of (Soderhall and Smith, 1983) to determine the phenoloxidase activity. The haemolymph was drawn from the animal and a thin layer of haemolymph was made on slide and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 1hour at 4^{0}C. The smear was washed in phosphate buffer thrice (15minutes each). It was incubate in 0.1% L-DOPA in phosphate buffer for 16 to 18 hours at room temperature. The slides were observed under a microscope (100x). The black staining of the granules indicated positive reaction and the percentage of positive cells were counted.

Nitroblue Tetrazolium Assay (NBT)

To determine cellular activity, NBT assay was performed. At the end of challenging study all the survived prawns from each treatment and control group were sampled to drawn the heamolymph. The haemolymph was drawn into pyrogen free eppendroff tube containing a drop of 3.8% sodium citrate. One drop of heamolymph (0.1 ml) was placed on glass slides and incubated for 30 minutes at room temperature (28^{O}C) on damp paper before being gently washed with phosphate buffered saline (PBS) having pH 7.5. A drop form 0.1 ml of 0.2 % Nitro Blue Tetrazolium in PBS was placed on slides and dried for another 30 minutes. The dried slides were stained by Wright's stain for 30 seconds and then washed with distilled water. The activated cells contained bluish granules when treated with

NBT dye while non-activated cells do not contain these bluish granules. The activated granular cells were counted under microscope at 400X. Granular cells were mostly spherical and contain large and highly refractive granules.

Statistical analysis

Mean weight gain and survival of prawn was calculated. The percentage values of NBT, proPO assay and mortality were transferred to logarithmic transformation than one-way ANOVA and Duncan multiple range teat was applied to analyse the data statistically at 0.05 level of significant.

Results and Discussion

Growth and survival of *M. rosenbergii* fed biogut containing diets

Mean weight gain and survival of prawn fed control diet, 1.0%, 1.5% and 2.0% biogut containing diet was 1.32gm, 1.82gm, 2.07gm,1.68gm and 65%, 75% 88% and 72% respectively (Table 2). The significant differences in weight gain and survival of prawn were observed.

Resistance of *M. rosenbergii* to white muscle disease virus

The clinical signs of poor feeding, anorexic, lethargy, exhibited ataxic swimming behavior of the prawns were observed especially after second and third days of post injection in control and treatment groups respectively. These clinical signs were identical to those found in prawn naturally infected with white muscle disease. Few juveniles of whitish appearance with multifocal or diffuse distribution in the cephalothorax, abdominal and /or tail muscles (Fig. 2) were noticed after third and fifth days of post injection resulting in high and slow mortality in control and treatment groups respectively. This whitish juvenile was observed to be cannibalized by healthy juveniles. Initially the whitish color was apparent only against a dark background. Later this whitish discoloration was gradually diffused both anteriorly and posteriorly from abdominal segments followed by high mortality. None of the prawn molted during the experiment. In eight days of challenging experiment with white muscle disease, the control group of shrimp fed on diet devoid of biogut succumbed to death 53.33 % within five days. The mortality of prawn fed control diet, 1.0%, 1.5% and 2.0% biogut containing diet was 53.33%, 46.66 %, 33.33% and 40% respectively. The relative percentage survival of prawn fed 1.0%, 1.5% and 2.0% biogut containing diet was 12.50%, 37.50 % and 24.95% respectively (Table 3).

The immune parameters of *L. vannamei*

Prophenoloxidase assay (PPO)

Phenoloxidase activity of prawn fed control diet, 1.0%, 1.5% and 2.0% of biogut containing diet was 36%, 48.7%, 62.30% and 52.70% respectively (Table 4). The significant difference in phenoloxidase activity was observed among the treatment groups and than control group.

Nitroblue Tetrazolium Assay (NBT)

Respiratory burst of prawn fed control diet, 1.0%, 1.5% and 2.0% of biogut containing diet was 40.70%, 51.30%, 65.0% and 51.70% respectively (Table 4). The significant difference in respiratory burst was observed among the treatment groups than control group.

The present study demonstrated the positive effect of probiotic feed supplement, biogut on the growth and survival of freshwater prawn, *M. roserbergii.*

Table.1 Composition of the basal diet for *M. rosenbergii*

Biogut (%)	Fish meal (%)	Ground nut oil cake (%)	Rice bran (%)	Tapioca flour (%)	Vitamin mineral mix (%)
0	24	60	9	6	1
1	24	60	8	6	1
1.5	24	60	7.5	6	1
2.0	24	60	7	6	1

Table.2 The mean survival and weight of *M. rosenbergii*

Biogut (%)	Survival (%)	Mean weight (g)
0	65^{a}	1.32^{a}
1.0	75^{c}	1.82^{cb}
1.5	88^{d}	2.07^{d}
2.0	72^{b}	1.68^{b}

Data in the same column with different superscripts are significantly different at $p<0.05$

Table.3 Mean mortality and relative percentage of survival (RPS) of *M. rosenbergii* challenged with white muscle disease virus

Biogut (%)	Mortality (%)	RPS (%)
0	53.32^{d}	--
1.0	46.0^{c}	12.50
1.5	33.32^{a}	37.50
2.0	40.0^{b}	24.95

(Data in the same column with different superscripts are significantly at $p<0.05$.)

Table.4 Phenoloxidase activity (proPO) and NBT reduction in *M. rosenbergii* challenged with white muscle disease virus

Biogut (%)	PO positive cells (%)	NBT positive cells (%)
0	36.0^{a}	40.70^{a}
1.0	48.7^{b}	51.30^{b}
1.5	62.30^{d}	65.0^{d}
2.0	52.70^{c}	51.70^{cb}

Data in the same column with different superscripts are significantly different at $p<0.05$.

Fig.1 Healthy juveniles of *M. rosenbergii*

Fig.2 Juveniles of *M. rosenbergii* infected with white muscle disease showing whitish discoloration and opacity of the cephalothorax, abdominal and tail muscles

The addition of probiotic, biogut has resulted in a significantly higher weight gain of prawns over the control group. The reason for the higher growth in prawn fed with biogut supplemented diet could be due to improved intestinal gut flora for better digestion and assimilation of feed, reduced tank contamination, noxious gases and enhance resistance against diseases. Similarly, *the* beneficial effects of soil extract (Maeda and Liao, 1992) and microencapsulated diet containing killed *Vibrio* cells (Itami *et al.*, 1991) on the growth and survival of penaeid larvae, *P. monodon* have been well documented. While *P. monodon* growth and survival was greater for shrimp treated with probiotic bacteria (Rengpipat *et al.*, 2000). Probiotic should be fed starting at an early age for optimal improvement of indigenous gut micro flora (Rengpipat *et al.*, 1998). Since *Bacillus* S11 is a long-term resident in probiotic-treated shrimp guts it should provide a longer-term immunostimulant for shrimp compared with glucan or other such immunostimulants (Sung *et al.*, 1994). In present experiment the best growth of prawn obtained at 1.5% level of inclusion in the diet. The result of present study are comparable to result obtain by other workers.

The survival of shrimp, *P.indicus* fed with probiotic, Lacto-sace supplement was 100% in each treatment groups against 77.66% in control (Uma *et al.*, 1999). The mean survivals of shrimp after 90 days culture were 35.6 ± 7.8% and 31.3 ± 15.3% in the probiotic and control groups, while the mean survivals of shrimp after 30 days culture were 34.2 ± 3.6% and 24.2 ± 5.1% in the probiotic and control groups, in both experiments cannibalism associated with clear water apparently caused low survival (Rengpipat *et*

al., 1998). *Bacillus* S11, a saprophytic strain appears harmless to shrimp culture system, while providing greater shrimp survival during normal culture and following disease challenge by luminescent bacterial challenges (Rengpipat *et al.,* 1998). Prawns are generally known to be cannibalistic with larger individuals often preying upon the small ones. The growth pattern was non-uniformal in *M. rosenbergii* and often addressed by the size grading of the juveniles at an early stage or selective harvest methods (Karplus *et al.,* 1989; Sandfier and Smith, 1985). Therefore the fact that large size distribution exists in the different tanks of the treatments and control might have led to the lower survival of prawn. Effective probiotic treatments may provide broader-spectrum and greater non-specific disease protection as a result of both serological immunity enhancement and competitive exclusion in shrimp guts. Rengpipat *et al.,* (1998) reported that isolation of a bacterial probiont, *Bacillus* S11, from healthy *P. monodon*, which reduced *P. monodon* mortality when challenged with *V.harveyi. Bacillus* S11, a saprophytic strain appears harmless to shrimp culture system, while providing greater shrimp survival during normal culture and following disease challenge by luminescent bacterial challenges (Rengpipat *et al.,* 1998). Probionts will proliferate in rearing water thus providing a better environment for shrimp by reducing the level of certain of pathogens in the culture water (Moriarty, 1998). Uma *et al.,* (1999) reported that probiotic, Lacto-sace supplement improves the growth, survival and disease resistance of white shrimp, *P. indicus.* Rengpipat *et al.,* 2000 reported that probiont *Bacillus* S11 provided both cellular and humoral immune defense responses in *P. monodon.*

During the 10-day challenge test of cumulative shrimp mortality in the probiotic treatment 45.7% was less than that of the control group 64.5 (Rengpipat *et al.,* 2000). In the present study, *M. rosenbergii* fed a diet containing probiotic, biogut at 1.5% showed increased resistance against white muscle disease. Therefore, probioptic like biogut, *Bacillus* S11 and lacto sace showed positive effects of protecting prawn against white muscle disease.

Recently, three approaches have been used to improve shrimp health and yields are use of specific disease-resistant shrimp, vaccination or immunostimulation of shrimp to promote immune response and probiotic use to stimulate immunity and to exclude pathogens. Since shrimp possess a non-specific immune response (Anderson, 1992) vaccination or immunostimulation may provide only short term protection against specific pathogens (Sung and Song, 1996; Sung *et al.,* 1996). These dual defense responses were elicited by the single immunogen, which resided in the shrimp's gut. This residency presumably further protected *P. monodon* against pathogenic bacterial infection by competitive exclusion. The relative importance of these defenses is still unknown, but they collectively conferred bacterial disease protection. It also appears that the use of this probiont was most effective when it was provided at an early age and continued during culture. Probiotic and other immunostimulant use are more desirable and environmentally benign compared to the use of antibiotics and chemicals. This suggests that probiotic treatment is an effective alternative for enhancing shrimp health. This study demonstrated promising results for immune response stimulation in *P. monodon*. Certain microbes can induce immune responses in crustaceans such as Yeast b-glucan (Sung *et al.,* 1994), yeast zymosan (Sung *et al.,* 1996) and dead bacterial cells (Adams, 1991; Itami *et al.,* 1991; Sung *et al.,* 1991) have stimulated immune responses in *P. monodon. Bacillus* S11 surface antigens, or their

metabolites might act as immunogenic for shrimp immune defense (Rengpipat *et al.*, 2000).

Bacillus S11 cell wall peptidoglycan might elicit an immune function in shrimps (Itami *et al.*, 1998) by acting on granulocytes for higher phagocytic activity. (Rengpipat *et al.*, 2000) reported that probiont *Bacillus* S11 provided both cellular and humoral immune defense responses in *P. monodon*. The precise mechanism of action of probiotic feed supplement in improving the disease resistance of shrimp is not known. Yet there is a possibility that the probiotic feed supplement may work as substance to activate the defense mechanism of shrimp as it has been reported in by itami *et al., (*1991) and Raa *et al.,* (1992). As shrimp possess a less developed immune system and are relatively more dependent on the non-specific immune processes such as phagocytosis, probiotic feed supplement could serve as an effective immunopotentiator. In the present study, *M. rosenbergii* fed a diet containing probiotic, biogut at 1, 1.5 and 2% was found to increase PPO and NBT activity against white muscle disease but better PPO and NBT activity was recorded at 1.5% of biogut treatment group. Therefore, probioptic like biogut, *Bacillus* S11 and Lacto sace are found to stimulate immuno response in freshwater prawn, *M. rosenbergii*. The use of probiotic in shrimp farming could greatly help to reduce the antibiotic use and environmental risks arising from the indiscriminate use of antibiotics. The result of the present study would provide a basis for future research.

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How to cite this article:

Prakash Pavadi, H. Shivananda Murthy, B.R. Honnananda and Choudhary, B.K. 2018. Enhancement of Growth, Immunity, Resistance and Survival of Freshwater Prawn, *Macrobrachium rosenbergii* against White Muscle Disease (WMD) Due to Dietary Administration of Probiotic and Biogut. *Int.J.Curr.Microbiol.App.Sci.* 7(04): 569-579.
doi: https://doi.org/10.20546/ijcmas.2018.704.067