*Fishery Technology*
2010, Vol. 47(2) pp: 173 - 178

# Probiotic Effects of *Bacillus* spp. on the Growth and Survival of Postlarvae of *Macrobrachium rosenbergii*

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Negative impact due to continued usage of antibiotics in aquaculture for growth promotion and disease control has necessitated research on alternative strategies such as development of probiotic strains, immunostimulants and vaccination. In this study, the probiotic effect of a *Bacillus* spp. on the growth and survival of postlarvae (PL) of *Macrobrachium rosenbergii* was studied. The *Bacillus* spp. under investigation was introduced in to the PL through culture tank water, feed and both. Changes in the bacterial load and flora, weight gain, percentage survival and changes in water quality parameters such as pH, dissolved oxygen, nitrite and ammonia were studied for a period of three months. There was no significant variation in the survival and weight gain of the PL of the experimental groups. However considerable improvement was noticed in the water quality parameters such as nitrite and ammonia concentration of the culture water in the experimental groups compared to the control group.

**Keywords:** ***Macrobrachium rosenbergii*, *Bacillus* spp., probiotics, growth, survival, water quality**

Antibiotics have played a major role in combating bacterial diseases of cultured aquatic organisms. However, their indiscriminate use can result in development of antibiotic-resistant bacteria in aquaculture system and transfer of drug resistance to human pathogens which makes the treatment of human diseases difficult (Chaithanya *et al.,* 1999). Research has focused on alternative methods of disease control to provide broader spectrum and greater non-specific disease protection by both immunity enhancement and competitive exclusion of pathogens. Many suggested probiotics as an effective and environment friendly tool to achieve the above objective resulting in the development of several probiotic species, including *Lactobacillus* spp. (Pollman *et al*., 1980; Jonsson, 1986), *Bacillus* spp. (Ogle & Inborr, 1987; Spriet *et al*., 1987; Rengpipat *et al.,* 2000), Vibrionaceae, *Pseudomonas*, lactic acid bacteria and yeast (Gatesoupe, 1999) and mixed cultures (Pollman *et al*., 1980; Lessard & Brisson, 1987). Considering the above, studies were undertaken to isolate bacterial strains from the natural habitat and feed of *M. rosenbergii* that could be used as probiotic in the culture operation of *M. rosenbergii*. In the present study, experiments were conducted to identify the probiotic effects of a bacterial strain of *Bacillus* spp. on the growth and survival of postlarvae (PL) of *M. rosenbergii*.

## Materials and Methods

A strain of *Bacillus* previously isolated from *M. rosenbergii* larvae collected from its natural habitat was used for the study. *Bacillus* was cultured in tryptic soy broth on a shaking incubator at room temperature. After 24 hours of incubation, the cells were harvested by centrifugation at 3000 rpm for 15 minutes. The cells were washed thrice in isotonic saline and resuspended in normal saline, for incorporation into culture water and feed. Culture purity and identity were

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routinely checked during this investigation. The centrifuged cells resuspended in normal saline were directly inoculated into the experimental tank to get an approximate concentration of $10^5$ cells $ml^{-1}$ of water. For the preparation of probiotic incorporated feed, the fresh *Bacillus* cells were thoroughly mixed with the commercial prawn feed manufactured by Higashimaru Feeds (India) Limited and the mixture was spread out and dried in an oven for 1–2 h at 37°C. Feed was then stored in clean, plastic bottles at 4°C until used. Probiotic incorporated feed had approximate bacterial concentration of $10^9$ cells $g^{-1}$.

The PL samples (PL 15) of *M. rosenbergii* were collected from Rosen Fisheries, Trichur, Kerala and were grown to 0.020–0.025 g in a rectangular Fibreglass Reinforced Plastic (FRP) tank of one tonne capacity. Artificial aeration was given during the culture period. Twenty five numbers of postlarvae at PL 30 stage were placed in 100 litre glass tank containing 50 litre water and were acclimatized for one week before the experiment. Three treatments were conducted for the PL *viz*., PL fed with probiotic supplemented prawn feed, probiotic introduced through culture water and probiotic incorporated through water and feed. The PL fed with commercial prawn feed without incorporation of bacterial strain was kept as control. The PL were fed with the corresponding feed according to the experimental set up viz., 10% of body weight. Aeration was continuously provided in all the experimental tanks, waste was removed on alternate days and about three litres of water in the tank was replaced with tap water every week. Live wet weight of the shrimp and total plate counts of heterotrophic bacteria from rearing tank water and prawn intestine were measured every 30 days till $90^{th}$ day.

Water samples were collected from the experimental tanks on a monthly basis and analysed for physico-chemical parameters such as pH, temperature, dissolved oxygen (DO), ammonia and nitrite. The pH was analysed using hand held digital pH meter (Eutech, Singapore), and temperature using mercury bulb thermometer. DO was analysed by Winkler's method, ammonia using phenol-hypochlorite method and nitrite by colorimetric method (APHA 1998).

Viable bacterial count of the water, feed and probiotics was analysed at one month intervals. The heterotrophic bacterial load in the gastrointestinal tract of PL of *M. rosenbergii* maintained in different experimental tanks was analysed at monthly intervals. Water, feed and probiotic samples were serially diluted up to $10^{-5}$ using sterile distilled water, following aseptic techniques. For the analysis of PL, the specimens were surface washed in a sterile 0.1% benzalkonium chloride (BZC) solution and then thoroughly rinsed in sterile distilled water three times before homogenising. Juvenile prawns were dissected using sterilized surgical scissors to remove intestines for microbial enumeration and identification. The samples were then homogenized aseptically in sterile all glass homogeniser and serially diluted upto $10^{-6}$. Aliquots of 0.2 ml from each dilution were spread plated in triplicate on Tryptone Soya Agar (TSA) to enumerate total viable count (TVC). Plates were incubated at 30°C for 24-48 hours. After incubation, plates with 30 to 300 colony-forming units (cfu) were selected for counting and isolation of bacterial cultures.

The isolates were purified and maintained on TSA slants for further characterisation and identified to generic level using the taxonomic key for identification by Buchanan & Gibbons (1984) and Prescott *et al*. (2002).

The results were subjected to statistical analysis, wherever required, using Analysis of Variance (ANOVA).

## Results and Discussion

Viable bacterial load associated with the commercial feed, culture water before

treating with probiotics, probiotic incorporated feed and that of the whole larvae at the beginning of the experiment are given in Table 1. Most of the reports showed the presence of viable count around $10^4$ to $10^6$ $ml^{-1}$ in the hatchery water (Anderson *et al*., 1989; Phaterpekar *et al*., 2002; Sahul Hameed *et al*., 2003). The fluctuation in the THB load depend on the method used in the hatchery system, quality of feed, removal of unconsumed feed, dead larvae and other suspended solids (Anderson *et al*., 1989; Aquacop, 1997). The TVC load of the PL before experiment was 1.95 x $10^5$ cfu $PL^{-1}$, while THB count of 1.64 x$10^8$ cfu $g^{-1}$ from *Penaeus indicus* and 3.50 x $10^8$ cfu $g^{-1}$ from *P. monodon* (ICAR, 1983) was reported. The bacterial load of feeds was similar to that reported by Muroga *et al*. (1987) as 1.2x$10^4$ cfu $g^{-1}$ for artificial feeds.

Table 1. Initial total viable count and percentage incidence of various genera of heterotrophic bacteria

| | Experimental tank water | Commercial feed | Postlarvae |
|---|---|---|---|
| **Total viable count** | cfu $ml^{-1}$ | cfu $g^{-1}$ | cfu $PL^{-1}$) |
| | 5.00x$10^4$ | 2.00x$10^5$ | 1.95x$10^5$ |
| **Genera** | **% incidence of various genera from samples** | | |
| **Gram Negative** | | | |
| *Acinetobacter* | 16.0 | ND | 28.5 |
| *Alcaligenes* | ND | ND | 5.7 |
| Enterobacteriaceae | 4.0 | ND | 14.3 |
| *Moraxella* | 32.0 | ND | 22.9 |
| *Vibrio* | ND | ND | 5.7 |
| **Gram Positive** | | | |
| *Bacillus* | ND | 36.7 | 5.7 |
| Coryneforms | 48.0 | 63.3 | 5.7 |
| *Micrococcus* | ND | ND | 2.9 |
| *Streptococcus* | ND | ND | 2.9 |
| Unidentified | ND | ND | 5.7 |
| Total | 100 | 100 | 100 |

There was an equal representation of gram positive and gram-negative genera in the water from the rearing tank before incorporation of the probiotic. There was a predominance of *Acinetobacter* and *Moraxella* among the gram negative bacteria present in the tank water. The bacterial flora of the commercial feed were entirely of gram positive bacteria belonging to the genera *Bacillus* and *Coryneformes*. The findings of the present study differ with the observations of Phatarpekar *et al*. (2002) who studied microflora of freshwater prawn hatchery and reported the predominance of *Alcaligenes*, *Pseudomonas*, *Streptococcus* and members of the family Enterobacteriaceae from hatchery reared larvae. One of the reasons for this variation may be the disinfection process of water that is used for hatchery operations that will help in the dominance of certain bacteria. It is possible that different conditions in rearing system favour the predominance of certain group of bacteria.

After introduction of the probiotic to the rearing system through water, feed and both, the TVC load of the rearing water was analysed. The initial load of rearing water was 5 x $10^4$ cfu $ml^{-1}$. Rearing water from all the experimental groups as well as control showed increase in TVC when compared to the initial load (Fig. 1). However, the TVC load of the rearing water from the treatment groups was significantly higher ($p<0.05$) when compared to that of control tank. This may be due to the incorporation of probiotic strains in the treatment groups. However, Rengpipat *et al*. (1998) reported a higher THB of about $10^{10}$ cfu $ml^{-1}$ after 42 days of experiment which was substantially higher than the bacterial load reported in this study.

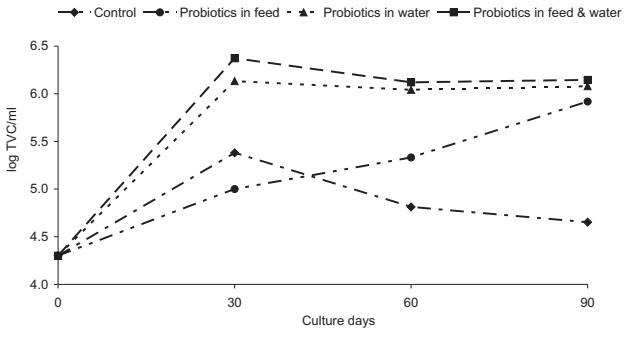


Fig. 1. The TVC load of water from different experimental group of *Macrobrachium rosenbergii*

The fluctuation in the range of THB load depends on quality of feed, removal of unconsumed feed and method used in the culture system (Anderson *et al*., 1989).

Physico-chemical parameters such as pH, temperature, DO, ammonia and nitrite of the rearing water in the various treatments were measured at monthly intervals for a period of 3 months. The pH (7.68 - 7.77), temperature (28.6 - 29.1°C), DO (5.07 - 5.76 mg $l^{-1}$), ammonia (0.032-0.074 mg $l^{-1}$) and nitrite (0.016-0.087 mg $l^{-1}$) concentration of rearing water from different treatment groups showed that the values were within the optimum range for culture of *M. rosenbergii*. There was significant ($p<0.001$) reduction in the nitrite and ammonia levels of the rearing water from the treatment group where probiotics were introduced through water and through both feed and water. Studies of Rengpipat *et al*. (1998; 2000) showed that there were no obvious effects of *Bacillus* S11 species on the water quality parameters in the culture of *P. monodon*. But in the present study pH, nitrite and ammonia of rearing water were found to decrease in the experimental set up where probiotic introduced through water and feed when compared with control set up. Ammonia is toxic at low concentrations and should therefore be removed from the water. The advantage of administering beneficial bacteria in the culture water is bioremediation for controlling water quality (Queiroz & Boyd, 1998; Gatesoupe, 1999), and biocontrol with the goal of being antagonistic to pathogens (Moriarty, 1998; Rengpipat *et al*., 1998; Gatesoupe, 1999; Skjermo & Vadstein, 1999).

The PL of *M. rosenbergii* were maintained in the experimental set up for 90 days. During this period TVC load of the PL was estimated at 30 days interval (Fig. 2). When compared to the initial load, there was 2-3 log increase in the TVC of PL from all treatment groups. Increase in the TVC load of PL from different treatment groups was comparable to previous observations (Rengpipat *et al*., 1998; 2000).

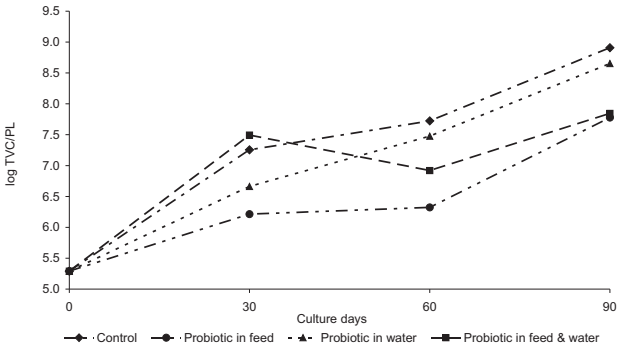


Fig. 2. The TVC load of individual prawn from different experimental groups of *Macrobrachium* rosenbergii

Percentage survival of *M. rosenbergii* PL maintained in various experimental set up as well as in the control was estimated at 30 days intervals for a period of 90 days (Table 2). At the end of the experimental period, 76% survival was observed in the experimental set up where probiotic was introduced through feed, 68% in the experimental set up where probiotic was introduced through water and also in combined mode viz., through water and feed. The percentage survival of PL in the control group was slightly lower (64%) at the end of the experiment. However, the enhancement in the survival of PL in the experimental groups was not statistically significant when compared to that of control. Previous studies by Vici *et al*. (2000) concluded that the application of bacteria and yeast to protect the

Table 2. Percentage survival of the postlarvae of *Macrobrachium rosenbergii* reared in different experimental set up

| Experimental set up | Percentage survival of prawn (n = 25), after | | |
|---|---|---|---|
| | 30 days | 60 days | 90 days |
| Probiotics introduced through feed | 92 (23)* | 92 (23) | 76(19) |
| Probiotics introduced through water | 80 (20) | 76 (19) | 68(17) |
| Probiotics introduced through feed and water | 88 (22) | 84 (21) | 68 (17) |
| Control | 92 (23) | 92 (23) | 64 (16) |

* Number of postlarvae

larvae of *M. rosenbergii* from vibriosis showed that the overall percentage survival of the group of larvae in the hatchery, administered with the bactericins and yeast cell preparation, was higher than that of the control group.

The weight gained by the PL in different treatment setup was estimated at 30 days intervals for a period of 90 days (Fig. 3). The weight gain of PL in the experimental group where probiotic was introduced through water was higher when compared to other two treatments and control. Similar studies conducted by Rombaut *et al*. (1999) showed that by the addition of pure bacterial strain in the rotifer culture, population growth rate increased. Venkat *et al.* (2004) also reported higher weight gain of PL of *M. rosenbergii* by incorporating two *Lactobacillus* species in feed.

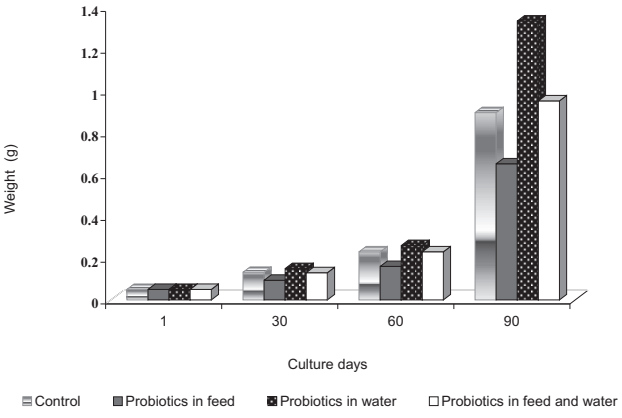


Fig. 3. Mean weight gain of the juveniles of *Macrobrachium rosenbergii* from the different experimental groups

The observation from the study indicate that the concept of introducing selected bacterial strains as probiotics to the culture systems might facilitate healthy growth and survival of *M. rosenbergii* larvae with minimum intervention in terms of antibiotics and other chemical treatments that could result in long term negative impact to the industry as a whole.

The authors wish to express their gratitude to the University Grants Commission (UGC), Govt. of India for the financial assistance (F.3-37/2002).

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