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ORIGINAL ARTICLE

Oral administration of formalin killed *Vibrio anguillarum* cells improves growth and protection against challenge with *Vibrio harveyi* in banana shrimp

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Significance and Impact of the Study: The study demonstrates the cross-protection offered by the oral feeding of formalin-killed *Vibrio anguillarum* against pathogenic *V. harveyi* challenge at the early developmental stages of banana shrimp, *Fenneropenaeus merguiensis*.

Keywords
Fenneropenaeus merguiensis, *V. harveyi* and *V. anguillarum*, vibrio bacterin.

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Abstract

Larval rearing in hatcheries and highly intensive grow-out culture practices followed in shrimp production systems favour the growth of potential pathogenic bacterial loads. This study reports the efficacy of formalin-killed vibrio bacterin on growth, survival and protection to challenge with virulent *Vibrio harveyi* and *Vibrio anguillarum* in juveniles of banana shrimp *Fenneropenaeus merguiensis*. Postlarvae 15 (0·24 ± 0·01 g) were administered orally in different concentrations of bacterial preparation (0, 10^6, 10^8, 10^{10} and 10^{12} CFU kg^{-1} feed) for a period of 6 weeks. Physicochemical and microbial quality of water in larval rearing tanks, and growth and survival of the postlarvae were monitored at regular intervals, and body composition was estimated at the end of the experiment. Shrimps were challenged with *V. harveyi* and *V. anguillarum*, and cumulative mortality was calculated. The group receiving 10^8 CFU kg^{-1} feed showed highest average weight gain (162·66 ± 22·94 mg) and survival (90·33 ± 4·5%) and lowest cumulative mortality following the challenge with *V. anguillarum* (26%) and *V. harveyi* (36·67%). The results of the study suggest that formalized vibrio administered orally to *F. merguiensis* postlarvae could induce both homologous and heterologous protection against *V. anguillarum* and *V. harveyi*. 'Vaccination' of shrimp postlarvae at hatcheries would help in preventing the losses due to vibriosis and the most susceptible stages of shrimp development.

Introduction

Vibriosis is one of the most economically important diseases affecting shrimp aquaculture both in hatcheries and in grow-out cultures ponds (Ruangpan 1998). Larval rearing practices especially followed in intensive shrimp hatchery operations harbour bacterial populations comprising both beneficial and pathogenic forms. A major threat in shrimp hatcheries is from pathogenic bacteria that gain entry into the larval rearing systems through the water supply or food source (Muroga *et al.* 1994). Additionally, *Vibrio* spp have the potential to cause economic losses in juveniles and adult shrimp especially under environmental stress (Saulnier *et al.* 2000). It has been reported in penaeid shrimp that environmental stress decreases immune response parameters such as hyalinocytes, granulocytes, total haemocyte counts, superoxide dismutase, respiratory burst and phenoloxidase activities, leading to an increased susceptibility to pathogens of *vibrio* spp.

Vibrio spp in shrimp is implicated in number of syndromes, such as oral and enteric vibriosis, appendage and cuticular vibriosis, localized vibriosis of wounds, shell disease, systemic vibriosis, septic hepatopancreatitis, tail rot disease, bacterial white tail disease and 'Bright-red' syndrome (Lightner 1996; Haldar *et al.* 2010; Soto-Rodriguez

et al. 2012; Zhou *et al.* 2012). Mass mortalities associated with different species of vibrio bacteria both in shrimp hatcheries and in grow-out cultures have been extensively reported worldwide (Karunasagar *et al.* 1994; Sahul Hameed 1995; Vandenberghe *et al.* 1999; Saulnier *et al.* 2000).

Following the regulatory restrictions on the use of antibiotics in aquaculture, the use of immune stimulants to control vibrio infections has been suggested (Rodríguez *et al.* 2007). Ability of formalin-killed vibrio bacteria to induce immunity following the administration as injection in adult and as microencapsulation in larval stages of shrimp has been reported (Devaraja *et al.* 1998; Teunissen *et al.* 1998; Krupesha Sharma *et al.* 2010; Wongtavatchai *et al.* 2010; Pope *et al.* 2011; Powell *et al.* 2011). However, the practical utility of this mechanism is useful only when the delivery methods are easily followed under field conditions. Further, most of the previous immune stimulation studies are limited to tiger shrimp *Penaeus monodon* and *Litopenaeus vannamei* and with the current drive for species diversification; it will be interesting to see similar phenomena in one of the candidate species banana shrimp, *Fenneropenaeus merguiensis*.

This work was carried out to study the effect of regular administration of formalin-killed *V. anguillarum* on survival, growth, microbial loads, proximate composition and resistance to challenge with homologous challenge with *V. anguillarum* and heterologous challenge with *V. harveyi* in banana shrimp *F. merguiensis* juveniles.

Results and discussion

Resistance to *V. anguillarum* and *V. harveyi* challenge

Effects of administration of bacterin at different concentrations on resistance to challenge with *V. anguillarum* and *V. harveyi* were evaluated separately at the end of the 6 weeks of experiment. The treatment groups showed significantly higher ($P \leq 0{\cdot}05$) resistance to challenge compared with controls, both *V. anguillarum* and *V. harveyi* experiments. In the *V. anguillarum* challenge experiment, mortality started 48 h postchallenge, and by fifth day, 100% of mortality was recorded in control groups. Among the treatments, group receiving 10^8 CFU kg^{-1} showed significantly lower mortality (26%), indicating higher resistance compared with the control group. At the end of the seventh day of observation, the cumulative mortality in treatment groups of 10^6, 10^{10} and 10^{12} CFU kg^{-1} was 40, 27·33 and 30·67%, respectively (Fig. 1).

In the *V. harveyi* challenge experiment, mortality started at 24 h postchallenge in PLs in all the groups and control group recorded 100% mortality by 3rd day postchallenge. Among the treatments, groups receiving bacterin at the concentration of 10^8 CFU kg^{-1} showed significantly lower mortality (36·67%), suggesting higher resistance compared with the control group. However, the resistance to challenge was not significantly different between the treatment groups except the group receiving 10^6 CFU kg^{-1}, with cumulative mortality of 60% at the end of seventh day of observation period (Fig. 2). In both the experiments, negative controls inoculated with bacteria-free buffer showed 100% survival at the end of challenge study.

In this study, general enhancement of immune status of the shrimp was demonstrated by the reduction in cumulative mortality in treatment groups following the experimental challenge with virulent *V. anguillarum* and *V. harveyi*. Higher resistance to challenge as an indicator

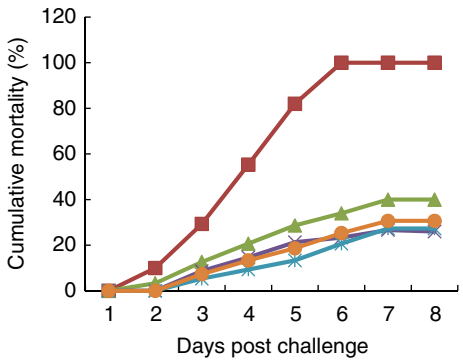

Figure 1 Percentage cumulative mortality of formalin-killed *Vibrio anguillarum* administered *Fenneropenaeus merguiensis* juvenile shrimp challenged with *V. anguillarum* (n = 100). (■) Group I (negative control), (▲) Group II (10^6 CFU kg^{-1}), (×) group III (10^8 CFU kg^{-1}), (×) group IV (10^{10} CFU kg^{-1}) and (●) group V (10^{12} CFU kg^{-1}).

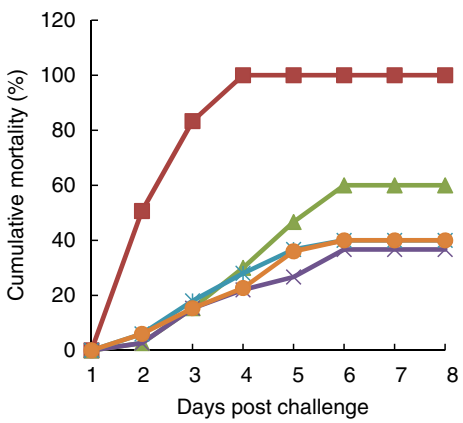

Figure 2 Percentage cumulative mortality of formalin-killed *Vibrio anguillarum* administered *Fenneopenaeus merguiensis* juvenile shrimp challenged with *V. harveyi* (n = 100). (■) Group I (negative control), (▲) group II (10^6 CFU kg^{-1}), (×) group III (10^8 CFU kg^{-1}), (×) group IV (10^{10} CFU kg^{-1}) and (●) group V (10^{12} CFU kg^{-1}).

of improved antimicrobial defence in shrimp has been reported previously for both bacterial and viral pathogens (Devaraja *et al.* 1998; Krupesha Sharma *et al.* 2010; Pope *et al.* 2011). Although the potential mechanism of resistance conferred is not clear, it is hypothesized that cell wall components of vibrio bacteria nonspecifically stimulate the antimicrobial factors in the shrimp immune system.

Presently, the concept of specific immunity and cross-protection in invertebrates is not clearly understood. The protection offered against the *V. harveyi* indicates that *V. anguillarum* induces cross-protection against *V. harveyi*; whether similar phenomenon is working with other related *vibrio* spp needs to be investigated. In a similar study, Aqua-Vac™ Vibromax™ (formalin-inactivated *V. anguillarum*, *V. harveyi, V. parahaemolyticus* and *V. vulnificus*) showed antibacterial activity against *V. parahaemolyticus* (Wongtavatchai *et al.* 2010), *V. anguillarum and V. harveyi* (Powell *et al.* 2011). This study reports the effect of continuous feeding of vibrio bacterin orally as feed topdressing for 6 weeks from PL_{15} stage in *F. merguiensis*. Commercial bacterin preparation Vibromax™ is marketed for use in shrimp hatcheries to control vibriosis, while in the current experiment indigenously developed formalin-killed *V. anguillarum* was used for 'vaccination/immunostimulation' in juvenile stage of shrimp and challenged with virulent *V. harveyi* and *V. anguillarum*.

Physicochemical and microbial quality of water

Physicochemical and the microbial quality parameters in this study were found within the optimal ranges in the penaeid shrimp larval rearing systems (data not shown). There was no deterioration of water quality with regard to pH, concentration of ammonia and nitrite in larval rearing tanks. Any variations in these parameters especially pH have been reported to influence the postlarvae physiology (Kanaujia and Mohanty 1999) and facilitate the growth and multiplication of microbial populations (Kennedy *et al.* 2006). Analysis of the data suggests the oral administration of bacterin as feed topdressing was safe for banana shrimp PLs at the concentrations tested in the study.

Shrimp larval growth and survival

The average body weight gain of the postlarvae fed with different concentrations of bacterin showed significantly higher weight gain compared with the control. However, among the treatment groups, Group III (10^8 CFU kg^{-1}) showed higher weight gain (162·66 ± 22·94 mg) than all the treatment groups. These results indicate that bacterin prepared from a concentration of 10^8 CFU kg^{-1} improved the growth of postlarvae and higher concentrations did not further enhance the growth. However, the average body weight gain at the end of the 6-week experiment was significantly higher in all the treatments (Table 1). Statistical analysis revealed a significant difference between the groups with respect to body weight gain at different sampling points. Further, significant improvement in average survival was observed in treatment groups compared with the control (Table 1). Among the different concentrations of the bacterin, Group III fed with 10^8 CFU kg^{-1} showed significantly higher ($P \leq 0{\cdot}05$) survival rate (90·33 ± 4·50%) while no significant difference was observed in other treatment groups.

In this study, overall performance improvement as suggested by average body weight gain and higher survival rate was observed in treatment groups compared with control groups. Similar results were observed following the oral administration of formalin-killed *V. vulnificus* and *P. monodon* (Song and Sung 1990) and PL stages of *L. Vannamei* (Wongtavatchai *et al.* 2010). By tracking the killed bacterium in the shrimp body, Sung and Song (1996) could not detect the antigen after 14 days of administration, indicating the elimination of vibrio antigen from the body. Hence, the regular administration of bacterin followed in this study might have helped for the continuous enhancement of immune system.

Proximate analysis

The proximate analysis of the whole body at the end of 6-week bacterin administration revealed highest lipid (29·84 ± 2·86 mg g^{-1}) and protein (62·72 ± 2·55 mg g^{-1}) content in Group III (10^8 CFU kg^{-1}). The lipid and protein composition in all the bacterin treated groups was significantly higher than the control. However, among the bacterin-administered groups, the lipid content in Group III was significantly higher (29·84 ± 2·86 mg g^{-1}) compared with Group II (20·98 ± 2·72 mg g^{-1}) and Group V (26·33 ± 1·88 mg g^{-1}) (Table 2).

Effectiveness of drugs and other biologicals depends on the route and schedule of administration in all living beings including aquatic animals. Successful application of immunostimulants has been reported in shrimp using different routes of administration such as injection, immersion, oral as water treatment or as feed topdressing. Oral administration as feed topdressing is the most convenient and practical method of drug application in aquaculture (Smith *et al.* 2003). Stability of the orally administered agents in oral cavity and the digestive track of the animals is considered one of the major drawbacks. The observed higher resistance to bacterial challenge despite the oral route of administration might be due to regular feeding followed in this study.

Table 1 Percentage survival and average body weight gain (g) of the *Fenneropenaeus merguiensis* PL (n = 20) after 6 weeks of feeding with different concentrations of formalin-killed *Vibrio anguillarum* (mean ± SD)

Experimental groups (CFU kg^{-1})	Survival (%)	Av. weight at 0 days	Average body weight gain (g)		
			14th day	28th day	42nd day
Control	82·00 ± 5·29	0·257 ± 0·03	0·084 ± 0·01^{a}	0·099 ± 0·01^{a}	0·109 ± 0·01^{a}
10^{6} CFU kg^{-1}	85·66 ± 3·05	0·240 ± 0·01	0·103 ± 0·00^{a}	0·119 ± 0·01^{ab}	0·132 ± 0·01^{ab}
10^{8} CFU kg^{-1}	90·33 ± 4·50	0·225 ± 0·01	0·109 ± 0·00^{a}	0·147 ± 0·00^{b}	0·162 ± 0·00^{b}
10^{10} CFU kg^{-1}	88·33 ± 1·52	0·229 ± 0·02	0·098 ± 0·00^{a}	0·131 ± 0·00^{ab}	0·156 ± 0·01^{b}
10^{12} CFU kg^{-1}	86·33 ± 3·21	0·258 ± 0·02	0·100 ± 0·00^{a}	0·143 ± 0·01^{b}	0·154 ± 0·01^{b}

Data in the same column having different superscripted letters are significantly different ($P \leq 0·05$).

Table 2 Proximate composition of lipid and protein in the carcass of *Fenneropenaeus merguiensis* PL (n = 20) immunostimulated with different concentrations of formalin-killed *Vibrio anguillarum* (mean ± SD)

Experimental groups	Lipid composition	Protein composition
Control	15·34 ± 3·40^{d}	54·97 ± 0·68^{b}
10^{6} CFU kg^{-1}	20·98 ± 2·72^{a}	58·95 ± 3·31^{bc}
10^{8} CFU kg^{-1}	29·84 ± 2·86^{c}	62·72 ± 2·55^{c}
10^{10} CFU kg^{-1}	28·29 ± 2·03^{bc}	58·50 ± 1·65^{bc}
10^{12} CFU kg^{-1}	26·33 ± 1·88^{b}	59·73 ± 2·59^{c}

Data in the same column having different superscripted letters are significantly different ($P \leq 0·05$).

In conclusion the study showed that oral administration of formalin-killed *V. anguillarum* as feed topdressing at the concentration of 10^{8} CFU kg^{-1} feed improved the growth and survival of *F. merguiensis* juveniles and enhanced resistance to challenge with *V. anguillarum* and *V. harveyi*. As the oral route of delivery is easy and practical, this method could be useful in controlling the vibriosis in early stages of shrimp grow-out cultures.

Materials and methods

Bacterial source, isolation and maintenance

The bacteria, *V. anguillarum* and *V. harvey,i* were isolated from a diseased shrimp collected from *P. monodon* culture ponds near Kalpakkam, Chennai, India. Both the bacteria were isolated on Tryptone soya broth (TSB; Himedia, Mumbai, India) containing 2% NaCl and identified based on biochemical characteristics (Alsina and Blanch 1994) and further characterized using 16S rRNA analysis (GenBank Accession No. JF 264473). The isolates were preserved in TSB (with 1·5% NaCl) containing 15% glycerol at −20°C. For use, an aliquot for each *vibrio* species was revived on nutrient broth and stored at 4°C.

Preparation of bacterin

Bacterin was prepared using *V. anguillarum* as per the method described previously (Azad *et al.* 2005). Briefly, the cultures were grown in 1·5% peptone water broth with 1% NaCl for 24–36 h under constant stirring at room temperature (25–30°C). The bacterial cells were harvested by centrifugation at 13 500 ***g***. Density of the bacterial cell was assessed using spectrophotometer (at wave length 575 nm) and simultaneously counting it by spread plate method just before the inactivation. The concentration was adjusted to 10^{12} CFU ml^{-1} and inactivated with formalin at the final concentration of 0·5%. Complete inactivation of the bacteria was confirmed by inoculating the Tryptone soya broth. The inactivated cells were stored at 4°C for further use.

Preparation and administration of experimental diet

Experimental diet was prepared by diluting the bacterin suspension in water to obtain the final concentration of 10^{6}, 10^{8}, 10^{10} and 10^{12} CFU kg^{-1} feed. The suspension was mixed with 0·1% guar gum (Merck, Germany) as binder and applied uniformly on the larval feed (CP 45%). Similarly, the control feed was prepared with binder solution without inclusion of the bacterin suspension. Bacterin-coated and control diets were fed to the respective groups of shrimp daily during the experimental period of 6 weeks.

Experimental design

Uniform-sized (0·24 ± 0·01 g) healthy (negative for WSSV in PCR test) *F. merguiensis* postlarvae (PL_{15}) were obtained from CIBA, Shrimp Hatchery, Chennai, India. Experiment was designed with groups I, II, III, IV and V for the administration of bacterin at the final concentration of 0, 10^{6}, 10^{8} 10^{10} and 10^{12} CFU kg^{-1} feed, respectively. Three replicates were kept for each group with 300 numbers of PLs in each replicate in 100-l fibreglass-reinforced

plastic (FRP) tanks. Postlarvae were fed with experimental diet three times a day during the study period. The tanks were cleaned daily for removing the uneaten food and wastes. All the tanks were observed daily for dead shrimp, if any, and were recorded. Concurrently, two experiments were separately conducted for challenging with *V. harveyi* and *V. anguillarum* at the end of the 6 weeks.

Analysis of physicochemical parameters and enumeration of bacterial load

Physicochemical parameters pH, salinity and temperature were recorded daily while ammonia and nitrite were estimated weekly in water samples of larval tanks using standard procedures (APHA 1989). For enumerating bacterial loads, water samples were collected aseptically from each tank, and total cultivable heterotrophic bacteria and presumptive *Vibrio* bacteria were determined using marine agar (MA) and TCBS supplemented with 1·5% NaCl, respectively.

Growth and survival

The growth was monitored once in 2 weeks by measuring the weight of randomly collected 20 PLs from each replicate tank, and average body weight gain was calculated for the tank. Animals collected for weighing were replaced back to the respective tanks without harming. The survival was recorded by counting the number of juveniles survived at the end of the experiment.

Estimation of body composition

At the end of the experiment, 20 animals were randomly taken from each replicates and pooled. The whole body was analysed for protein and lipid values using standard procedures (AOAC 1990).

Evaluation of resistance challenge with *V. harveyi* and *V. anguillarum*

Juveniles ($n = 100$) from each replicate tank of the experimental groups were challenged separately with fresh culture suspension of *V. harveyi* or *V. anguillarum* by immersion for 1 h at the final concentration of 10^6 and 10^{10} CFU ml^{-1}, respectively. Mortality of the larvae was monitored for 7 days, and cumulative mortality was estimated.

Statistical analysis

All the experiments were performed in triplicates, and the data were expressed as mean ± standard deviation. Statistical analyses for significance of difference between the treatments' mean values over different days of experimental feeding were performed using repeated measures ANOVA. Survival and proximate composition were analysed using one-way ANOVA. Difference between mean values was compared by Tukey's test at a significance level of $P \leq 0{\cdot}05$.

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Conflict of interest

The authors have no conflict of interests.

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