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Bio-confronting Efficacy of the *Bacillus* Probiotic Strains of NOVIB™ in Controlling Vibriosis on Low Saline Semi-Intensive Pond Culture System of the White Leg Shrimp, *Litopenaeus vannamei* (Boone, 1931)

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Keywords

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A B S T R A C T

The study implies on the efficacy of the *Bacillus* probiotic, Novib in controlling vibriosis on low saline *Litopenaeus vannamei* shrimp aquaculture pond at Muarmalla, East Godavari District, Andhra Pradesh, India. Novib was used since beginning in the test ponds. Control ponds were infected with *Vibrio* upon 42 days of culture. Physicochemical parameters of the ponds were also monitored. The laboratory report and visual observation of the animal confirmed the infection of the pond. Green and yellow colonies in the control ponds were enumerated to be 7.167±3.2 to 117.462±3.2 cfu/ml and 18.363±3.2 to 291.132±3.2 upon 49 days of culture respectively. Animals were observed to be with antenna cut, red discoloration of the body and minor necrosis in the tail. Feed intake was drastically dropped with mortality of 5-10 animals/day. Novib with the dosage of 500g/acre was then administered onto the pond water on a sunny day with aerators operated continuously on the first day. Novib of 10g/kg feed was given in two meals for a duration of 5 to 8 days and henceforth the pond was found to recover and gradually reduced mortality to zero in 5 days. Overall, administration of Novib on shrimp ponds has considerably improved the recovery of the shrimps with *Vibrio* infection and ensured healthy culture.

Introduction

Global aquaculture industry incurs $3 billion of annual economic loss due to increasing infectious diseases. Out of which, vibriosis is one of the major devastating bacterial diseases in *L. vannamei* shrimp farming caused by at least 14 species of *Vibrio* (Brock and Lightner, 1990). The term vibriosis describes a spectrum of diseases and includes oral and enteric vibriosis, appendage and cuticular vibriosis, localized vibriosis of wounds, shell disease, systemic vibriosis and septic hepatopancreatitis (Brock and Lightner, 1990). 90% mortality could be caused within a day after infection in *Penaeus monodon*, *P. japonicus* and *L. vannamei* by highly virulent strains such as *V. harveyi*, *V. vulnificus*, *V.*

penaeicida, V. parahaemolyticus, V. alginolyticus and *V. nigripulchritudo* (Lavilla-Pitago *et al.,* 1998). Sizemore and Davis, (1985) hypothesize that, outbreaks may occur when environmental factors trigger the rapid multiplication of bacteria which is usually tolerated at low levels within shrimp blood, or by bacterial penetration of host barriers. The shrimp exoskeleton provides an effective physical barrier to pathogens, however, *Vibrio* spp. are chitinoclastic bacteria associated with shell disease and may enter through wounds in the exoskeleton or pores (Jiravanichpaisal and Miyazaki, 1994; Alday-Sanz *et al.,* 2002).

Numerous technology based techniques have been proposed towards efficient control of vibriosis. However, many proposals were futile due to lacking factors like less cost effectiveness on field, less accuracy and other genetic factors. Existing mitigation strategies includes, vaccination strategy, development of Specific Pathogen Free (SPF) shrimps and antibiotics. However, the lack of a vertebrate – like adaptive immune response mediated by B and T cells limits the use of *Vibrio*-specific vaccination strategies in shrimp (Hill, 2005), SPF status is a temporary condition which was not passed on genetically and was lost once the SPF brood stock were transferred to a commercial facility, and the use of prophylactic antibiotics in aquaculture is banned in many countries due to emergence of antibiotic-resistant microbes (Cabello, 2006; FAO-WHO, 2006) respectively.

Efficient mitigation strategy which is effective, eco-friendly, cost effective and multi-functional is the need of the hour for the rising war against vibriosis and to ward off infections. Probiotics are of great deal in this concern. Though they are not therapeutic agents, they directly or indirectly alter the composition of the microbial community in the rearing environment or in the gut of the host. It is likely that they function by competitive exclusion, that is, they antagonize the potential pathogen by the production of inhibitory compounds or by competition for nutrients and/or space (Verschuere *et al.,* 2000). It is also likely that probiotics stimulate a humoral and/or cellular response in the host. In this mode of farming, probiotics are usually introduced as part of the feeding regimen or applied directly to the water. NOVIB™ is a probiotic product comprising of two efficient *Bacillus* strains namely *Bacillus amyloliquefaciens* and *Bacillus cereus* which are potential anti-vibrio strains. The study emphasizes on the efficacy of Novib on controlling vibriosis in low saline semi-intensive shrimp culture system.

Materials and Methods

Study site

Six shrimp ponds of equal size (1 acre each) located in Muarmalla (16.674˚ N, 82.167˚ E), East Godavari district, Andhra Pradesh (Fig. 1) were selected for the study during the period of March to June 2017.

Product composition

Novib is a commercial probiotic product from the R&D facility of Tablets (India) Limited, Chennai, certified by Coastal Aquaculture Authority (CAA) of India (CAA Reg. No. CAA/F16/PRO/00015), Government of India. The composition of Novib per 500gms is presented in table 1.

Experimental design

L. vannamei shrimp pond was properly prepared prior to stocking. Bore water with salinity ranging from 13 – 17 psu was pumped onto the shrimp ponds. Post larvae (PL) at the stage of 11 were subjected to laboratory analysis for monitoring the health status of the PLs including White Spot Syndrome Virus

(WSSV), *Enterocytozoon hepatopenaei* (EHP), and *Vibrio* infection. With results being negative for all the aforementioned tests, PLs were stocked at a density of 40/m^2 with the water depth of 1.5m and pond area of 1 acre each. Water exchange was not entertained in the ponds. Physico-chemical parameters of the water were carried out once in a week and was analysed. 500gm/acre of Novib probiotic was brewed with 3 kg of country jaggery for 3 hrs and applied onto the test ponds once in a week since the beginning of the culture as preventive dose in test ponds. Also, Novib was added as the part of feeding regimen with 10gm/kg feed in 2 meals twice a week in test ponds. Control ponds were left untreated initially until symptoms of infection appears. Control pond condition was monitored and feed were skipped for two meals and further feeding was reduced to 60% of the feeding module maintained. Novib was then applied onto the control ponds after the observation of infection in control ponds. Efficacy of Novib in treating the infested ponds was then studied by applying Novib to the control ponds in water and feed. Changes were then observed and recorded.

Sampling analysis

Physico-chemical analysis

The physicochemical parameters of water were analysed in the ponds pre and post usage of Novib at four different points/spots. Water samples were collected between 07.00 and 08.00 hrs for *in situ* examination and laboratory analysis. Collected samples were examined for pH, temperature, and salinity on the spot. The salinity in the ponds was recorded *in situ* by means of a portable hand-held optical refractometer (Atago, Japan) and was cross-checked in the laboratory using Mohr-Knudsen method. pH was measured using electronic pH pen (Erma, Japan), temperature was measured using standard Celsius thermometer. The dissolved oxygen concentration was estimated by modified Winkler's method as described by Strickland and Parsons (1972). The total hardness of the water was estimated by complexometric titration using EDTA (Vogel, 1978) and alkalinity was measured as per APHA (1998). Transparency was measured based on the penetration of light using a Secchi disc.

Statistical analysis

The data were presented as mean ± SE. All statistical calculations were performed using SPSS for Windows version 11.5 (SPSS Inc, Chicago, IL, USA). All column charts were plotted using Origin 6.1(OriginLab Corporation, Massachussets, USA).

Results and Discussion

Water quality parameters

The physico-chemical parameters of the water play a critical role in maintaining the quality of the pond water and health status of the shrimp and were carefully measured on regular intervals. Shrimp farmers usually manage the water quality parameters by the application of various components like lime and sodium carbonate. The records of the parameters including pH, temperature, salinity, dissolved oxygen (DO), total hardness and total alkalinity of the pond during the study period were discussed below.

pH in the control pond ranged from a minimum of 7.813±0.3 to 8.326±0.3 and was observed to be ranging from 7.926±0.3 to 8.413±0.3 in the test ponds (Fig. 2) with no significant. Temperature was properly monitored and recorded. Temperature in the control and test ponds ranged from 24.912±0.4 to 34.301±0.4 ˚C (Fig. 3). Salinity was recorded once in a week and it was observed to be ranging from 14.167±0.3 to 21.413±0.3

psu (Fig. 4). The dissolved oxygen concentration was properly monitored and recorded exhibiting the values ranging from 2.881±0.2 to 3.987±0.2 ppm in the control ponds. The DO value was observed to be 3.426±0.2 to 4.931±0.2 ppm in the test ponds (Fig. 5). The total hardness ranged from 1112.261±3.2 to 1432.376±3.2 mg/L in the control ponds and 998.162±3.2 to 1187±3.2 mg/L in the test ponds (Fig. 6). Alkalinity was checked and documented wherein the alkalinity in the control ponds ranged from 129.367±1.5 to 161.289±1.5 ppm and the values in the test ponds ranged from 131.567±1.5 to 152.248±1.5 ppm (Fig. 7).

Bio-confronting efficacy of *Bacillus* probiotic, Novib against *Vibrio* species

Efficacy of *Bacillus* probiotic strains of Novib in controlling *Vibrio* infection was tested. Test ponds administered with Novib at regular intervals on water and feed since the initial stage of the culture remained free from *Vibrio* infection across the culture days. However, control ponds which were left untreated developed *Vibrio* population in the water and animal (Fig. 8). Pond water when plated onto TCBS agar plate exhibited growth of green and yellow colonies of *Vibrio.* Shrimps when visually examined showed signs of infection including antenna cut, green fluorescence on tail and red discoloration of the body.

Based on microbiological examination of the pond water samples until 49 days of culture, green colonies in the control ponds ranged from 7.167±3.2 to 117.462±3.2 cfu/ml (Fig. 9) and no *Vibrio* population was recorded in the test ponds treated with Novib. Yellow colonies in the test and control ponds were monitored and documented. The number of yellow colonies on TCBS agar plate ranged from 18.363±3.2 to 291.132±3.2 in the control ponds (Fig. 10) and no *Vibrio* population was observed in the test ponds treated with Novib.

Control ponds upon infestation with the *Vibrio*, when observed to be with increasing population of bacterial load was then treated with Novib in water and feed for 5 to 7 days. No feeding was given to the shrimps for two meals followed by only 60% of feeding for upto 7 days. Meanwhile, 500gm/acre of Novib was brewed with 3kg of country jaggery and was applied over the pond water in a sunny day with constant operation of the aerator. In addition, 10gm/kg of feed was added as part of the feeding regimen in two meals for 5 to 7 days. *Vibrio* count was examined on daily basis post application of Novib. The green and yellow colonies were observed to decrease daily with zero colonies on the fifth day of application (Fig. 11). Still, application was continued for 3 more days in order to prevent further infection relapses. Quality of the water was also observed to be good with better bloom development and transparency record.

Table.1 Composition of Novib per 500 gms

Ingredients	Quantity	
***Bacillus subtilis* ABPL 154**	3.5×10^{9}	gms
***Bacillus cereus* ABPL 155**	12.5×10^{9}	gms

Fig.1 Map showing the study area Muarmalla, East Godavari district, Andhra Pradesh

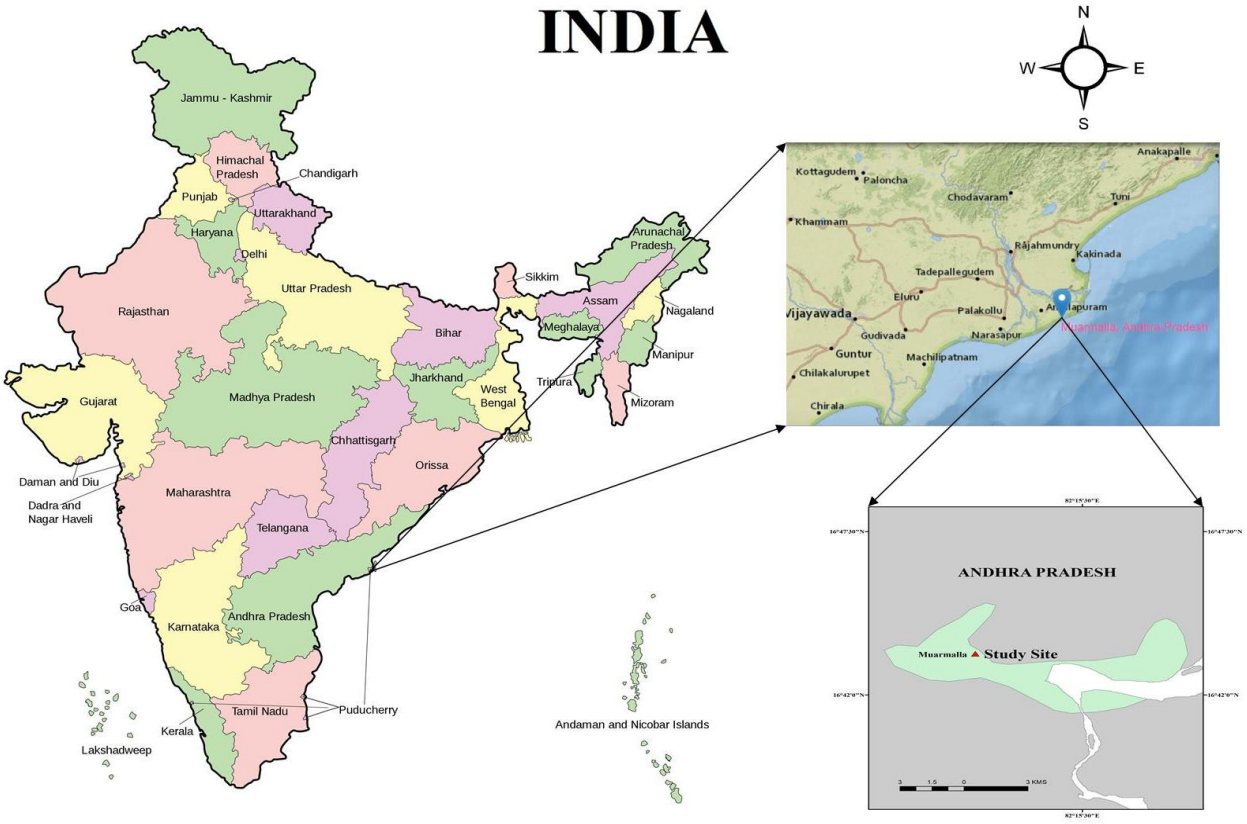
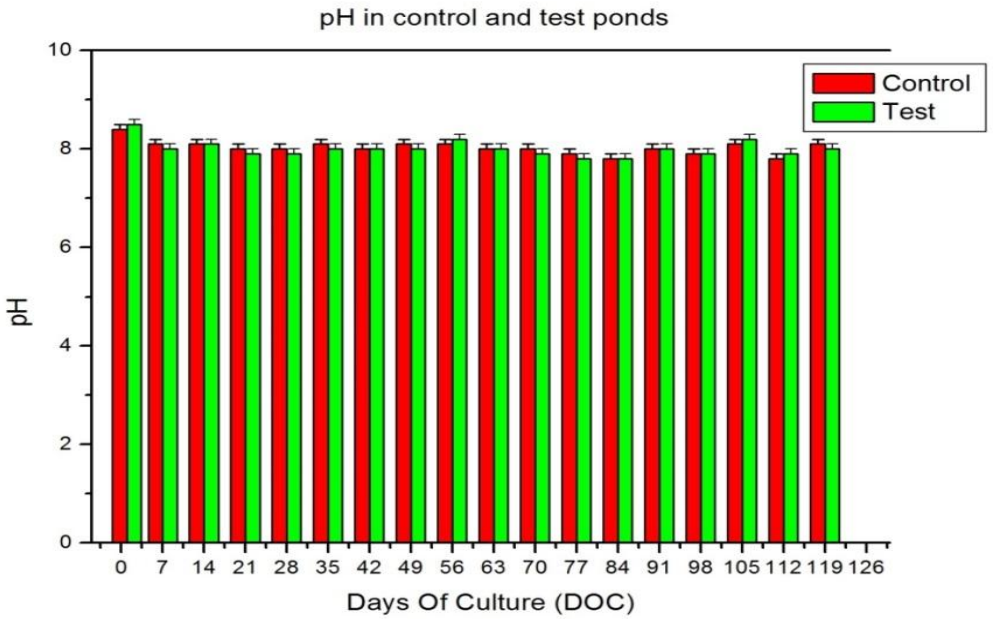
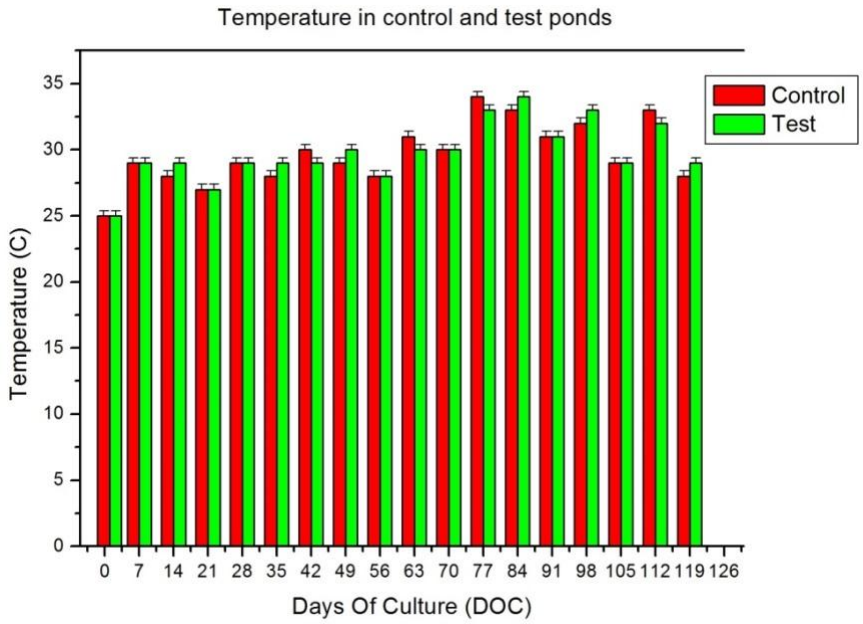


Fig.2 Figure showing the record of pH on the *L. vannamei* shrimp ponds during the study period



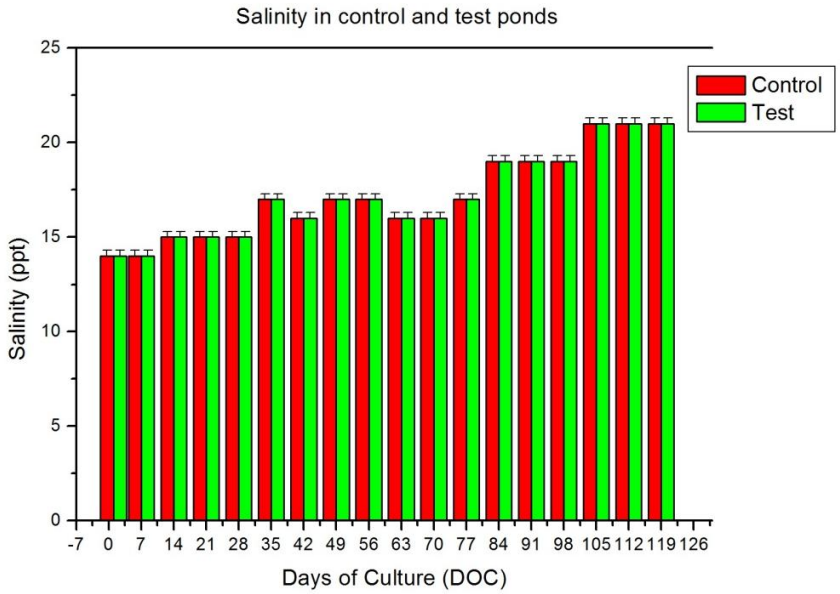
*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.3 The record of temperature on the *L. vannamei* shrimp ponds during the study period



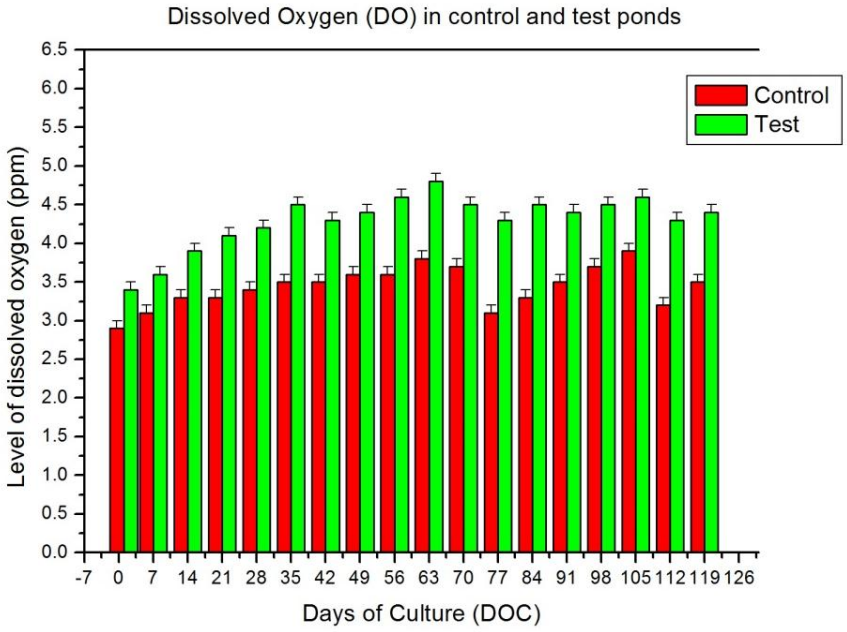
*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.4 Figure showing the record of salinity on the *L. vannamei* shrimp ponds during the study period



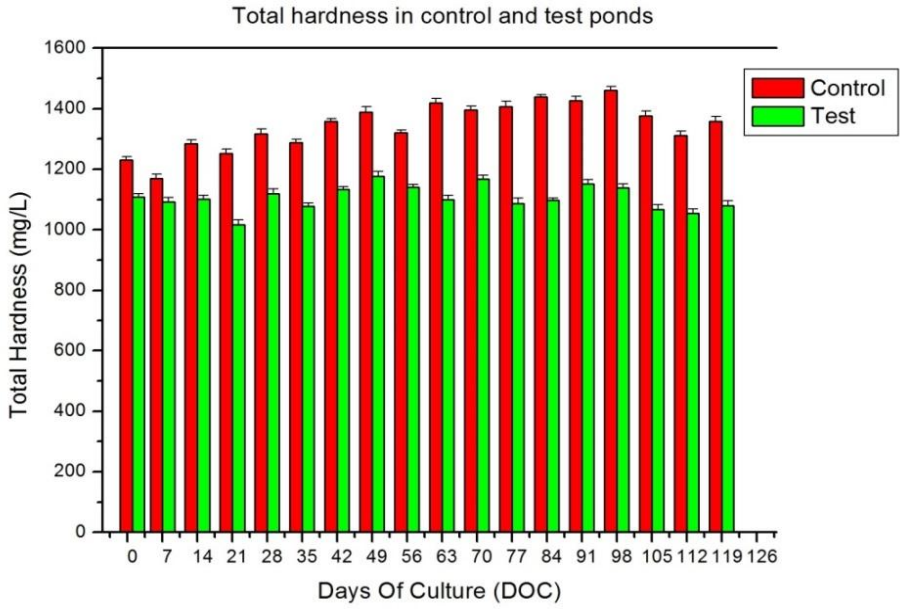
*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.5 Figure showing the record of Dissolved Oxygen (DO) on the *L. vannamei* shrimp ponds during the study period



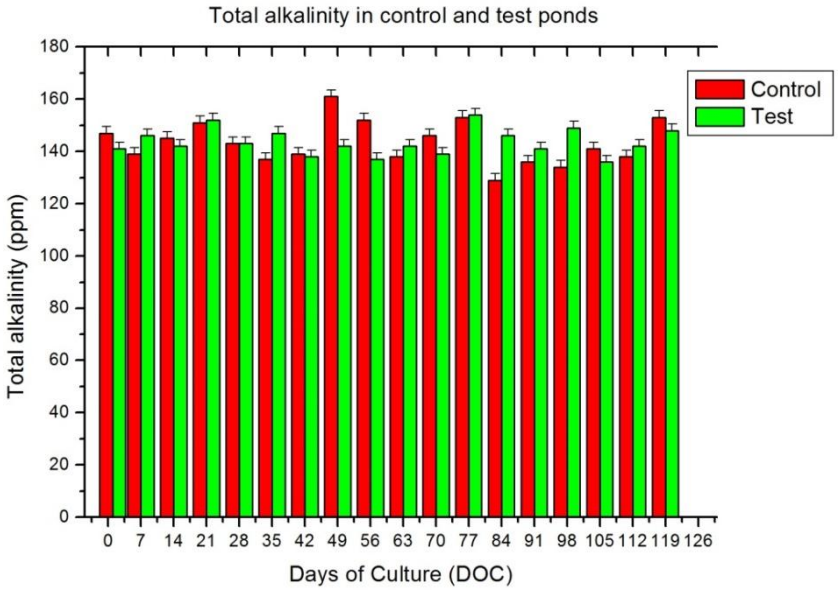
*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.6 Figure showing the record of total hardness on the *L. vannamei* shrimp ponds during the study period



*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.7 Figure showing the record of total alkalinity on the *L. vannamei* shrimp ponds during the study period



*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.8 Figure showing the *Litopenaeus vannamei* shrimps infected with Vibrio species (A) in control pond and uninfected healthy shrimps (B) from test ponds treated with Novib

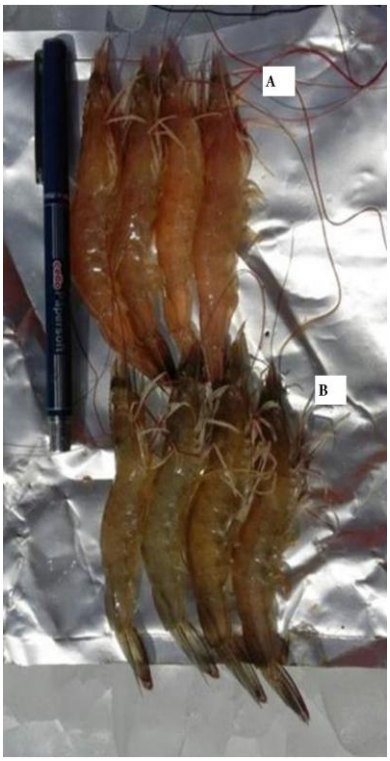
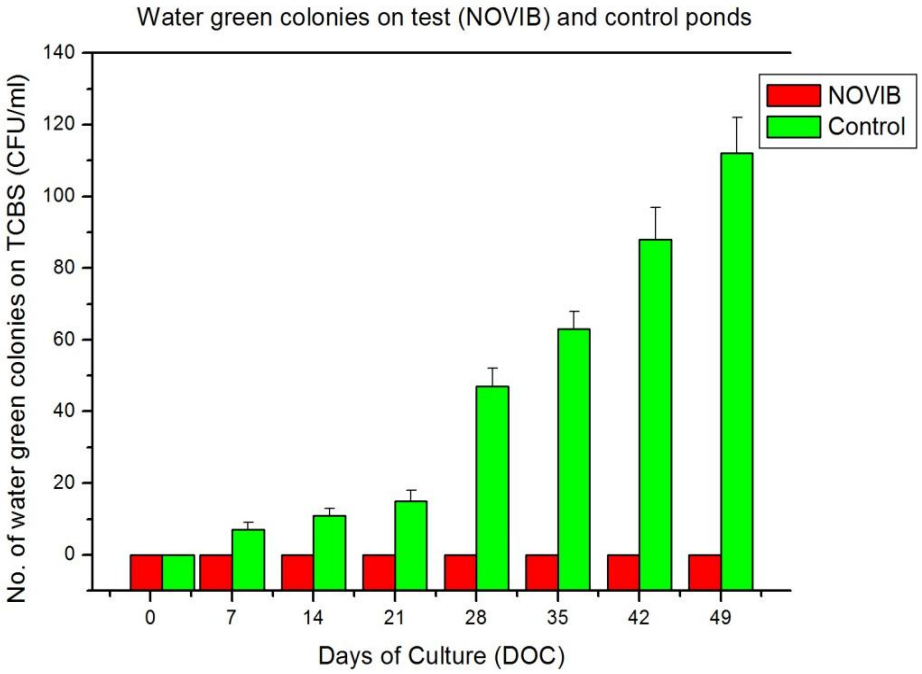
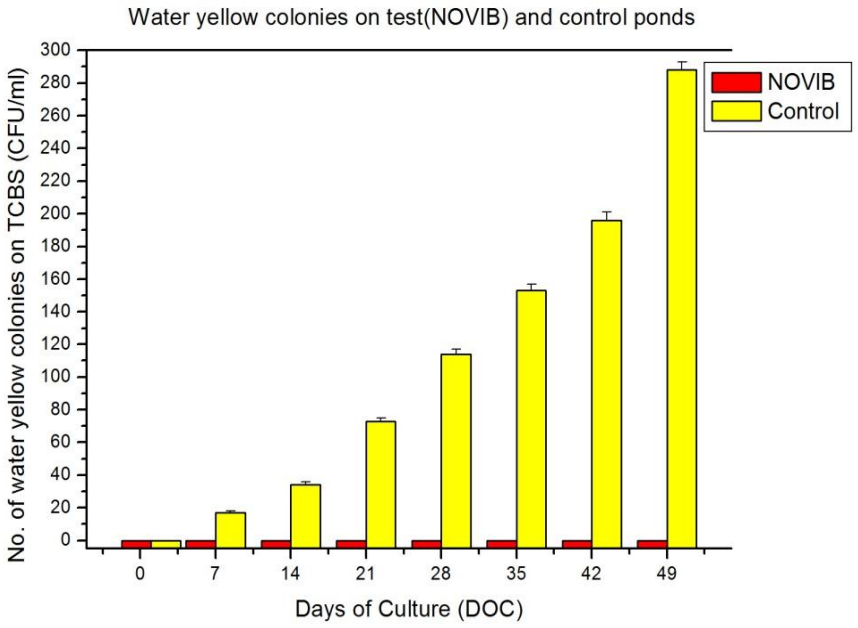


Fig.9 Figure showing the efficacy of Novib in controlling the green colonies of Vibrio (test) on *L. vannamei* shrimp culture ponds



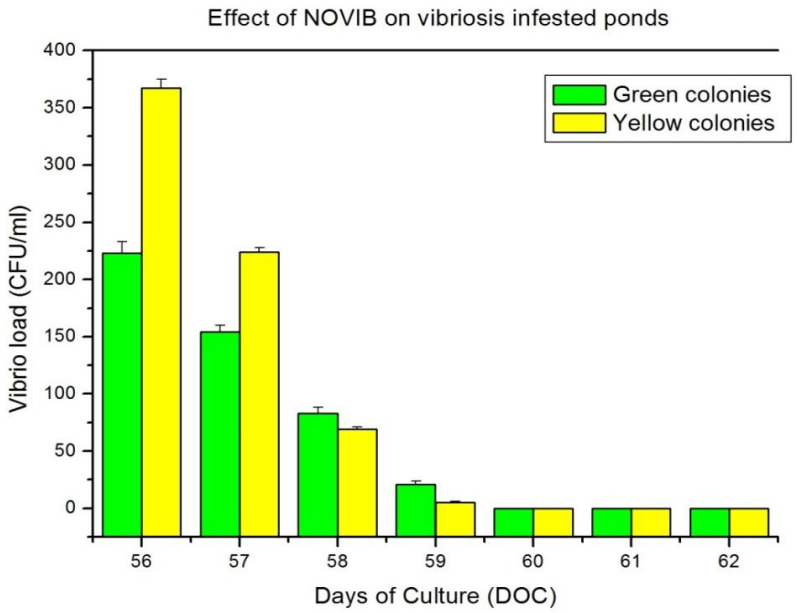
*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.10 Figure showing the efficacy of Novib in controlling the yellow colonies of Vibrio (test) on *L. vannamei* shrimp culture ponds



*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Fig.11 The efficacy of Novib in controlling the green and yellow colonies of Vibrio infested *L. vannamei* shrimp culture ponds



*Results are presented as means with standard errors of three shrimp ponds for each group (mean ± SE; n = 3)

Serious economic losses in various countries have been reported and disease outbreaks remain as one of the major reason for the same. Global losses due to shrimp diseases are noted. *Vibrio* species occur as the dominant flora in various developmental stages of *Penaeus monodon* and have been described as the causal pathogens (Lightner 1996; Sung *et al.,* 2001). Increased concern about antibiotic-resistant micro-organisms (Amabile *et al.,* 1995) has led to emergence of alternative disease prevention methods, including the use of non-pathogenic bacteria as probiotic bio-control agents (Austin *et al.,* 1995; Moriarty 1997). *Bacillus* spores have been used as biocontrol agents to reduce vibrios in shrimp culture facilities (Skjermo and Vadstein 1999; Rengipipat *et al.,* 2000). *Bacillus* constitutes a large part of the microflora of the gills, skin and intestinal tracts of shrimps (Sharmila *et al.,* 1996). *Bacillus* species are often antagonistic against other micro-organisms, including fish and shellfish pathogenic bacteria (Gatesoupe 1999; Rengipipat *et al.,* 2000).

The control of fish and shellfish pathogenic *Vibrio*, particularly using non-pathogenic bacterial strains and disease prevention, has received much attention during the last decade (Sugita *et al.,* 1998; Rengipipat *et al.,* 2000). Yasuds and Taga (1980) suggested that probiotic bacteria would be of use not only as food but also as biological controllers of fish disease and activators of nutrient regeneration. Beneficial bacteria are helpful in nutrient recycling and organic matter degradation and thus clear the environment (Moriarty, 1997).

The present study showed that the *Bacillus amyloliquefaciens* and *Bacillus cereus* probiotic strains of Novib are capable of inhibiting the growth of pathogenic *Vibrio* species developed in *L. vannamei* shrimp aquaculture ponds. Ponds treated with Novib since the initial days of the culture remained healthy with notable growth and survival in the test ponds. But in the case of control ponds where the pond was left untreated showed signs of *Vibrio* infection, which was

then proved by the laboratory report. After diagnosis, Novib was used in the infected control ponds and *Vibrio* infected shrimp pond recovered from the infection with the application of Novib over feed and water and has retained the survival. Rengpipat (1998) challenged shrimps with a shrimp pathogen, *Vibrio harveyi* by immersion for 10 days; all probiotic treated groups had 100% survival, whereas the control group had only 26% survival which suggested competitive exclusion by probiotic *Bacillus* S11. The inhibitory effects of *Bacillus* sp. may be due to the production of antibiotics, bacteriocins, lysozymes, proteases, and hydrogen peroxide and the alteration of pH values by the production of organic acids (Verschuere, 2000).

Rengpipat *et al.,* (1998) stated that tiger shrimp inoculated with *Bacillus* S11, which had previously demonstrated its inhibitory effect *in vitro* against *V. parahaemolyticus* and *V. harveyi*, resulted in greater survival of *P. monodon* challenged with pathogenic luminescent bacteria. Vaseeharan and Ramasamy (2003) found that growth of pathogenic *V. harveyi* in tiger shrimp was controlled by the probiotic effect of *B. subtilis* BT23 *in vitro* and *in vivo*. Disease resistance was improved and accumulated mortality was reduced by 90% when juvenile *Penaeus monodon* were exposed to *B. subtilis* BT23 isolated from shrimp culture ponds before a challenge with *V. harveyi*.

Proper feeding strategy exists as a key factor in infection control and pond parameter maintenance. Recovery of the shrimps in ponds diagnosed with *V*ibrio infection was found to be swifter when feed control was coupled with the administration of Novib in water and feed.

It has been reported that probiotic supplemented feed improve food conversion efficiency by preventing intestinal disorders and predigestion of anti-nutritional factors present in the feed ingredients (Venkat *et al.,* 2004).

Overall, probiotics are the key preventive measure component which has to be administered on to the shrimp since early days of the culture to immunise the shrimps and the pond. Also, it is recommended that, preventive doses have to be given in order to culture the shrimps with minimal infection favouring the growth and ensuring a successful culture.

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