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ORIGINAL ARTICLE

Effect of orally administered vibrio bacterin on immunity, survival and growth in tiger shrimp (*Penaeus monodon*) grow-out culture ponds

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Significance and Impact of the Study: Administration of vibrio bacterin in feed as a top dressing induced immune stimulation as indicated by higher levels of total haemocyte count and prophenoloxidase. Further reduction in percentage of animals with anatomical deformities suggests the protection against subclinical bacterial infections. The overall improvement in the production parameters like, average daily gain, survival, feed conversation ratio and production in different shrimp stocking densities under commercial farming conditions suggested the possible development of an immune stimulant product based on the inactivated vibrio bacteria for improved health and production in *Penaeus monodon* shrimp farming.

Keywords
bacterin, immunostimulant, *P. monodon*, shrimp deformities, survival and production, vibrios.

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Abstract

Vibriosis is one of the important diseases causing economic loss to the shrimp industry worldwide. The present study reports field observations on the immune stimulatory effect of vibrio bacterin in commercial tiger shrimp (*Penaeus monodon*) grow-out culture ponds ($n = 62$) which were grouped under three stocking densities; low (6–8 nos per m^2), medium (9–11 nos per m^2) and high (12–14 nos per m^2). The bacterin was administered in feed as a top dressing at final concentration equivalent to 2×10^8 CFU per kilogram feed twice a week throughout the culture period. In 20 representative ponds, total haemocyte count and prophenoloxidase activity in shrimp were significantly ($P < 0{\cdot}05$) higher and anatomical deformities like, antennae cut ($5{\cdot}02 \pm 2{\cdot}42$), tail rot ($5{\cdot}10 \pm 1{\cdot}74$), rostrum cut ($4{\cdot}49 \pm 2{\cdot}19$) and soft shell ($10{\cdot}05 \pm 5{\cdot}77$) were significantly lower compared to controls in all the studied stocking densities. Significant ($P < 0{\cdot}05$) improvement in production parameters like survival and production (kg ha^1) was observed in all treatment ponds while similar improvement in average daily gain and feed conversion ratio could be observed in groups with low and medium stocking densities. Results of the study suggest that, oral administration of vibrio bacterin improves the immunity, reduces anatomical deformities and enhances the production in commercial shrimp culture operations.

Introduction

Asia tops the world fishery production with the share of aquaculture reaching to 55% in 2014 and it is estimated that contribution of Asian countries to global aquaculture production may reach 91% by 2022 (FAO 2016). Intensification in shrimp farming to meet the rising demand of international market has led to deterioration of culture environment imposes stress on Penaeid shrimp adversely affecting their immune system and increasing their susceptibility to opportunistic pathogens (Moullac and Haffner 2000). Bacterial diseases especially, vibriosis cause mass mortalities leading to significant economic loss both in hatcheries and grow-out shrimp culture systems (Austin and Zhang 2006). The disease is manifested either as oral/enteric like appendage, cuticular and localized wounds, shell disease as well as systemic diseases like, septic hepatopancreatitis, tail rot disease, bacterial white tail disease or 'Bright-red' syndrome (Haldar *et al.* 2010; Rodriguez-Soto *et al.* 2012; Zhou *et al.* 2012).

Following the restrictions on use of antibiotics in aquaculture, alternative strategies like, application of natural antimicrobial agents, probiotics and immunostimulants have been suggested to control bacterial infections (Selvin *et al.* 2004; Wang *et al.* 2009; Peraza-Gomez *et al.* 2014). Being invertebrates shrimp depend almost entirely on innate immunity to fight the invading pathogens and application of immune stimulating agents would help in enhancing the disease resistance. Several immune stimulating agents from chemicals, plants, animals and microbial origin were reported to improve the immune parameters and confer resistance to challenge in *Penaeid* shrimp (Azad *et al.* 2005; Peraza-Gomez *et al.* 2014). Further, beneficial effects of live microbes and inactivated microbial products on health, welfare and nutrition in fish/shellfish have been well documented (Merrifield and Ringo 2014). Efficiency of microbial products like, inactivated bacteria, glucans, peptidoglycans and lipopolysaccharides in stimulating immune response and conferring resistance to pathogen challenge has been reported (Klannukaran *et al.* 2004; Azad *et al.* 2005; Tung *et al.* 2009; Sharma *et al.* 2010). Several studies have reported the ability of vibrio bacterin to induce immune regulatory factors in *penaeid* shrimp conferring resistance to pathogenic vibrio (Tung *et al.* 2009; Sharma *et al.* 2010; Pope *et al.* 2011; Patil *et al.* 2014), WSSV challenge (George *et al.* 2006; Sharma *et al.* 2010) and improved growth and survival (Azad *et al.* 2005). This bacterin contains pathogen-associated molecular patterns (PAMPs) which include lipopolysaccharide and peptidoglycan. These PAMPs act as immune stimulants and activate pathogen recognition receptors and prophenoloxidase (proPO) system. (Tafalla *et al.* 2013; Wang *et al.* 2013). Though several researchers have documented the efficacy of vibrio bacterin in stimulating immune response and protecting the shrimp against bacterial challenge in controlled laboratory experiments, similar studies demonstrating the performance under the commercial farming conditions are limited (Patil *et al.* 2013). The present work reports the effect of oral administration of formalin inactivated Vibrio sp. on nonspecific immunity, anatomical deformities, growth, survival and production in commercial tiger shrimp, *Penaeus monodon* grow-out cultures.

Results and discussion

Pond sediment and water quality

The physico-chemical parameters of pond sediment and water were found within the optimal ranges in all the ponds studied (Table 1). There was no significant difference in the levels of water and pond sediment quality parameters between the treatment and control ponds throughout the culture period.

Effect on immunological status

In the present study, it was observed that the levels of total haemocyte count (THC) and proPO in harvested adult shrimp from ponds administered with bacterin-coated feed were significantly higher ($P \leq 0{\cdot}05$) than that of shrimp receiving control feed (Fig 1a). Earlier studies suggested that invertebrate haemocytes are the first line of defence and higher the number of these cells in circulation better the resistance to virulent challenge and their reduced number is reported to be correlated with

Table 1 Microbiological and Physico-chemical parameters of pond sediment and water samples collected from different farmer's *Penaeus monodon* culture ponds

Parameter	Range	
	Treatment	Control
Pond sediment		
Total bacterial count ($\times 10^7$ CFU per millilitre)	34·5–100·17	34–128·2
Total *Vibrio*count ($\times 10^3$ CFU per millilitre)	28·17–94·5	21·6–91
Available N (kg ha^{-1})	146·4–229·26	146·18–189·92
Available K (kg ha^{-1})	2227·34–5003·71	2842·68–4718·71
Available P (kg ha^{-1})	159·86–355·03	214·66–373·91
Pond sediment pH	8·33–8·61	7·87–8·42
Pond sediment EC (dSsq.cm^{-1})	3·37–16·58	5·69–8·46
Organic carbon (%)	0·38–0·68	0·35–0·69
Water		
Total bacterial count ($\times 10^5$ CFU per millilitre)	2·37–7·27	4·42–7·18
Total *Vibrio* count ($\times 10^2$ CFU per millilitre)	1·31–4·48	1·56–4·1
Total alkalinity (ppm)	161·85–205·72	156·32–229·36
pH	7·51–8·33	8·16–8·41
N02-N (ppm)	0·03–0·28	0·03–0·25
TAN (ppm)	0·02–0·18	0·03–0·19
Available. PO4 (ppm)	0·01–0·06	0·01–0·05
Salinity (ppt)	13·8–33·92	15·22–33·69
Ca^{++} (ppm)	165·49–354·06	164·6–324
Mg^{++} (ppm)	802·47–1200·99	809·42–1296·96

enhanced susceptibility to infection (Johansson *et al.* 2000; Sritunyalucksana and Söderhäll 2000; Sritunyalucksana *et al.* 2005). Invertebrate haemocytes are reportedly responsible for variety of defence responses like phagocytosis, encapsulation, melanization and coagulation (Johansson *et al.* 2000), release of humoural defence molecules such as proPO, antimicrobial peptides and clotting factors. Additionally, these haemocytes are also involved in several metabolic functions, development and nutrient utilization in invertebrates (Wood and Jacinto 2007). Perazzolo *et al.* (2002) suggested that haemocyte numbers could be considered as an indirect measure of antibacterial defence ability of the host. Hence in the present study, overall beneficial effect of vibrio bacterin administration could in part be attributed to higher levels of haemocytes.

The proPO activity (unit min^{-1} ml^{-1}) in shrimp from treatment ponds at different stocking densities is shown in Fig. 1b. Phenoloxidase system plays a key role in shrimp immunity by enabling haemocyte attraction and inducing phagocytosis, melanization, cytotoxic reactant production, particle encapsulation and the formation of nodules and capsules in response to invading pathogens (Amparyup *et al.* 2013). Thus, the activation of proPO levels is considered as an indicator of immune stimulation. Significantly higher proPO levels observed following the administration of vibrio bacterin indicate the immune stimulatory effect as observed previously under controlled laboratory experiments in different shrimp species (Sharma *et al.* 2010; Patil *et al.* 2013). Total haemocyte count and the proPO system are considered key indicators of innate immunity of invertebrates against pathogen infection. Upon activation of PAMPs, prophenol-activating enzyme converts proPO to phenoloxidase which finally acts against pathogens by producing melanin (Tafalla *et al.* 2013).

Effect on anatomical deformities

Significant reduction in anatomical deformities such as antennae cut, tail rot, rostrum cut and soft shell was observed in treatment ponds compared to control (Table 2). Health of the shrimp can be monitored by recording the presence or absence of body deformities like, antennae cut, tail rot, rostrum cut and soft shell (Fegan and Clifford 2001; Haldar *et al.* 2010). The occurrence of deformities was considered as symptoms of subclinical vibrio infections (Lightner and Redman 1998) and the lower levels of these deformities in shrimp from treatment ponds could be attributed to enhanced nonspecific immunity by the vibrio bacterin conferring protection against subclinical bacterial infections.

Effect on production

All the treatment ponds showed significant ($P < 0{\cdot}05$) improvement in survival (%) and production (kg ha^{-1}) compared to their respective controls while similar improvement in average daily gain (ADG) and feed conversation ratio (FCR) could be observed in groups with low and medium stocking densities (Fig. 2). Among three groups based on stocking densities, maximum survival ($86{\cdot}55 \pm 11{\cdot}61\%$) was observed in high stocking density group followed by low and medium densities. The low and medium stocking density groups showed improved ADG compared to their respective controls (Fig. 2b). The

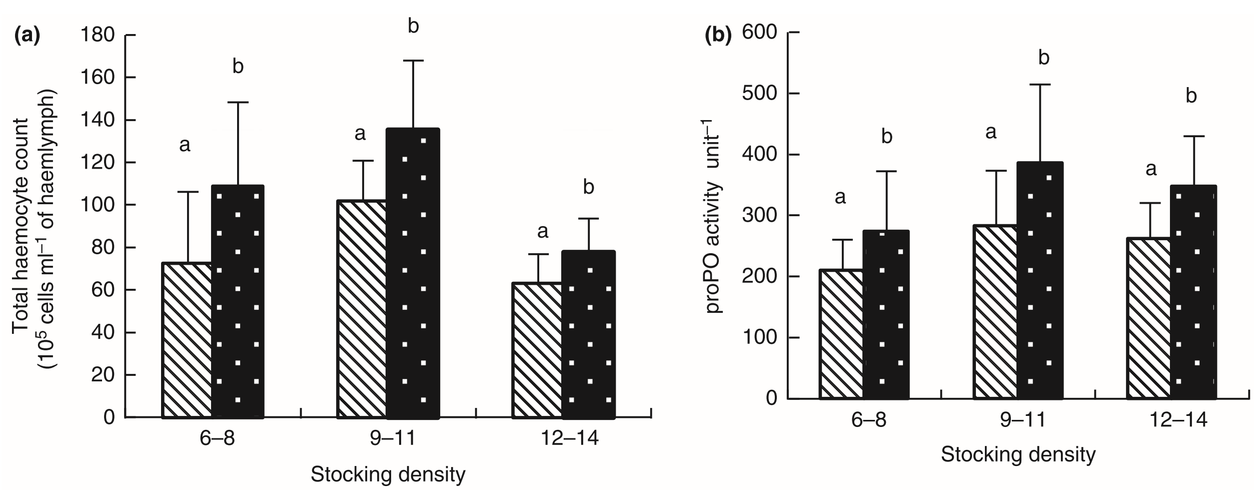

Figure 1 (a) Total haemocyte count (nos × 10^5 cells per millilitre of haemolymph) and (b) prophenoloxidase (proPO) activity (U min^{-1} ml^{-1} of haemolymph). Treatment (■) and control (▧).

Table 2 Effect of vibrio bacterin administration on percentage of animals with appendage deformities (mean ± SD) in *Penaeus monodon* culture ponds ($n = 20$)

Deformities in shrimp ($n = 100$)		
	Treatment (%)	Control (%)
Antennae cut	$5{\cdot}02^{a} \pm 2{\cdot}42$	$16{\cdot}87^{b} \pm 6{\cdot}17$
Tail rot	$5{\cdot}10^{a} \pm 1{\cdot}74$	$19{\cdot}22^{b} \pm 9{\cdot}16$
Rostrum cut	$4{\cdot}49^{a} \pm 2{\cdot}19$	$10{\cdot}15^{b} \pm 3{\cdot}07$
Soft shell	$10{\cdot}05^{a} \pm 5{\cdot}77$	$22{\cdot}96^{b} \pm 10{\cdot}29$

Means in the same row with different superscript are significantly different ($P < 0{\cdot}05$).

lower FCR value observed in the treatment groups indicated improved feeding and lower feed loss leading to reduction in cost of production and higher economic benefit. Production data in the present study showed the overall improvement in treatment ponds in all the groups with different densities. The production increased by 32, 19 and 28% over control at low, medium and high stocking densities respectively. The higher production performance by shrimp in treatment ponds could be attributed to improved defence system following administration of vibrio bacterin leading to improvement in survival and ADG which ultimately led to higher production yields. Controlled experimental studies following oral administration of formalin inactivated *V. vulnificus in* postlarval stages of pacific white shrimp (Wongtavatcha *et al.* 2010) and subadult stages of banana shrimp (Patil *et al.* 2013) support the evidence of increase immune defence system leading to higher production attributes.

Materials and methods

Preparation of the bacterin

Bacterin was prepared using *V. anguillarum* grown in 1·5% peptone water broth with 1% NaCl for 24 to 36 h under constant stirring condition at 30°C in 20 litre capacity fermentor (Murphye Scientific Co., Mysore, India). The bacterial cells were harvested by centrifugation at 13,500 ***g*** (Sorvall RC5B Plus; Thermo Fisher Scientific, MA, USA) and inactivated with formalin (0·5%). Briefly, formalin (formaldehyde 37%) was added at the rate of 0·5% (v/v) to the bacterial suspension and incubated at room temperature overnight with constant mixing on magnetic stirrer. Formalin was neutralized by adding equal amount (0·5%) of sodium bisulphite (v/v). Inactivation of the bacteria was confirmed by plating on the Tryptose Soya Agar and absence of any growth following incubation for 48 h was considered as complete inactivation. Density of the bacterial cell was assessed using spectrophotometer and concentration adjusted to 10^{10} CFU per millilitre. The suspension was stored at 4°C till further use.

Administration of experimental diet

The bacterin suspension was diluted with phosphate buffered saline (137 mol ml^{-1} sodium chloride, 2·7 mol ml^{-1} potassium chloride and 9·8 mol ml^{-1} phosphate buffer) to get the final concentration (equivalent to 2×10^{8} CFU per kilogram feed). The suspension was mixed with 0·1% guar gum (HiMedia, Mumbai, India) as binder and applied uniformly on the commercial feed (CP Aquaculture (India) Private Limited, Chennai, Tamil Nadu, India) with 35% crude protein, 5% fat, 5% crude fibre, 15% ash and 11% moisture. The control feed was prepared using only the binder solution. The bacterin-coated or binder-coated control feed were air dried under shade for 2 h before feeding.

Study design

Penaeus monodon culture ponds ($n = 62$) with water spread area between 0·7 and 1·0 ha, located in Navsari, Surat and Valsad districts of Gujarat, west coast of India were selected for the study. Prior consent from the farmers ($n = 3$) was obtained to use the immunostimulant in their culture ponds. Farm 1 had three control ponds and 10 treatment ponds with low stocking density (6–8 nos per m^2), Farm 2 had six control ponds and 27 treatment ponds with medium stocking density (9–11 nos per m^2) and Farm 3 had three control ponds and 13 treatment ponds with high stocking density (12–14 nos per m^2). All the study ponds were stocked with hatchery reared *P. monodon* postlarvae (PL 20). The experimental diet was administered throughout the culture period for two consecutive days in a week from stocking to harvest (140 days). For intensive monitoring of environmental quality, immune response parameters and anatomical deformities, 20 ponds were randomly identified among the 62 experimental ponds. Three control ponds from each farm representing the three stocking densities, three treatment ponds each from Farm 1 and Farm 2 representing the low and medium stocking density while four ponds were identified from Farm 3 representing the high stocking density. Pond sediment and water samples were collected every 15 days for physico-chemical analysis and live animal samples were collected at harvest for immunological studies and to record the anatomical deformities. Growth and survival data were collected from all the study ponds ($n = 62$) by periodical cast net sampling and feed consumption data were collected from farm record during entire study period.

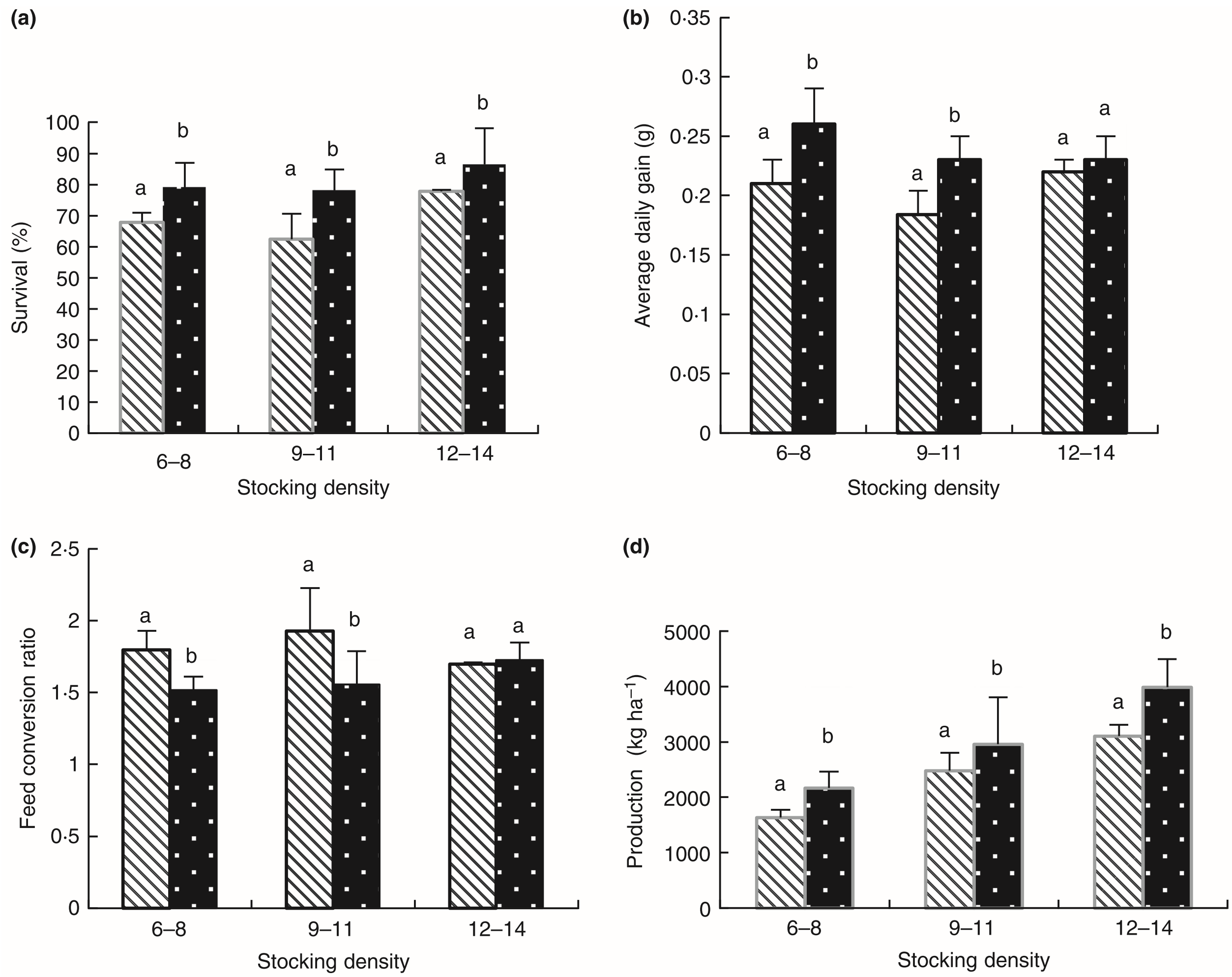

Figure 2 Production details showing mean value and standard deviation. Treatment (▪) and control (▧). (a) Survival; (b) Average Daily Gain; (c) Feed Conversion Ratio and (d) Production.

Monitoring for environmental quality, health and immunity production

All the intensively monitored ponds in study were periodically tested for water (pH, salinity, alkalinity, hardness, total ammonia nitrogen, nitrite) and pond sediment (organic carbon, pH, EC, available nitrogen, available phosphorus and available potassium) quality parameters following standard procedures (APHA 2012). To evaluate the effect of bacterin administration on the shrimp immune response, 20 animals from each intensely monitored pond were randomly selected at harvest and evaluated for THC and ProPO activity. Haemolymph was collected into a sterile vial containing ice-cold EDTA-free anticoagulant (27 mol ml^{-1} trisodium citrate, 385 mol ml^{-1} sodium chloride, 115 mol ml^{-1} glucose, pH 7·5) diluted to ratio 1 : 2 haemolymph to anticoagulant (v/v) and mixed gently to avoid coagulation. Collected haemolymph was divided into two parts; one half of the haemolymph was used for THC in Neubauer's Chamber and expressed as numbers per millilitre of haemolymph (Sritunyalucksana *et al.* 2005). The other half of the haemolymph sample was subjected to preparation of Haemocyte Lysate Supernatant (HLS) and stored at −80°C for proPO assay (Ji *et al.* 2009). The proPO activity of prepared HLS was measured by recording the change in absorbance due to oxidation of L-DOPA (L-3,4-dihydroxyphenylalanine) as substrate (1·5 mg ml^{-1} in 0·1 mol l^{-1} potassium phosphate buffer pH-6·6) at wave length 490 nm in a spectrophotometer (Shimazdu UV-1700; Kyoto, Japan). One unit of enzyme activity is measured by a linear increase in absorbance of 0·001 min^{-1} ml^{-1} haemolymph (Huang *et al.* 2010). At the time of harvest, one hundred of shrimp samples from each of the intensively monitored 20 ponds were individually checked for general health and anatomical abnormalities like, tail rot, rostrum cut, antenna cut and soft shell and percentage deformities were recorded.

Recording the production at harvest

Production parameters like average body weight (g), average body weight gain (g), survival (%), FCR and production (kg ha^{-1}) were recorded at the time of harvest in all the study ponds.

Data collection and statistical analysis

The data were expressed as mean ± SD. Significance between two treatments (with and without bacterin administration) at three stocking densities was evaluated using independent sample *t*-test at 0·05 level of significance. All statistical analyses were carried out in SPSS ver. 17.0.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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