Lat. Am. J. Aquat. Res., 41(1): 126-137, 2013
DOI: 103856/vol41-issue1-fulltext-10

Research Article

Effect of a commercial probiotic on bacterial and phytoplankton concentration in intensive shrimp farming (*Litopenaeus vannamei*) recirculation systems

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ABSTRACT. The aim of this study was to evaluate the effect of a commercial probiotic on the bacterial and phytoplankton concentration in intensive shrimp farming (*Litopenaeus vannamei*) with a recirculation system, for one culture period in Rio Grande do Norte, Brazil. Ponds, with mean area of 2.6 ha, were stocked with a density of 98 shrimp m^{-2}. A commercial probiotic was prepared following the manufacture's specifications and sprayed on the surface of the ponds seven days prior to stocking and then on a weekly basis until harvest. The same procedures were used with all treatments (control and probiotic), with regard to feeding, liming, fertilization, use of molasses and monitoring of water quality. Field data were analyzed using ANOVA, the Tukey test and Chi-square tests. No significant differences between treatments were found for water quality data, but treatment means showed significant differences for total heterotrophic bacteria in the sediment ($5.181 \pm 0.34x10^4$ cfu g^{-1} and $5.749 \pm 0.67x10^4$ cfu g^{-1}), total heterotrophic bacteria in surface water ($4.514 \pm 0.95x10^4$ cfu m L^{-1} and $4.136 \pm 0.81x10^4$ cfu m L^{-1}) and positive sucrose in surface water ($2.438 \pm 0.72x10^4$ cfu m L^{-1} and $2.203 \pm 0.76x10^4$ cfu m L^{-1}), respectively, for the control and probiotic treatment. Significant differences were also observed throughout the weeks for total heterotrophic bacteria in the sediment, positive and negative sucrose in the sediment, total heterotrophic bacteria in surface and bottom water, and Pyrrophyta percentage values between 10 and 16 weeks. These results showed that the probiotic causes changes in the total heterotrophic bacteria in the sediment and percentage values of Pyrrophyta concentration, improving the environmental quality of the sediment and water in ponds with closed recirculation systems.

Keywords: *Litopenaeus vannamei*, heterotrophic bacteria, *Vibrio*, microalgae, aquaculture, Brazil.

Efecto de un probiótico comercial sobre la concentración de bacterias y fitoplancton en cultivo de camarón (*Litopenaeus vannamei*) con sistemas de recirculación

RESUMEN. El objetivo de esta investigación fue evaluar el efecto de un probiótico comercial sobre la concentración de bacterias y fitoplancton en el cultivo intensivo de camarón (*Litopenaeus vannamei*) con sistemas de recirculación, para un ciclo de cultivo en Rio Grande do Norte, Brasil. La siembra de camarones se hizo con una densidad de 98 camarones m^{-2} en viveros de 2,6 ha. Se preparó un probiótico comercial siguiendo las especificaciones del fabricante y rociado sobre la superficie de los estanques siete días antes de la siembra, y después se aplicó semanalmente hasta la cosecha. Los mismos procedimientos con respecto a la alimentación, encalado, fertilización, uso de la melaza y monitoreo de la calidad del agua se utilizaron en todos los tratamientos (control y probiótico). Los datos fueron analizados utilizando el análisis de varianza, prueba de Tukey y Chi-cuadrado. Para los datos de calidad de agua no hubo diferencias significativas entre los tratamientos, sin embargo, las medias de los tratamientos mostraron diferencias significativas para bacterias heterotróficas totales en el sedimento ($5.181 \pm 0.34x10^4$ cfu g^{-1} y $5.749 \pm 0.67x10^4$ cfu g^{-1}), bacterias

heterotróficas totales en la superficie de agua ($4.514 \pm 0.95x10^4$ cfu m L^{-1} y $4.136 \pm 0.81x10^4$ cfu m L^{-1}) y sacarosa positiva en la superficie de agua ($2.438 \pm 0.72x10^4$ cfu m L^{-1} y $2.203 \pm 0.76x10^4$ cfu m L^{-1}), respectivamente para los tratamientos de control y probiótico. También se observaron diferencias significativas a lo largo de las semanas para bacterias heterotróficas totales en el sedimento, sacarosa positiva y negativa en el sedimento, bacterias heterotróficas totales en la superficie y fondo del agua, sacarosa positiva y negativa en el fondo del agua, y Pyrrophyta entre 10 y16 semanas. Los resultados mostraron que el probiótico causa modificaciones sobre bacterias heterotróficas totales en el sedimento y de porcentaje de la concentración de Pyrrophyta, mejorando la calidad ambiental del sedimento y del agua en estanques con sistemas de recirculación cerrada.

Palabras clave: *Litopenaeus vannamei*, bacterias heterotróficas, *Vibrio*, microalgas, acuicultura, Brasil.

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INTRODUCTION

Brazil has an extensive shoreline as well as climatic, hydrobiological and topographical conditions favorable to shrimp farming, which began on a commercial scale in the late 1980s. Despite these good conditions and stable annual temperatures, especially in the northeastern region of the country, the increase in shrimp production has occurred in a diversified way due to strict environmental regulations.

Aquaculture has been gaining importance in overall revenue from stock breeding in Brazil, reflected by the increase of 43.8% between 2000 and 2009, compared to breeding of other animals in the same period, such as pigs (12.9%), chickens (9.2%) and cattle (-8.2%). In 2010, production from aquaculture in Brazil was 479,398.6 ton, while the production from marine shrimp was 69,422.4 ton (MPA, 2012).

Aquaculture, especially shrimp farming, has been the target of constant pressure from non governmental organizations and environmental agencies. However, adequate management strategies have contributed towards making the activity sustainable (Brito *et al*., 2010).

Ponds are ecosystems in which microorganisms and shrimp are engaged in a variety of ecological interactions, from competition and predation to pathogenesis and commensalism (Moriarty, 1997). Microorganisms dominate cultivation sites due to the richness of these environments in terms of food sources, which favors their growth and reproduction. Microbiology is a new science in aquaculture and the understanding of microbial processes in this field is indispensable to the future progress of the industry.

Decamp & Moriarty (2006), report that the main bacterial genera tested as probiotics in aquaculture are *Vibrio, Pseudomonas, Bacillus* and different lactobacilli. However, other organisms, such as fungi and yeasts, have also been used for probiotic purposes (Devajara *et al*., 2002; Ribeiro *et al*., 2008). Research on probiotics for aquatic animals is increasing with the demand for sustainable, environmentally friendly aquaculture (Wang *et al*., 2005; Farzanfar, 2006; Ravi *et al*., 2007; Decamp *et al*., 2008; Li *et al*., 2008; Gomez *et al*., 2009; Guo *et al*., 2009; Liu *et al*., 2009; Ninawe & Selvin, 2009; Peraza-Gómes *et al*., 2009; Pai *et al*., 2010; Shen *et al*., 2010; Silva *et al*., 2011, 2012; Souza *et al*., 2011; Zhang *et al*., 2011).

Although the beneficial effect of the application of certain bacteria on human, pig, cattle and poultry nutrition has long been recognized, the use of such probiotics in aquaculture is a relatively new concept (Farzanfar, 2006), and the effectiveness of these products in commercial shrimp farming is not yet clearly established. The aim of this study was to evaluate the effect of a commercial probiotic on bacterial and phytoplankton concentration in intensive shrimp farming (*Litopenaeus vannamei*), with a recirculation system, for one culture period in Rio Grande do Norte, Brazil.

MATERIALS AND METHODS

Experimental design and farm site

The experiment lasted 141 days in eight grow-out ponds of equal size (2.6 ha each), selected at a commercial shrimp farm (Aquarium Aquaculture Brazil Ltda.), located on the left bank of the Apodi River in the municipality of Mossoró, state of Rio Grande do Norte, northeastern Brazil (5º11”S, 37º20”W), to assess the effect of a commercial probiotic compared with the traditional management practice (control). Each treatment had four replicates.

Pond management

Twenty days prior to stocking, all eight ponds (with beds consisting of clayey and saline soil) were emptied. The intake and drainage gates were sealed and the ponds received two consecutive bacteria applications from screens of 500 and 1000 µm. Wet areas were treated with chlorine (100 ppt). The

treatment of the soil was performed by mechanical tilling and application of dolomitic limestone (1,500 kg ha^{-1}). After five days, the ponds were filled to a water level of 1.0 meter. The water was fertilized with urea and triple superphosphate (3.0 mg L^{-1} of nitrogen and 0.3 mg L^{-1} of phosphorus). Three subsequent fertilizations (1.0 and 0.1 mg L^{-1} of nitrogen and phosphorus, respectively) were performed every three days prior to stocking. This procedure is the traditional management for successive culture soon after harvesting.

During the experiment, top fertilizations with urea (total 230 kg $pond^{-1}$) and triple super phosphate (total 23.5 kg $pond^{-1}$) were performed, and the alkalinity of the water was corrected weekly with the application of dolomitic limestone (total of 8,062 kg $pond^{-1}$). Molasses (total of 4,160 kg $pond^{-1}$) was also added weekly, to adjust C: N ratio, from the sixth week to the end of the experiment. All ponds had artificial aeration through the use of paddle wheels (10 HP ha^{-1}).

Probiotic application

A probiotic composed of *Bacillus* spp. and *Lactobacillus* yeasts was administered to the ponds seven days prior to stocking (probiotic treatment), using the following criteria: dilution in water at a proportion of 75.0 g L^{-1}; placement of solution in two-liter plastic bottle; agitation and rest for four hours in the shade; further agitation and also sprinkling on ponds. Assuming a total aerobic count of $2.2x10^8$ colony-forming units (cfu g^{-1}) (specified as the minimal count by the manufacturer), the initial quantity administered to the ponds was 4.5 kg. Supplementary applications (162.5 kg $week^{-1}$ $pond^{-1}$) were performed over 16 consecutive weeks.

Shrimp rearing

Twelve-day-old Pacific white shrimp (*Litopenaeus vannamei*) (about 22,650,000) were acquired from a commercial hatchery and cultured in raceways (0.25 ha) with liners and depth of 1.5 m for 30 days. Commercial feed with 40% crude protein, 10% crude lipid, 7.5% moisture (max.), 5% fiber (max.), 3% calcium (max.), 1.45% phosphorus (min.), 4,000 (U.I.) vitamin A, 2,000 (U.I.) vitamin D3, 150 (U.I.) vitamin E and 130 mg vitamin C (pellets from 0.4 to 1 mm in diameter) was applied three times a day (08.00, 12.00 and 16.00 h). Subsequently, about 20,320,000 juveniles (2.09 ± 0.3 g) were stocked in experimental units (grow-out ponds of 2.6 ha without liners) at a density of 98 shrimp m^{-2}, fed a commercial feed with 35% crude protein, 7.5% crude lipid, 10% moisture (max.), 5% fiber (max.), 3% calcium (max.), 1.45% phosphorus (min.), 4,000 (U.I.) vitamin A, 2,000 (U.I.) vitamin D3, 150 (U.I.) vitamin E and 130 mg vitamin C (pellets between 2 and 2.5 mm in diameter), offered at the same three times of day using 100 trays per hectare.

Microbiological samples

Total heterotrophic bacteria (THB) and presumptive analyses of *Vibrio* spp. were performed every two weeks, by sampling the sediment, surface and bottom water, and shrimp. All samples were collected between 05.00 and 07.00 h. The samples were placed in isothermal chests and immediately transported to the Environmental Microbiology and Fishery Laboratory of the Institute of Ocean Sciences, Federal University of Ceará (Brazil). The time elapsed between collections and processing was approximately four hours. The water samples were collected in sterile glass bottles (500 mL) at a depth of 40 cm (surface water) and 0.4 cm above the sediment (bottom water) from two different locations (input and drainage) in each pond. Sediment samples (0.1 m depth in soil) were collected in the same sterile glass bottles and at the same two locations (input and drainage) in each pond. Shrimp specimens were sampled randomly using a nylon cast net with an area of 8.0 m^2. The shrimp (35 from each pond) were placed in plastic bags containing water from the pond, artificially saturated with oxygen to keep them alive until reaching the laboratory.

Bacteriological analysis

The preparation of the sample dilutions and bacteriological assays of the surface and bottom water, sediment and shrimp were performed with the two water samples separately and averaged followed the methods described by Downes & Ito (2001) and APHA (2005). Water bacteriological analysis with appropriate sample dilutions was carried out (10^{-1} to 10^{-5}) with sterilized saline solution (2.5% NaCl). Aliquots of 0.1 mL of the serial dilutions were inoculated. Each sediment sample was mixed with a magnetic agitator for 30 min and allowed to rest for 2 h. Around 1 g of uniform sediment sample was suspended in 25 mL of sterilized saline solution (2.5% NaCl). One milliliter of the homogenate was serially diluted (10^{-1} to 10^{-5}) and inoculated. Around 1 g of macerated shrimp hepatopancrea and muscle was suspended in 25 mL of sterilized saline solution (2.5% NaCl). One milliliter of the homogenate was serially diluted (10^{-1} to 10^{-5}) and inoculated. Standard count agar (TSA, Oxoid, UK) for THB and thiosulphate–citrate–bile sucrose (TCBS) agar (Oxoid) as well as sensitivity to the vibriostatic agent (0/129) (Oxoid)

were used to identify the types of bacteria. Each analysis was performed in duplicate by the spread plate method.

To count the total heterotrophic bacteria (THB) of sediment, water and shrimp, all the inoculated plates were incubated at 35°C for 48 h and colony forming units (cfu) were counted with a Quebec Darkfield Colony Counter (Leica Inc., Buffalo, New York) equipped with a guide plate ruled in square centimeters. Readings obtained with 25 and 250 colonies on a plate were used to calculate bacteria population numbers, recorded as cfu per sample unit. For vibrio counts of pond sediment, water and shrimp, all the inoculated plates were incubated at 35°C for 18 h and colony forming units (cfu) were counted. Again, readings obtained with 25 and 250 colonies on a plate were used to calculate bacterial population numbers, recorded as cfu per sample unit.

Phytoplankton and Cyanobacteria

Once a week vertical sampling was performed using plastic bottles with a volume of 600 mL for phytoplankton and Cyanobacteria collection. The water was filtered through a cylindrical-conical net (mesh: 15 μm) to 15 mL, providing a 40-fold more concentrated sample. The phytoplankton and Cyanobacteria was fixed with formalin (4%), buffered with borax (1%) and stored in 10-mL plastic recipients. A Sedgewick-Rafter chamber and optical stereomicroscope with magnification of 800x were used for qualitative and quantitative analyses through the identification and quantification of microalgae and Cyanobacteria samples, respectively. The phytoplankton and Cyanobacteria concentration was expressed as individuals per milliliter (cells mL^{-1}), estimated based on the sample preparation methods described by Pereira-Neto *et al.* (2008), according to the following formula:

$$C = [(nm / nq) \times 1000] / F$$

where C is phytoplankton or Cyanobacteria concentration; *nm* is the number of organisms found in the chamber; *nq* is the number of quadrants analyzed in the chamber; and *F* is the dilution (60) correction factor.

The main groups considered in the identification were: Bacillariophyta, Chlorophyta, Pyrrophyta and Cyanobacteria (Hoek *et al*., 1995; Stanford, 1999).

Water quality

Temperature and dissolved oxygen were measured daily (05.00 and 15.00 h) with a digital oximeter (YSI model 550, Yellow Springs, Ohio, USA). Salinity (YSI model 30, Yellow Springs, Ohio, USA) and pH (YSI model 100, Yellow Springs, Ohio, USA) were measured twice a week. All samples were collected from the ponds' drainage gates.

Statistical analysis

The Shapiro-Wilk and Bartlett tests ($\alpha = 0.05$) were performed to verify the normality of the data and the homogeneity of the variances, respectively. Field data were analyzed using two-way ANOVA (treatments x week) for THB, *Vibrio* spp., phytoplankton and Cyanobacteria concentration. When a significant difference was detected, the Tukey test ($P < 0.05$) was used. The Chi-square test ($P < 0.05$) used for phytoplankton and Cyanobacteria percentage. The data were analyzed using the Assistat Version 7.6 Program (Assistat Analytical Software, Campina Grande, Paraiba, Brazil).

RESULTS

Temperature and dissolved oxygen varied respectively from 26.7 to 28.7ºC and 2.82 to 9.14 mg L^{-1}. Salinity and pH varied respectively from 21.1 to 22.2 ppt and 7.6 and 7.0. There were no significant differences between treatments in any of these variables during the experiment ($P > 0.05$).

The total heterotrophic bacteria (THB) and *Vibrio* spp. concentration are summarized in Table 1. The mean sediment THB was significantly higher ($P < 0.05$) in the probiotic treatment than the control during the experiment (Fig. 1a). The mean concentrations of sucrose-positive and sucrose-negative *Vibrio* spp. in the sediment were not significantly different between treatments ($P > 0.05$), but showed significant differences between weeks ($P < 0.05$) (Figs. 1b and 1c). The THB concentrations in the sediment during the cultivation period varied between $4.56x10^4$ to $5.6x10^4$ cfu g^{-1} for the control and $4.74x10^4$ to $6.9x10^4$ cfu g^{-1} for the probiotic treatment. The concentration of sucrose-positive *Vibrio* spp. ranged from $2.35x10^3$ to $3.9x10^3$ cfu g^{-1} for the control and from $2.43x10^3$ to $4.06x10^3$ cfu g^{-1} for the probiotic treatment. Finally, the concentration of sucrose-negative *Vibrio* spp. ranged from $1.7x10^3$ to $2.92x10^3$ cfu g^{-1} for the control and from $1.92x10^3$ to $4.64x10^3$ cfu g^{-1} for the probiotic treatment.

The mean THB concentration in the surface water was significantly higher ($P < 0.05$) in the control than in the probiotic treatment (Fig. 2a). However, the mean concentrations of sucrose-positive and sucrose-negative *Vibrio* spp. were not significantly different between the treatments ($P > 0.05$). The THB concentrations in the surface water during the cultivation

Table 1. Total heterotrophic bacterial concentration and *Vibrio* spp. in intensive shrimp farming (*Litopenaeus vannamei*) with recirculation system.

Variables	Treatments	
	Control	Probiotic
Sediment THB (x10^4 cfu g^{-1})	5.181 ± 0.34*	5.749 ± 0.67
Sediment sucrose positive (x10^3 cfu g^{-1})	3.010 ± 0.47	2.909 ± 0.55
Sediment sucrose negative (x10^3 cfu g^{-1})	2.178 ± 0.47	2.421 ± 1.00
Surface water THB (x10^4 cfu mL^{-1})	4.514 ± 0.95*	4.136 ± 0.81
Surface water sucrose positive (x10^3 cfu mL^{-1})	2.438 ± 0.72*	2.203 ± 0.76
Surface water sucrose negative (x10^3 cfu mL^{-1})	2.204 ± 0.61	2.182 ± 0.85
Bottom water THB (x10^4 cfu mL^{-1})	4.596 ± 0.83	4.343 ± 0.61
Bottom water sucrose positive (x10^3 cfu mL^{-1})	2.591 ± 1.00	2.451 ± 0.93
Bottom water sucrose negative (x10^3 cfu mL^{-1})	2.183 ± 0.83	2.188 ± 0.53
Shrimp THB (x10^4 cfu mL^{-1})	2.320 ± 2.26	2.610 ± 2.65
Shrimp sucrose positive (x10^3 cfu mL^{-1})	3.888 ± 1.24	3.965 ± 1.42
Shrimp sucrose negative (x10^3 cfu mL^{-1})	2.862 ± 0.84	2.978 ± 0.86

CFU: colony-forming units; THB: total heterotrophic bacterial; Control: without the use of a probiotic; Probiotic: the probiotic, composed of *Bacillus* spp. and *Lactobacillus* yeasts, was administered to the ponds (2,604.5 kg $pond^{-1}$); Values are expressed as means (log_{10}) and SD (n = 8). * Significantly different means in the same line by the Tukey test ($P < 0.05$).

period varied between 2.95x10^4 to 5.67x10^4 cfu mL^{-1} for the control and between 2.89x10^4 to 5.09x10^4 cfu mL^{-1} for the probiotic treatment. The concentration of sucrose-positive *Vibrio* spp. ranged from 1.7x10^3 to 3.49x10^3 cfu mL^{-1} for the control and from 1.7x10^3 to 3.69x10^3 cfu mL^{-1} for the probiotic treatment, and the concentration of sucrose-negative *Vibrio* spp. varied from 1.7x10^3 to 3.1x10^3 cfu mL^{-1} for the control and from 1.7x10^3 to 4.01x10^3 cfu mL^{-1} for the probiotic treatment.

The mean THB concentration in the bottom water was not significantly different between the treatments ($P > 0.05$). However, there were significant differences ($P < 0.05$) during some of the cultivation weeks (Fig. 2b). In turn, the mean concentrations of sucrose-positive and negative *Vibrio* spp. were not significantly different between the treatments ($P > 0.05$), but again there were significant differences ($P < 0.05$) during some of the weeks (Figs. 2c and 2d).

The THB concentrations in the bottom water during the cultivation period varied from 3.43x10^4 to 5.42x10^4 cfu mL^{-1} for the control and from 3.43x10^4 to 5.09x10^4 cfu mL^{-1}, for the probiotic treatment. The concentration of sucrose-positive *Vibrio* spp. ranged from 1.7x10^3 to 4.43x10^3 cfu $mL^{-1}$for the control and from 1.7x10^3 to 4.28x10^3 cfu mL^{-1} for the probiotic treatment, while the similar ranges for the sucrose-negative *Vibrio* spp. were 1.7x10^3 to 4.02x10^3 cfu mL^{-1} for the control and 1.7x10^3 to 2.97x10^3 cfu mL^{-1} for the probiotic treatment.

The mean concentrations of THB and sucrose-positive and sucrose-negative *Vibrio* spp. in the shrimp did not present significant differences between the treatments ($P > 0.05$). The THB concentrations in the shrimp during the cultivation period ranged from 1.94x10^4 to 2.69x10^4 cfu g^{-1}) for the control and from 1.72x10^4 to 2.87x10^4 cfu g^{-1} for the probiotic treatment. The concentration of sucrose-positive *Vibrio* spp. varied from 2.47x10^3 to 5.34x10^3 cfu g^{-1} for the control and from 1.7x10^3 to 5.89x10^3 cfu g^{-1} for the probiotic treatment, while the corresponding ranges for the sucrose-negative *Vibrio* spp. were from 1.7x10^3 to 4.19x10^3 cfu g^{-1} for the control and from 1.7x10^3 to 3.69x10^3 cfu g^{-1} for the probiotic treatment.

The phytoplankton and Cyanobacteria concentration and percentage values are summarized in Table 2 and Figure 3, respectively. Cyanobacteria were the most abundant organisms, followed by Chlorophyta, Bacillariophya and Pyrrophyta. There was a significant difference in mean percentage values of Pyrrophyta between the control and the probiotic treatments ($P < 0.05$) between the 10th and 16th weeks. Bacillariophyta concentration varied from 8,400 to 96,000 cells mL^{-1} and 5,600 to 135,900 cells mL^{-1}, Chlorophyta from 11,000 to 392,200 cells mL^{-1} and 11,900 to 269,500 cells m L^{-1}, Cyanobacteria from

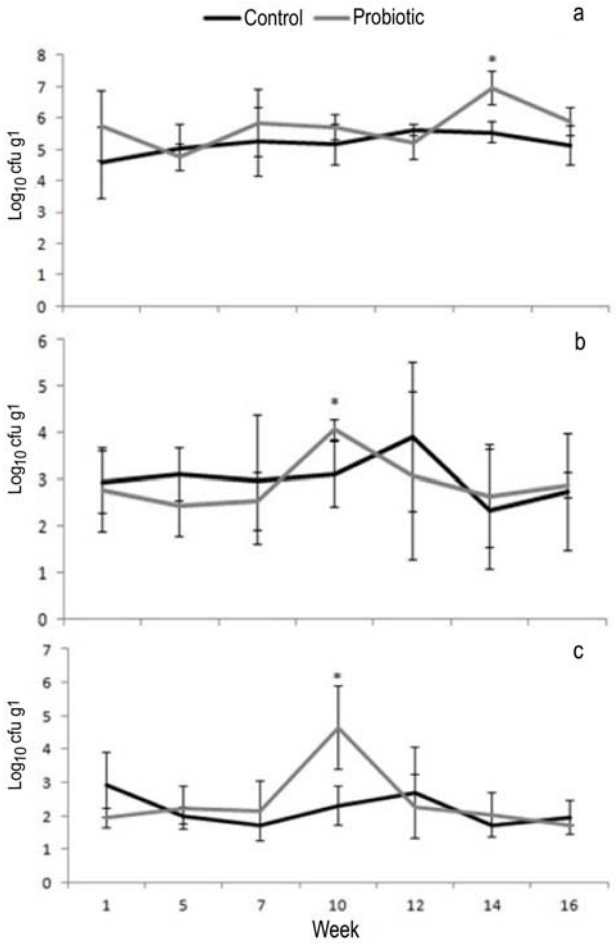

Figure 1. Total heterotrophic bacteria and *Vibrio* spp. in intensive shrimp farming (*Litopenaeus vannamei*) with recirculation system. a) total heterotrophic bacteria (THBx10^4 cfu g^{-1}) in sediment, b) *Vibrio* spp. (sucrose positive x10^3 cfu g^{-1}) in sediment, and c) *Vibrio* spp. (sucrose negative x10^3 cfu g^{-1}) in sediment. * Significantly different by the Tukey test ($P < 0.05$).

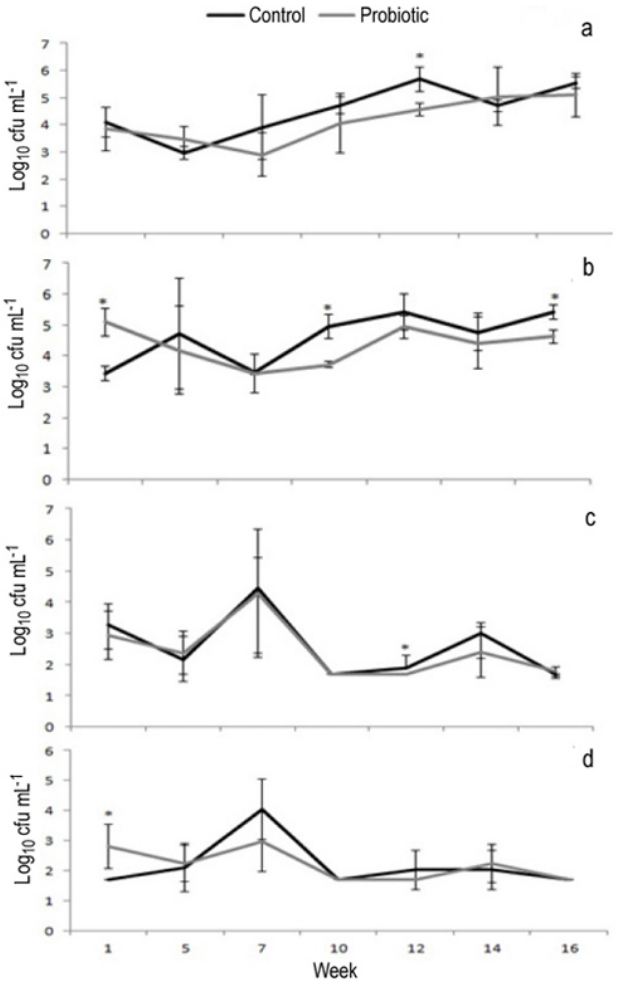

Figure 2. Total heterotrophic bacteria and *Vibrio* spp. in intensive shrimp farming (*Litopenaeus vannamei*) with recirculation system. a) Total heterotrophic bacteria (THBx10^4 cfu mL^{-1}) in surface water, b) total heterotrophic bacteria (THBx10^4 cfu mL^{-1}) in bottom water, c) *Vibrio* spp. (sucrose positive x10^3 cfu mL^{-1}) in bottom water and d) *Vibrio* spp. (sucrose negative x10^3 cfu mL^{-1}) in bottom water. *Significantly different by the Tukey test ($P < 0.05$).

4,400 to 544,100 cells m L^{-1} and 4,300 to 383,400 cells m L^{-1}, Pyrrophyta from 1,700 to 106,600 cells m L^{-1} and 0 to 54,100 cells mL^{-1} in the control and probiotic treatment, respectively.

DISCUSSION

Devajara *et al*. (2002), studying the effects of two probiotic products on ponds of a shrimp farm raising *P. monodon*, found that the sediment treated with the product containing *Bacillus* sp. and *Saccharomyces* sp. had a significantly greater concentration of total bacteria (1.24x10^6 cfu g^{-1}) in comparison to the other treatments. Hari *et al.* (2004, 2006), analyzing the concentrations of THB in the sediment of indoor tanks stocked with *Penaues monodon* with carbohydrate addition, reported densities between 24.8 and 53.9x10^6 cfu m L^{-1}, and between 41.5 and 72.5x10^7 cfu m L^{-1} in outdoor tanks.

The concentrations of total *Vibrio* spp. in the sediment in the control and probiotic treatment were similar. However, in the 10th week there were higher densities of both sucrose-positive and sucrose-negative *Vibrio* spp. in the probiotic treatments. The dominance of THB, the low concentrations of total *Vibrio* spp. and the prevalence of S^+ *Vibrio* spp. in the control and probiotic treatment may indicate that the *Bacillus* spp., *Lactobacillus* yeasts administered proliferated in the sediment of these ecosystems,

Table 2. Phytoplankton and Cyanobacteria concentration in intensive shrimp farming (*Litopenaeus vannamei*) with recirculation system.

Variables	Treatments	
	Control	Probiotic
Bacillariophyta (cells mL^{-1})	43,000 ± 35,800	43,400 ± 25,700
Chlorophyta (cells mL^{-1})	48,200 ± 66,600	81,000 ± 112,200
Pyrrophyta (cells mL^{-1})	19,000 ± 18,900	42,500 ± 39,200
Cyanobacteria (cells mL^{-1})	95,700 ± 93,900	150,600 ± 172,700

Control: without the use of a probiotic; Probiotic: the probiotic, composed of *Bacillus* spp. and *Lactobacillus* yeasts, was administered to the ponds (2,604.5 kg $pond^{-1}$); Values are expressed as means and SD (n = 16). * Significantly different means in the same line by the Tukey test ($P < 0.05$).

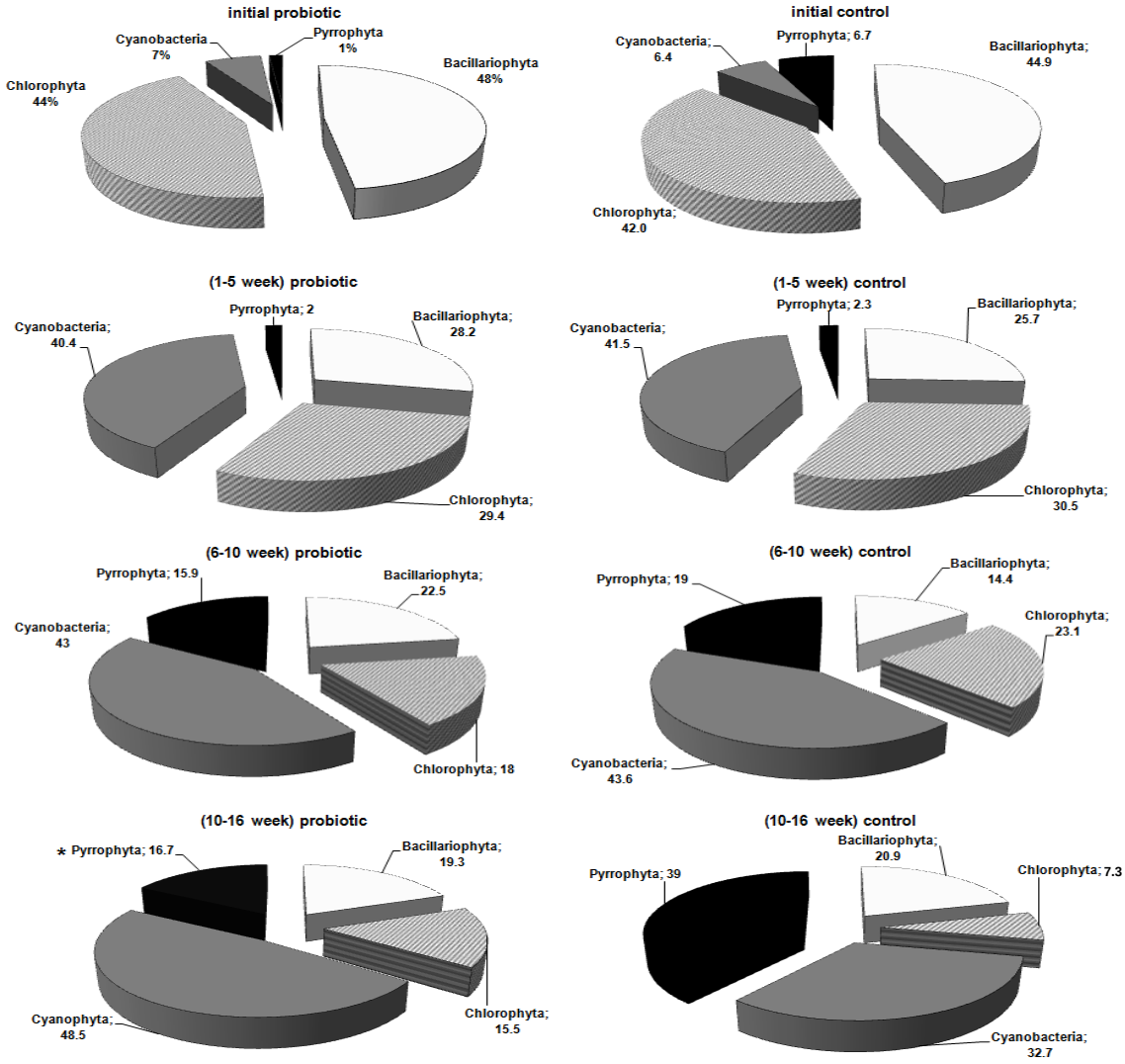

Figure 3. Phytoplankton and Cyanobacteria (%) in intensive shrimp farm (*Litopenaeus vannamei*) with recirculation system. *Significantly different by the Chi-square test ($P < 0.05$).

suggesting that the application of the probiotic and molasses contributed toward the occurrence of the succession and dominance of this community. Administering strains of *Bacillus* spp. in ponds containing *P. monodon*, Moriarty (1998) found that the proportion of pathogenic luminous S^- decreased in the sediment of the probiotic treatment. Vaseeharan & Ramasamy (2003), Nakayama *et al.* (2009) and Zhang *et al.* (2009) showed *in vitro* and *in vivo* antagonistic effect of *Bacillus* against pathogenic *Vibrio*. In the present study, despite the relatively larger mean concentrations of THB and *Vibrio* spp. in the sediment of the control, these differences were only statistically significant for the THB.

Devajara *et al.* (2002) found mean THB concentrations ranging from $1.78x10^4$ to $6.82x10^4$ cfu m L^{-1} in the bottom water of probiotic ponds and a mean density of $2.4x10^4$ cfu m L^{-1} in control ponds, and similar presumptive concentrations of *Vibrio* spp. in control ponds ($6.26x10^2$ cfu m L^{-1}) and probiotic ponds (5.57 to $6.16x10^2$ cfu m L^{-1}). Hari *et al*. (2004) reported THB in the water between 12.1 to $26.9x10^4$ cfu mL^{-1} (indoor experiment) and 40.6 to $57.1x10^5$ (farm trial) and Hari *et al.* (2006) reported figures between 20.8 to $37.1x10^4$ cfu mL^{-1}. Studies in shrimp farming in Brazil, such as in the states of Pernambuco (Mendes *et al*., 2009), Ceará (Costa *et al.*, 2009; Vieira *et al.*, 2010), and Santa Catarina (Mouriño *et al*., 2008), have reported *Vibrio* spp. in the water, sediment and shrimps.

Wang *et al.* (2005) found average counts of *Bacillus* sp., ammonifying bacteria and protein mineralizing bacteria were significantly higher in ponds with application of commercial probiotics compared to the control ponds. In the control ponds, an increase in presumptive *Vibrio* was observed and the average density was up to $2.09x10^3$ cfu m L^{-1}, whereas this was only $4.37x10^3$ cfu m L^{-1} in the treated ponds.

In the present study, the results for THB in the surface water were quite similar in the two treatments in terms of both variation and mean concentrations, suggesting that the substrate availability in the water was similar in both treatments. The abundance of microorganisms is a function of the substrate supply, increasing with the increase in supply and dropping quickly when the supply is depleted, after which the community rests and then resurges with a new input of substrate. Hari *et al.* (2006) reported carbohydrate addition had an effect on the THB in the water column and sediment. These THB concentrations recorded in both the control and probiotic treatment in the present study thereby support the hypothesis that the regular supply of carbohydrate (molasses) positively contributed to the survival and proliferation of the THB.

The statistical analysis of the data on the THB in the surface water revealed similarity between the two treatments. However, the analysis of the data on *Vibrio* spp. revealed that, despite the lack of a significant difference, faster reduction occurred in the concentration of S^+ *Vibrio* spp. over time in the probiotic treatments. This indicates that greater concentrations may have occurred in other niches of these ecosystems, increasing the demand for specific substrates and consequently limiting the stability and growth of these bacteria in the surface water, or it may indicate that the substrate availability was insufficient to maintain the concentrations of these organisms.

Data on the THB in the bottom water of ponds in studies involving probiotics are not common in the literature, as the majority of authors only refer to the surface water and water column. In the first few weeks of the study, no definite behavioral tendency was observed in the THB community of the bottom water in either treatment. However, unlike what occurred in the sediment, the mean THB concentrations in the bottom water from the 15^{th} week through to the end of the experiment were always higher in the control than in the probiotic treatment.

The variation in mean concentrations of total *Vibrio* spp. in the bottom water of both treatments displayed no definite tendency or dominance in either treatment. The mean S^+ *Vibrio* spp. values in the bottom water were slightly higher in probiotic treatment over the first nine weeks of the study, with equal values occurring in the 10^{th} week, at which point the values became slightly higher in the control. With the exception of the first week and last two weeks of the experiment, mean concentrations of S^- *Vibrio* spp. in the bottom water were always higher in the probiotic treatment, likely indicating the influence of the administered *Bacillus* spp. and *Lactobacillus* in controlling this bacterial community.

The shrimp THB concentration with probiotic were always much higher than those observed for animals in the control. However, the concentrations of total shrimp *Vibrio* spp. increased from the 13^{th} week in both treatments and these were mainly represented by proliferation and dominance of S^+, especially in the control. The higher concentrations of THB and sucrose-positive and negative *Vibrio* spp. in the shrimp in the probiotic treatment are probably related to the administration of the probiotic product along with the addition of molasses.

We found a stronger relationship between the bacterial communities of the sediment and shrimp, in

both treatments, from the intermediate phase to the end of the experiment. This finding suggests that due to the benthic behavior of *L. vannamei* and its close relationship with the sediment, especially during molting, the bacterial microbiota of these animals was much more related to the bacterial diversity of the sediment than the water column.

Probably the installed artificial aeration power (10.0 hp ha^{-1}) was not sufficient to ensure adequate availability of dissolved oxygen in the proposed production system, resulting in anaerobic conditions in the early hours every day in both treatments, thus severely limiting the efficiency of probiotic bioregulators in the closed system using recirculated water. Specific studies to stimulate the development of natural bacterial microbiota of shrimp ponds, through the use of substrates such as molasses and others, are important.

Plankton responds to high nitrogen and phosphorus levels, toxic contaminants and low dissolved oxygen, making them excellent indicators of environmental conditions of ponds, because they are susceptible to changes in water quality (Casé *et al.*, 2008). Bacillariophyta and Chlorophyta are considered to be beneficial because they are a source of food and nutrients for zooplankton and shrimp. However, Cyanobacteria and Pyrrophyta negatively affect water quality by producing compounds that are toxic to some aquatic animals (Jú *et al.*, 2008).

The initial dominance and prevalence of Bacillariophyta in the farming of *L. vannamei* in comparison to the initial prevalence of other phytoplankton organisms and Cyanobacteria was shown Yusoff *et al.* (2002) in shrimp ponds treated with a commercial bacterial product, and by Yusoff *et al.* (2010) in shrimp ponds with unpolluted water. These results certainly resulted from the fertilization process, which employed a nitrogen to phosphorus (N:P) ratio of 10:1, corresponding to the requirements of marine microalgae.

In the present work, Bacillariophyta accounted for 48% and 44.9% of the composition in the probiotic treatment and control, respectively, at the start of the experiment. At the end of the cycle these figures declined to 19.3 and 20.9%, respectively. In contrast, Cyanobacteria went from 6.7 and 6.4% of the initial phase to 48.5 and 32.7% in the final phase, in the probiotic and control, respectively. In percentage values, Cyanobacteria were the most abundant and Bacillariophyta were the second most abundant in ponds at the end of the experiment. Some authors, such as Yusoff *et al.* (2002, 2010), Alonso-Rodriguez & Paez-Osuna (2003), Casé *et al*. (2008), Pereira-Neto *et al*. (2008), Santana *et al.* (2008), Brito *et al.* (2009), Melo *et al.* (2010), Maia *et al.* (2011) and Sun *et al.* (2011), have also reported the dominance of Cyanobacteria in marine shrimp farms throughout the cultivation cycle.

The majority of problems related to water quality in aquaculture systems are due to inadequate production and management of plankton, the result of which is the dominance of Cyanobacteria, especially genera that form harmful blooms, such as *Shizothrix calcicola*, *Microcystis*, *Oscillatoria* and *Anabaena* (Pérez-Linares *et al*., 2003; Zimba *et al*., 2006) These genera are relatively poor oxygen producers and can generate compounds that are toxic to the farmed animals.

According to Yusoff *et al.* (2010), with the onset of eutrophication of water bodies, the Bacillariophyta population decreases and Cyanobacteria and Pyrrophyta persist. In the present study, the application of the probiotic significantly influenced the Pyrrophyta concentration between 10^{th} and 16^{th} week. Different factors working together certainly contributed to the Pyrrophyta blooms and local dominations, such as the static condition imposed by the lack of water renewal as well as the increase in organic matter and phosphate and nitrogen nutrients and the competitive advantages of these microorganisms over other plankton groups, especially with regard to nutrition mechanisms and adverse environmental conditions, such as a high degree of turbidity, increased salinity and a reduction in temperature.

The effects of the domination and blooms of Pyrrophyta and other organisms that are harmful to aquaculture systems, lakes and oceans have been reported for different regions of the world (Gómez, 2003; Yan *et al*., 2003; Drira *et al*., 2008). The resulting problems cited in these studies include eutrophication, fixation, anoxia, lowered availability of dissolved oxygen, release of ammonium, production of slime layers and gill obstruction as well as reduced growth and survival rates and mass death.

Given the nutritional value of Bacillariophyta and Chlorophyta and their combined, similar and relatively high participation in both treatments throughout the experiment, it is likely that this natural alimentary biomass stimulated the growth and survival of *L. vannamei*, with positive results in terms of production, productivity and health, in similar fashion in both the control and probiotic treatment.

CONCLUSIONS

The use of a commercial probiotic influenced the THB of the sediment and Pyrrophyta percentage values,

improving the environmental quality of the sediment and water in grow-out ponds stocked with *Litopenaeus vannamei* in a closed recirculation system. However, further study is necessary on selecting autochthonous bacterial strains and applying adequate concentrations of this microbiota to improve the ecological conditions and productivity of shrimp farms.

ACKNOWLEDGEMENTS

Alfredo Olivera is a research fellow of the Brazilian Council for Scientific and Technological Development (CNPq).

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Received: 11 April 2012; Accepted: 27 February 2013

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