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Evaluation of *Pseudomonas* sp. PM 11 and *Vibrio fluvialis* PM 17 on immune indices of tiger shrimp, *Penaeus monodon*

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Abstract

Occurrence of widespread epizootics among cultured stock of shrimp has put research programmes on preventive approaches such as application of probiotics on a high priority in aquaculture. In the present study two bacteria, *Pseudomonas* sp. PM 11 and *Vibrio fluvialis* PM 17 were selected as candidate probionts from a pool of bacteria isolated from gut of farm reared sub-adult shrimp and tested for their effect on the immunity indicators of tiger shrimp. Sub-adult shrimp, weighing 14 to 22 g were treated in separate experiments with *Pseudomonas* sp. PM 11 and *V. fluvialis* PM 17 @ 10^3 bacterial cells ml^{-1} in the experimental shrimp culture tanks. One set of experimental animals was treated every 3 days and another set of animals every 7 days with each of the candidate probionts. Estimation of immunological indicators such as haemocyte counts, phenol oxidase and antibacterial activity showed declining trends. The haemocyte counts dropped from 31×10^3 to 65×10^3 ml^{-1} on the first day to $4–16\times10^3$ ml^{-1} on the 45th day. Similarly, the phenol oxidase activity declined from 12–32 units on the first day to 11–14 units on 45th day of the experiment. Antibacterial activity of haemolymph reduced to 46–67 percent on the 45th day of the experiment. The results of the study suggest that, the criteria used for the selection of putative probiotic strains in the present study, such as predominant growth on primary isolation media, ability to produce extracellular enzymes and siderophores, did not bring about the desired effect *in vivo* and improve the immune system in shrimp. Hence, new protocols have to be evolved for selection of microbe(s) as putative probiotics and that, detailed understanding of proven probiotics, employed presently on empirical basis may provide a clue on the selection procedure.

Keywords: Antibacterial activity; Haemocyte counts; *Penaeus monodon*; Phenol oxidase; *Pseudomonas*; Probiotic; *Vibrio fluvialis*

Shrimp production has been affected severely by infectious diseases caused by bacteria and viruses during the past several years, as seen with white-spot disease [1–3], infectious hepatopancreatic and lymphoid organ

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necrosis [4], monodon baculovirus disease [5] and vibriosis [6]. A number of preventive approaches such as the use of vaccines [7], immunostimulants [8] and probiotics [9] have been explored in order to reduce the losses due to diseases and mortality of cultured stock. Application of probiotics in aquaculture was envisaged in the early 1970s, whereby the gut microflora of shrimp could serve as food despite keeping them healthy [10]. A number of bacteria and mixtures of bacteria have been sold as probiotics in the aquaculture sector. However, scientific evidence on their efficacy is very scanty and often not available [11].

Certain characteristics, such as the ability to produce adhesins, inhibitory substances like bacteriocines, antibacterial substances and siderophores, competition with pathogens for chemicals and energy, and the ability to boost the immune response, in addition to being non-pathogenic to the target animal have been suggested as traits required in an organism to be a candidate probiotic [12]. However, in most instances selection of bacteria as putative probiotics thus far has been based on an empirical approach.

The defense system in shrimp depends on a non-specific immune response to fight infectious diseases [13]. A number of microbial cell components such as muramyl dipeptides, lipopolysaccharides, Freund's complete adjuvant, β-glucans, and heat killed bacterial preparations are reported to possess immunostimulatory properties [8]. However, studies on the use of live bacterial cells as probiotics directed to improve the immune system are scanty [14]. The objective of this study was to isolate and screen the bacteria from shrimp gut for preferred in vitro criteria and then test them for their ability to improve the immune system in sub-adult tiger shrimp.

Bacteria were isolated and identified from the gut contents of farm-reared sub-adult tiger shrimp, *P. monodon*, of 12.7–16.7 g and were screened for desirable traits such as predominance in the gut microenvironment, ability to secrete extracellular macromolecule digesting enzymes, and to produce iron sequestering compounds like siderophores [15–17]. Based on these traits, two bacterial isolates, *Pseudomonas* sp. PM 11 and *Vibrio fluvialis* PM 17 were selected as candidate probionts.

The two-candidate probiotic bacteria selected were cultured in 25 ml tryptone soya broth (Hi-Media, Mumbai, India) and incubated overnight at 30 °C on a rotary shaker at 120 rpm. After 16–18 h of incubation, the cells were harvested by centrifugation at 7000 g at 4 °C and washed three times in sterile phosphate buffered saline (PBS, pH 7.4). The pellet was re-suspended in 25 ml of sterile PBS and the total viable count of the bacterial suspensions was estimated by serial dilution and spread plate technique.

Healthy shrimp of 14–22 g obtained from a commercial shrimp farm were used in the experiment. Four shrimps were kept in each of the 20 tanks of 200-L capacity and divided into five groups. Water (80–90%) was exchanged daily using filtered seawater. Water quality was maintained at optimal levels during the experiment (pH: 7.6–7.9 and salinity: 28–32 ppt). Shrimp were fed standardised pellet feed at 5% of biomass, split into two feeding intervals. Shrimp were acclimatised for a period of one week before starting the experimental inoculation of candidate probionts. To the first group of four tanks, the candidate probiont *V. fluvialis* PM 17 was inoculated every 3 days, and to the second group of four tanks every 7 days to a final concentration of 10^3 cfu ml^{-1} in the rearing water. Similarly, *Pseudomonas* sp. PM 11 was inoculated to a final concentration of 10^3 cfu ml^{-1} in the rearing water at 3 days and 7 days intervals to the third and fourth groups of tanks respectively. The last set of four tanks was maintained as a control. Immunity indicators of shrimp, viz., haemocyte counts, phenol oxidase activity and antibacterial activity of haemolymph were studied at fortnightly intervals for a period of 45 days.

About 100–150 μl of haemolymph was collected from each of the experimental animals from the ventral sinus, using a sterile disposable 26-guage needle fitted to a tuberculin syringe containing 100 μl of anticoagulant (30 mM trisodium citrate, 388 mM sodium chloride, 115 mM glucose, 10 mM ethylene diamine tetra acetic acid) solution [18]. The haemolymph samples were immediately kept in ice and subjected to further study. Haemocyte counts in the haemolymph samples were obtained by counting them in a haemocytometer under 400× magnification. The haemolymph samples from each treatment group were pooled and used for preparation of the haemocyte lysate fraction (HLF). The pooled haemolymph samples were centrifuged at 4600 g for 10 min at 4 °C. The supernatant was collected in a fresh sterile tube and used

for assay of antibacterial activity. Haemocytes were washed in Tris buffered saline (TBS: 50 mM Tris, 100 mM NaCl, 10 mM EDTA) and re-suspended in 100 µl of TBS. The suspension was sonicated for 20 s using a micro-tipped probe fitted to a Vibracell ultrasonic processor (Sonics & Materials Inc, USA). The resultant suspension was centrifuged at 12,000 g at 4 °C and the supernatant (haemocyte lysate fraction or HLF) was collected in a fresh tube for further assay. The HLF prepared as given above was assayed for phenol oxidase (PO) activity spectrophotometrically using L-3,4-dihydroxy phenylalanine (L-DOPA) (Hi-Media, Mumbai, India) as substrate [19]. The PO assay was performed in flat-bottomed microtitre plates [20]. The dopachrome formed was measured by reading the absorbance at 490 nm using a MULTISCAN ELISA reader (Labsystems, Finland) at 1 min and 3 min. Phenol oxidase activity was expressed in units defined as the amount of enzyme giving an increase in absorbance of 0.001 min^{-1}. Antibacterial activity of haemolymph samples collected from probiont-treated shrimp was tested on a fish pathogenic bacterial isolate, *Vibrio anguillarum* as described in an earlier study [20]. The antibacterial activity of the haemolymph was expressed as percentage inhibition of bacteria.

The haemocyte counts in shrimp groups, treated with the two candidate probionts as the control shrimp showed profound variations. However, their counts generally showed a decreasing trend as the experiment progressed. Before the commencement of bacterial fortification of shrimp culture water (day one), the average haemocyte counts in the haemolymph samples ranged from 31×10^3 to 65×10^3 ml^{-1}, and after 45 days of bacterial treatment, the haemocyte count came down to as low as $4–16\times10^3$ ml^{-1} (Fig. 1). The average phenol oxidase levels also exhibited high fluctuations among the four groups and ranged from 12 to 32 units during the first day of the experiment, with the level dropping to 11–14 units on the 45th day of the experiment (Fig. 2). The average antibacterial activity of the haemolymph samples also decreased from 100% on day one to as low as 46–67% over the 45-day experimental period (Fig. 3). In order to assess the significance of probiotic-treated shrimp compared to untreated controls, a *t*-test was performed [21] and no significant variation in PO, haemocyte counts and antibacterial activity of haemolymph could be found between the experimental and control animals ($P > 0.05$).

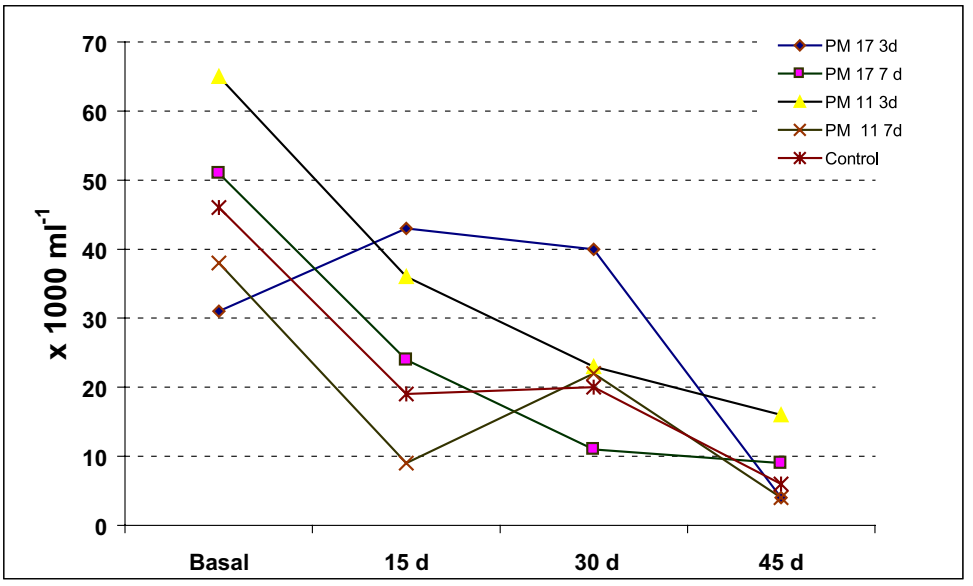

Fig. 1. Haemocyte counts in the haemolymph samples of sub-adult *P. monodon* subjected to treatment with *V. fluvialis* PM 17 and *Pseudomonas* sp. PM 11 as candidate probiotics.

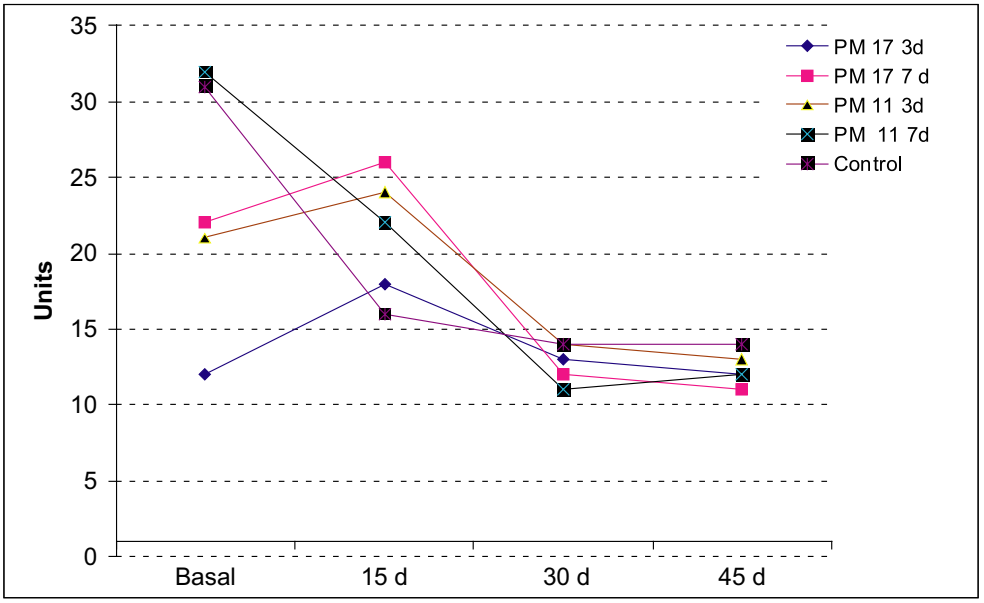

Fig. 2. Phenol oxidase activity in the haemolymph samples of sub-adult *P. monodon* subjected to treatment with *V. fluvialis* PM 17 and *Pseudomonas* sp. PM 11 as candidate probiotics.

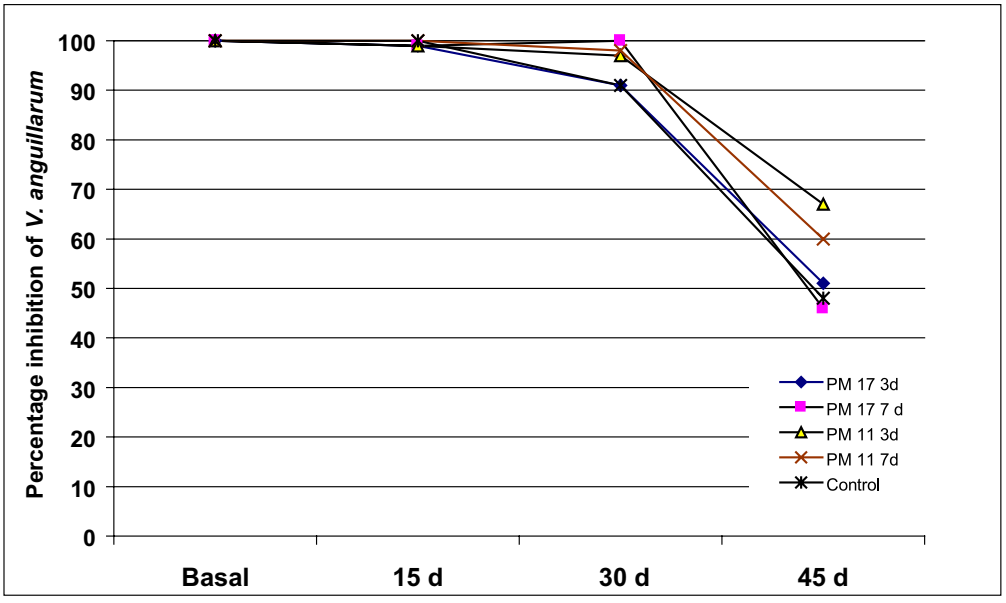

Fig. 3. Antibacterial activity of haemolymph samples of sub-adult *P. monodon* subjected to treatment with *V. fluvialis* PM 17 and *Pseudomonas* sp. PM 11 as candidate probiotics.

These data are in contrast to an earlier report, where a strain of *Bacillus* sp. S11 isolated from a healthy shrimp was reported to enhance immune responses in shrimp. Activation and increase in the phagocytic, prophenol oxidase and antibacterial activity of the haemolymph accompanied by the increase in the growth rate and survival was reported upon treatment of shrimp with *Bacillus* sp. S11 over a 90-day trial [14]. This study employed a very high bacterial concentration of probiotic bacteria, wherein, they mixed one volume of *Bacillus* sp. S11 with three volumes of commercial feed in the pond treatment experiment. In the present study, a dose of 10^3 bacterial cells of candidate probiont per ml of seawater was used and this concentration of bacterial cells was maintained in the experimental tanks in order to keep the total bacterial load within the ranges normally found in near shore water samples and fish and shrimp rearing ponds, which usually are of the order of 10^3–10^6 ml^{-1} [22,23]. So why did not the organisms and the two doses tested in the present study induce immune enhancement in shrimp. Possibly the bacteria did not colonise the gut, or, if they did colonise the gut they did not help in improving the immune system of shrimp. It remains to be seen if an increase in the periodicity of treatment would help in immune enhancement in shrimp. However, increasing dosage in the culture tanks would not be appropriate since their numbers would exceed levels occurring in natural aquatic systems. Bacterial biomass mixed with feed might help in colonisation of gut, and bring about enhancement of immune system of shrimp as reported in an earlier study [14]. However, difficulties of bacterial viability associated with feed preparation have to be overcome. Furthermore, microbes that are sought as candidate probionts have to be examined for the presence of adhesins, which would help in colonisation of gut epithelium in the treated shrimp [24].

Microorganisms possessing antimicrobial activity capable of inhibiting pathogens in vitro have been employed as probiotics by some investigators [25,26]. Harmless bacteria with the ability to produce siderophores have also been used as probiotics to compete and overgrow pathogenic microbes [27]. The microorganisms able to produce siderophores are reported to exert an inhibitory effect on other microflora by depriving iron in their microenvironment [28]. The two bacterial isolates in the present study, despite possessing desirable traits in vitro, such as the ability to produce extracellular enzymes and siderophores, did not bring about any desired probiotic effects. So perhaps bacteria isolated from the gut of shrimp do not necessarily help in enhancing immune responsiveness of shrimp. The present study has indicated that new protocols have to be evolved for selection of microbe(s) as putative probiotics and that, detailed understanding of proven probiotics, which have been employed presently on an empirical basis may provide a clue as to the appropriate selection procedure.

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