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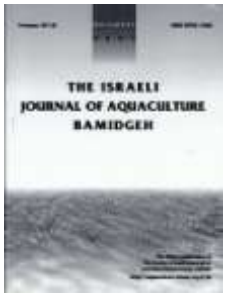

Effect of Formalin-Killed *Vibrio anguillarum* Administration on Immunity and Resistance to *Vibrio harveyi* in Pond-Reared Banana Shrimp *Fenneropenaeus merguiensis*

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Abstract

The present study reports the effects of oral administration of formalin-killed *Vibrio anguillarum* on immune response parameters and resistance to *Vibrio harveyi* in banana shrimp, *Fenneropenaeus merguiensis*. The *Vibrio* bacterin was administered as a feed top-dressing at 10^8 cfu/kg feed, twice a week, for 120 days. Shrimp seed, produced at the institute's hatchery, were stocked in 1500-m^2 earthen ponds at the Navsari Agricultural University experimental station in Gujarat and adult shrimp (12±2.53 g) were collected at the end of the experimental period to determine immune response and protection against challenge with virulent *V. harveyi*. Total hemocyte counts, granulocyte counts, bacterial clearance tests, and phagocytic assays were used to evaluate *in vivo* and *in vitro* immune responses. Animals fed the bacterin had significantly (p<0.05) higher hemocyte and granulocyte counts and *in vivo* and *in vitro* immune responses than those fed the control feed. Bacterin-fed animals had significantly higher (p<0.05) relative survival (43-50%) than the control shrimp. Results suggest that oral administration of *V. anguillarum* bacterin can induce humoral and cellular immune responses and protect against *V. harveyi* infection in shrimp cultured in earthen ponds.

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Introduction

Marine production is economically important to India with an annual export value of US$2.67 billion (MPEDA, 2011). However, since the mid-1990s, shrimp culture, which represents some 46% of India's total marine export value, has been suffering heavy losses due to recurring viral diseases caused by white spot syndrome virus (WSSV). Infectious diseases, especially bacterial infection caused by *Vibrio* spp., are the major problem for sustainable shrimp culture worldwide (Ruangpan, 1998; Flegel, 2001). Intensification of aquaculture and bans on the use of antibiotics necessitate the development of safe, effective, and economic alternatives for control of bacterial infections in shrimp culture ponds.

One approach to overcoming disease problems in shrimp aquaculture is to develop feed additives such as immune stimulants that increase shrimp defense against potential pathogens (Smith et al., 2003; Citarasu et al., 2006; Ganguly et al., 2010). Agents with immune response modifying effects in invertebrates include complex carbohydrates, chemicals, nutritional factors, animal and plant extracts, and bacteria and their products (Hou and Chin, 2005; Citarasu et al., 2006; Pholdaeng and Pongsamart, 2010; Tayag et al., 2010). Safe and effective immuno-prophylactic agents for aquaculture include products of microbial origin such as killed bacteria (bacterin), glucans, peptidoglycans, and lipopolysaccharides that are used to stimulate the immune system (Devaraja et al., 1998; Itami et al., 1998; Takahashi et al., 2000; Klannukarn et al., 2004; Azad et al., 2005; Sharma et al., 2010).

Vibrio bacterins can induce immune regulatory factors in shrimp (Itami et al., 1989; Adams, 1991; Devaraja et al., 1998; Sharma et al., 2010; Pope et al., 2011). Administration of *V. alginolyticus* biofilm stimulates shrimp immunity and confers challenge resistance to *V. alginolyticus* and WSSV (George et al., 2006; Sharma et al., 2010). Administration of killed *Vibrio* bacteria cells to penaeid shrimp induces resistance to challenge by some species of virulent bacteria (Itami et al., 1989; Song and Sung, 1990; Adams, 1991; Sung et al., 1991; Sharma et al., 2010). Further, survival and growth are improved in shrimp receiving *Vibrio* bacterin (Sung et al., 1991; Teunissen et al., 1998; Zafran et al., 1998) and in *Penaeus monodon* postlarvae administered heat-killed *V. anguillarum* by immersion (Azad et al., 2005). *Vibrio* bacterin can modulate immune-related molecules such as chemokinetic cell migration factors (Itami et al., 1989), bactericidins (Adams, 1991), and prophenoloxidase activating enzyme 1 (Jang et al., 2011).

To be used commercially, agents showing immune response modifying effects under controlled laboratory conditions need to be evaluated under pond culture conditions. The success of immune stimulation for disease resistance depends mainly on dose, schedule, and route of application. Hence, in the present work we study the effects of oral administration of formalin-killed *V. anguillarum* on nonspecific immunity and resistance to *V. harveyi* challenge in banana shrimp *Fenneropenaeus merguiensis* reared in earthen ponds.

Materials and Methods

Ponds and shrimp. The study was conducted at the Danti Experimental Station of Navsari Agricultural University, Navsari, Gujarat, India. Four 1500-m^2 ponds were prepared following standard procedures. Briefly, the ponds were flushed three times with tidal water after application of lime to remove the organic load accumulated at the pond bottoms. Then the ponds were filled with tidal water and bleached with bleaching powder (300 kg/ha). After 10 days incubation, lime was applied at 100 kg/ha and ponds were fertilized with a concoction of fermented juice (5 kg rice bran, 5 kg jaggery, and 100 g yeast mixed in 100 l sea water). After one week the pond water turned light green, signifying the development of algae bloom that was maintained thereafter until harvest. Banana shrimp *Fenneropenaeus merguiensis* seed (PL20) produced at the Muttukadu Experimental Station of the Central Institute of Brackishwater Aquaculture (CIBA) near Chennai were stocked into the four ponds at 10/m^2 and fed a commercial tiger shrimp feed (40% crude protein) as per the standard feeding chart. Twice weekly, two test

ponds (T1 and T2) were fed the feed top-dressed with bacterin while the two control ponds (C1 and C2) continued to be fed the feed without bacterin.

From 45 days after stocking, water was exchanged every ten days. Agricultural lime (5 kg/ha), dolomite (5 kg/ha), zeolite (2 kg/ha), commercial probiotics for water (90 g/ha), and soil (3-4 l/ha) were added. The pond bottoms were periodically disturbed by chain dragging to maintain aerobic conditions at the soil-water interface. The optimum dissolved oxygen level (4-5 ppm) was maintained by two 5-hp aerators per pond, 8 h/day. Water losses due to seepage and evaporation were compensated by pumping pre-treated (bleaching powder 300 kg/ha) water from reservoirs.

Bacteria. Vibrio anguillarum and *V. harveyi* were isolated from diseased *Penaeus monodon* culture ponds near Kalpakkam, Chennai. The bacteria were isolated on 1.5% tryptone soya broth (TSB; Himedia, Mumbai) containing 2% NaCl and identified by biochemical characteristics and 16S rRNA analysis. The isolates were preserved in 1.5% TSB containing 0.15% glycerol at -20°C. For use, an aliquot of each bacteria was revived on nutrient broth and stored at 4°C. *Vibrio anguillarum* was used to prepare the bacterin and *V. harveyi* was used for the challenge study.

Preparation of the bacterin. Vibrio anguillarum was 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*, then processed and deactivated with formalin (0.5%). Density of the bacterial cells was assessed with a spectrophotometer and the concentration was adjusted to 10^{10} cfu/ml. The suspension was stored at 4°C until further use.

Preparation of diets. The experimental diet was prepared by diluting the bacterin suspension in pond water to a final concentration of 10^8 cfu/kg feed. The suspension was mixed with guar gum (0.1%) as a binder, applied uniformly on the shrimp feed, and dried in the shade for 3-4 h. The control feed was prepared similarly, with the binder solution but without the bacterin suspension. The bacterin-coated diet was fed to the treated shrimp on two consecutive days each week during the culture period. Trays were checked daily to determine feed consumption and to adjust the feed ration.

Water quality. Water samples were periodically collected and analyzed for pH, salinity, CO_3, HCO_3, Ca, Mg, alkalinity, TAN, NO_2, NO_3, and PO_4. Soil samples were analyzed for organic C, and available N and P as per standard APHA procedures. Water temperature, air temperature, and rainfall were recorded daily. Average values of dissolved oxygen (4.1±0.5 ppm), pH (8.1±0.6), temperature (28±1.4°C), salinity (29.1±1.8 ppt), ammonium (0.09±0.03 ppm), nitrite (0.01±0.008 ppm), and phosphate (0.2±0.06 ppm) in the treated ponds did not significantly differ from the control ponds and were within the optimum ranges for shrimp culture.

Shrimp sampling. Every 15 days from day 45 after stocking, shrimps were sampled by cast netting to assess their growth and health condition. Length, weight, sex ratio, abnormalities, and molting stage were noted.

Immunological parameters. On day 120, 150 shrimp were collected from each pond using a cast net and transported to the laboratory for measurement of immunological parameters. Shrimp (12±2.3 g) from the treated (T1 and T2) and control (C1 and C2) ponds were kept separately for one week in aerated 1-ton fiber reinforced plastic tanks for acclimatization during which they were fed their respective feeds. Then, the animals were randomly divided into triplicate groups of 50, each, for a total of 12 tanks (4 treatments × 3 replicates). Five animals were sampled from each replicate for hematology (total hemocyte and granular hemocyte counts), 25 for bacterial clearance and phagocytic activity assays, and 10 for *Vibrio* challenge.

Hemolymph was collected from the base of the first pleopod abdominal segment using a sterile 1-ml syringe with a 25-gauge needle. One hundred ml of hemolymph was drawn into the syringe containing equal amounts of precooled sterile anticoagulant (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6) as described by Söderhäll and Smith (1983). To determine total hemocyte count (THC), 100 µl of the hemocyte anticoagulant mixture was diluted with an equal amount of formaldehyde (4%) and allowed to stand for 30 min, about 50 µl of the fixed hemocyte

suspension was loaded onto a hemocytometer (Neubauer chamber), and cells in 5/25 squares (volume of 1 square = 0.2 x 0.2 x 0.1 mm^3) were counted using a compound microscope. THC/ml was expressed as 5 x count x 10^4 x dilution factor. To count granular hemocytes (GH), thin smears were prepared with a drop of hemolymph suspension on glass slides. The smeared slides were immersed in hematoxylin stain for 5-10 min followed by dehydration with 95% ethanol (10 dips) and 100% ethanol (10 dips), rinsed by submersion in xylene, and observed under oil immersion in a compound microscope. Two hundred hemocytes were counted and the proportion of granulocytes was recorded as GH/ml, i.e., granulocyte cell count/200 x THC.

Bacterial clearance and phagocytic activity. To determine bacterial clearance and phagocytic activity, shrimp were injected in the ventral sinus with 25 µl *V. anguillarum* bacterial suspension, resulting in 10^6 cfu/shrimp. Five shrimp from each group were bled and 100 µl hemolymph was collected from the ventral sinus in syringes containing equal amounts of sterile anticoagulant. Hemolymph samples and the original inoculums were subjected to bacterial enumeration by the standard dilution and plating method. Clearance efficiency, defined as the percent inhibition (PI) of *V. anguillarum*, was calculated as PI = 100 - 100[(cfu in test group)/(cfu in control group)], where cfu = colony forming units. Phagocytic activity was determined according the procedure described by Itami et al. (1992). Briefly, 100 µl hemolymph was mixed with 500 µl sterile anticoagulant and the viable cell number was adjusted to 10^6/ml. The hemocyte suspensions were incubated 1 h with heat-inactivated *Vibrio* bacterial preparation (10^6/ml). After incubation, the cells were fixed with 100% methanol and stained with Giemsa stain. At least 200 hemocytes were counted per sample. Phagocytic activity (%) was calculated as 100(phagocytic hemocytes/total hemocytes).

Evaluation of resistance to Vibrio harveyi. Shrimp from each group were challenged with *V. harveyi* by injecting each animal with 100 µl fresh bacterial culture suspension containing 10^8 cfu/ml. Mortality was monitored daily for 10 days and cumulative percent mortality was calculated.

Statistical analyses. The significance of differences between treatment and control groups in immune response parameters was analyzed using the two sample *t* test where $p \leq 0.05$.

Results

Production and survival were significantly higher in shrimp fed the bacterin-coated feed than in shrimp fed the uncoated control (Table 1). Shrimp in both treated ponds had over twice the total hemocytes and 2.5 times more granocytes than shrimp in the control ponds (Fig. 1). Mortality started in the control group 24 h after challenge with virulent *V. harveyi* but only on day 2 in the treated groups and was significantly higher in the control shrimp than in the treated. None of the shrimp in the unchallenged controls died.

Table 1. Production of banana shrimp *Fenneropenaeus merguiensis* in ponds with and without oral administration of an immunostimulant *Vibrio anguillarum* bacterin.

	Pond			
	Control		*Bacterin-treated*	
	1	*2*	*3*	*4*
Pond area (m^2)	1500	1500	1500	1500
Stocking density (no./m^2)	10	10	10	10
Days of culture	136	139	136	139
Avg body wt (g)	18.4	14.2	17.7	16.9
Survival (%)	39	58.3	63.5	76
Production (kg/ha)	717.3	862.7	1124	1284.7
Total hemocyte count (10^5 cells/ml)	82±17.11[a]	79.86±23.47[a]	177.20±16.68[b]	160.46±19.51[b]
Granocyte count (10^5 cells/ml)	52.26±22.91[a]	58.8±15.27[a]	127.46±12.72[b]	110.93±25.73[b]
Bacterial clearance efficacy (%)	32.93±9.39[a]	27.2±12.41[a]	74.4±13.6[b]	77.53±3.76[b]
Phagocytic activity (%)	16.93±2.61[a]	18.0±1.0[a]	28.86±2.20[b]	25.54±2.54[b]
Mortality after challenge to *Vibrio harveyi*	93.66 ±5.77[a]	96.66±5.77[a]	46±11.4[b]	50±10[b]

$p<0.05$

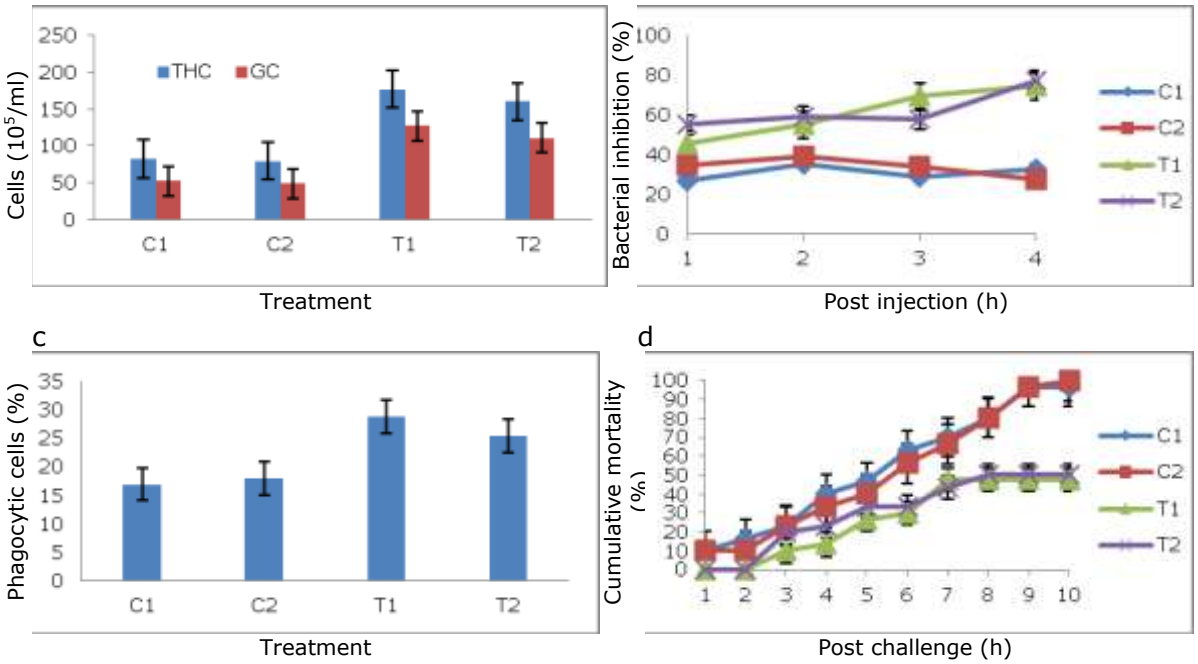

Fig. 1. Total (a) hemocyte (T) and granulocyte (GC) counts, (b) bacterial clearance, (c) phagocytosis, and (d) cumulative mortality in banana shrimp (*Fenneropenaeus merguiensis*) grown in control ponds (C1 and C2) and in treated ponds (T1 and T2) where shrimp received feed coated with *Vibrio* bacterin (mean±SD, vertical bars indicate standard deviations).

Discussion

Growth and survival were significantly improved in pond-reared shrimp that received a diet coated with *Vibrio* bacterin, similar to results in laboratory experiments (Itami et al. 1989; Song and Sung, 1990) and a field study with bacterin prepared from *V. harveyi* (Klannukarn et al., 2004).

Lipopolysaccharide (LPS), a component of the bacterial cell wall, has an immunostimulatory effect in crustaceans under experimental conditions (Takahashi et al. 2000). Bactericins and other humoral factors, possibly lectin, are induced following exposure to heat-killed *Vibrio* (Adams, 1991). The immune stimulatory activity of killed *Vibrio* can be attributed to the ability of LPS in gram-negative cell wall binding to pattern recognition proteins, stimulating the prophenoloxidase system of semigranulocytes and granulocytes (Sritunyalucksana and Soderhall, 2000). Oral administration of LPS induces virus-inactivating activity in hemolymph in kuruma shrimp *Penaeus japonicus* (Takahashi et al., 2000) and imparts resistance to WSSV challenge in *P. monodon* (George et al., 2006). Specific immune priming in *Litopenaeus vannamei* follows the administration of *V. harvyei* bacterin, although this was not demonstrated after challenge with unrelated bacterium (Pope et al., 2011), supporting the theory of vaccination against vibriosis. The efficacy of *Vibrio* bacterin varies widely (Song and Sung, 1990; Alabi et al., 1999), possibly due to differences in route, dose, or schedule of administration, culture conditions, or the age of the animals used in the study.

In crustaceans, total hemocyte count is an indicator of immune status and a reduced number of hemocytes is correlated with enhanced susceptibility to infection (Le Moullac et al. 1998). In shrimp, defense responses such as phagocytosis, encapsulation, melanisation, and coagulation originate in hemocytes (Johansson et al., 2000) and the number of hemocytes is an indirect measure of antibacterial defense (Perazzolo et al., 2002). Further, humoral defense molecules such as prophenoloxidase, antimicrobial peptides, and clotting factors are released from hemocyte granules, stimulating phagocytosis and other immune system responses to invading pathogens (Söderhäll and Cerenius, 1998; Sritunyalucksana and Söderhäll, 2000; Jang et al., 2011). Hence, the

number of granular cells may be a more specific measure of the potential defense capacity of these molecules. Since immunity and antimicrobial defense in shrimp depends on a number of complex interrelated cellular and humoral activities, the efficiency of bacterial clearance after challenge with a specific pathogen might be a simple method of measuring the summative potential of these activities (Sritunyalucksana et al., 2005). In the present study, total hemocytes, granocytes, bacterial clearance, and phagocytic index were used as indicators of cellular and humoral immunity in *F. merguiensis*. The hematological parameters and immune responses were significantly better in bacterin-treated shrimp than in control shrimp. The clear correlation between bacterial clearance and the phagocytic index indicate the role of phagocytosis in bacterial clearance.

The treated shrimp had a faster and enhanced immune response as indicated by the bacterial clearance and phagocytic assays. Bacterial clearance was improved until the end of fourth day. In a similar study, enhanced immune parameters were recorded on an hourly basis and the immune stimulation saturation point of bacterial clearance ability was reported at 90 min post challenge (Sritunyalucksana et al., 2005). The long duration of the bacterial clearance capacity of treated shrimp in the present study could be due to continued feeding of bacterin for the entire culture period as opposed to the one-time or short period of administration in previous studies (Song and Sung, 1990; Sung et al, 1991; Devaraja et al., 1998; Teunissen et al., 1998; Azad et al., 2005).

In earlier studies, bacterin-administered shrimp were challenged with the same bacterium that was used to prepare the bacterin (Itami et al., 1989; Devaraja et al., 1998; Klannukarn et al., 2004; Sharma et al., 2010). In the present study, *V. anguillarum* was used for the bacterin and *V. harveyi* for the challenge. Since *V. harveyi* is the most important *Vibrio* causing major economic losses, it would be appropriate to study its resistance against *V. harveyi* bacterin. The cross protection of *V. anguillarum* against *V. harveyi* suggests that bacterin prepared from *V. anguillarum* might also induce considerable resistance against other pathogenic *Vibrio* such as *V. parahaemolyticus*, *V. alginolyticus*, and *V. fischeri*. The present findings suggest that application of a bacterin as a top coating in feed improves the immunity of cultured shrimps against potent *Vibrio* such as *V. harveyi*, similar to results in concrete tanks and pond-reared *P. monodon* fed pellets top dressed with bacterin from *Vibrio harveyi* (Klannukarn et al., 2004).

In the present study, the production rate of shrimps in the treated group was better than in the control, irrespective of the number of culture days. In addition, the bacterin preparation improved the overall immune status of banana shrimp grown in earthen ponds. The better production performance of the treated shrimp could be attributed to the improved defense system and resistance to challenge. The oral administration of bacterin as a feed top-dressing in shrimp growout ponds was effective and, therefore, a simple and cost-effective method for improving health and production in commercial shrimp culture systems.

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