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Effect of probiotic (*Bacillus* spp.) addition during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei*

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Abstract

The effect of the addition of *Bacillus* probiotic during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei* was examined in three separate experiments: (I) $Nauplius_{4-5}$ to $Zoea_3$, which were exposed to probiotic in the water (P_w), only in the microalgae (P_m), in the water and microalgae (P_{wm}) and a control with no probiotic (C); (II) $Mysis_1$ to $Mysis_3$, which were exposed to probiotic in the water (P_w), only in *Artemia* (P_a), in the water and *Artemia* (P_{wa}) and a control (C); (III) PL_1 to PL_{10}, which were exposed to similar treatments for mysis experiment. The use of probiotic *Bacillus* spp. resulted in an increase in the survival and growth of zoea and mysis phases, especially when the probiotic was added only in the water. For postlarvae, the use of the probiotic had no influence on the zootechnical parameters, however, there was a reduction in the count of presumptive *Vibrio* both for water and shrimp.

Keywords: *Bacillus*, bacteria, larvae, probiotics, *Vibrio*

Introduction

Bacterial diseases are considered to be a major cause of mortality in shrimp larviculture (Wyban & Sweeney 1991; Wilkenfeld 1992) resulting in a decrease in quality, quantity and regularity of shrimp production (Bachère, Mialhe, Noël, Boul, Morvan & Rodriguez 1995; Mialhe, Bachère, Boulo & Cadoret 1995; Selvin & Lipton 2003). A large number of Gram-negative bacteria of the genus *Vibrio* have been reported to cause losses in penaeid larviculture. These opportunistic bacteria are normally present in the culture facilities, as well as in the larval gut flora and in the live feed, and usually cause disease under sub-optimal culture conditions (Decamp, Moriarty & Lavens 2008).

In an attempt to control bacterial infections or even the presence of potential pathogens in hatchery systems, antibiotics have been used in Latin America and Southeast Asia, where there are few restrictions on the use of these products (Gomez-Gil, Roque & Turnbull 2000). However, excessive use of antibiotic can lead to the emergence of resistant bacteria and increase its virulence (Moriarty 1999; Verschuere, Rombaut, Sorgeloos & Verstraete 2000). Thus, the use of probiotics in the culture of aquatic organisms has been increasing with the demand for more environment-friendly aquaculture practices (Gatesoupe 1999).

Probiotics are defined as 'live microorganisms, which when are consumed in adequate amounts, confer a health benefit for the host' (Reid, Sanders, Gaskins, Gibson, Mercenier, Rastall, Roberfroid, Rowland, Cherbut & Klaenhammer 2003). However, in aquaculture probiotics can be administered either as a food supplement or as an additive to the water (Moriarty 1998). In this context, Verschuere *et al.* (2000) define probiotics for

aquaculture as a live microbial adjunct which has a beneficial effect on the host or ambient microbial community. Several benefits have been associated with the use of probiotics in the culture of aquatic organisms such as creation of a hostile environment for pathogens by the production of inhibitory compounds and supply of essential nutrients and enzymes resulting in enhanced nutrition of the cultured animal. Furthermore, the use of probiotics provides an improvement of water quality, lower incidence of diseases by enhanced immune response of host species and higher survival (Boyd & Massaaut 1999; Gatesoupe 1999; Gomez-Gil *et al.* 2000; Verschuere *et al.* 2000; Irianto & Austin 2002; Balcázar, de Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Múzquiz 2006).

Bacillus bacteria have been widely used as probiotics in aquaculture, as observed for shrimp *Litopenaeus vannamei* and *Penaeus monodon* (Decamp *et al.* 2008), and some fish as *Ictalurus punctatus* (Queiroz & Boyd 1998), *Oncorhynchus mykiss* (Nikoskelainen, Ouwehand, Bylund, Salminen & Lilius 2003) and *Scophthalmus maximus* (Gatesoupe 1994). These bacteria constitute a diverse group of rod-shaped, Gram-positive bacteria, characterized by their ability to produce a robust spore (Ninawe & Selvin 2009). *Bacillus* compete for nutrients and thus inhibit the rapid growth of *Vibrio* and other bacteria, thereby limiting the growth of resistant bacteria, thus further reducing the transfer of resistant genes between bacteria (Hong, Duc & Cutting 2005). Furthermore, many different antibiotic compounds are naturally produced by a range of *Bacillus* species (Moriarty 1998). Studies have shown that when *Bacillus* were administered as probiotics for *P. monodon*, growth and survival were improved and immunity was enhanced (Rengpipat, Phianphak, Piyatiratitivorakul & Menasveta 1998; Rengpipat, Rukpratanporn, Piyatiratitivorakul & Menasaveta 2000). Similarly, Ziaei-Nejad, Rezaei, Takami, Lovett, Mirvaghefi and Shakouri (2006) observed an increase in survival and growth of *Fenneropenaeus indicus* in treatments where *Bacillus* was added. Nevertheless, few studies have been proposed to investigate different approaches to administrate probiotics according to each shrimp development phases and larviculture practices.

The objective of this study was to evaluate different ways of applying the probiotic (*Bacillus* spp.) in three separate experiments during larvae and postlarvae culture of the white shrimp *L. vannamei*.

Materials and methods

Experimental animals

The different larval stages used in each experiment were obtained from the spawning of domesticated broodstock in a commercial hatchery (Maricultura Netuno S/A) located in Pernambuco, Brazil. These larvae were transferred to our laboratory at the previous stage of development needed for each experiment [Nauplius$_{3\text{-}4}$, Zoea$_3$ (Z_3) and Mysis$_3$ for experiments I, II and III respectively]. Larvae were maintained in a fibreglass tank (80 L) under constant aeration, temperature of 27–28°C and salinity 34–35‰ until metamorphosis. The water was supplemented with the microalgae *Chaetoceros calcitrans* at a concentration of 12×10^4 cells mL^{-1}. Food consisted of specific commercial diets (INVE-Frippak) for *L. vannamei* larvae (Zoea and Mysis), offered every 2 h. Additionally, in Mysis stage, frozen *Artemia* nauplii was offered *ad libitum* once daily.

Experimental design

Each treatment had four replicates consisting of 5-L plastic containers which were used as experimental tanks. Before the beginning of experiment, water was sterilized with chlorine for 24 h and filtered through a 3-μm cartridge filter. The tanks were maintained under constant aeration and ambient temperature of 27–28°C.

The commercial probiotic used in this study (INVE Sanolife® MIC, INVE, Belgium, www.inve.be) contained spores of *Bacillus subtilis*, *B. licheniformis* and *B. pumilus* in the concentration of 5.0×10^{10} CFU g^{-1}. The concentration of probiotic used in the water followed manufacturer's recommendations for shrimp larviculture, where it was added daily at a concentration of 0.5 ppm (2.5×10^4 CFU mL^{-1}) for Nauplius$_{4\text{-}5}$ ($N_{4\text{-}5}$) to Zoea$_2$ and 1.0 ppm (5.0×10^4 CFU mL^{-1}) from Z_3 to PL$_{10}$.

Feed diets for *L. vannamei* (INVE-Frippak) specific to Zoea (1 CAR), Mysis (2CD), PL$_{1\text{-}4}$ (3CD) and PL$_{5\text{-}10}$ (PL+300) were added every 2 h in all treatments from each experiment. During all experiments, the water quality factors such as temperature, dissolved oxygen, pH and salinity were monitored daily using a multiparameter sensor (YSI 556). Nitrite (NO_2) and ammonia (NH_3) concentrations were measured using photocolorimetry (ALFAKIT-AT10P) on the first and last day

of experiment I and II, and the first, fifth, 10th day of the experiment III.

Experiment I

In this experiment, the effect of the probiotic on the growth and survival of the early larval stages $N_{4\text{-}5}$ to Z_3 was examined. Larvae were stocked in experimental tanks at a density of 100 nauplii per litre. The experiment lasted 4 days, and there was no renewal of water during this period. The treatments consisted of the daily addition of probiotic only in the water (P_w), only in the microalgae (P_m), in the water and microalgae (P_{wm}) and a control (C), where no probiotic was administered. The probiotic used along with microalgae was added daily at 2 ppm (10.0×10^4 CFU mL^{-1}) into the culture of *C. calcitrans* which was in its exponential phase (third day of culture). This probiotic and algae mix was added daily to the treatments. Microalgae without probiotic addition was cultured in similar conditions for all experiments. The microalgae *C. calcitrans* cultured with or without probiotic was maintained at a concentration of 20×10^4 cells mL^{-1}.

Experiment II

In this experiment, the effects of the addition of probiotic was examined on stages $Mysis_1$ to $Mysis_3$ (M_1-M_3). Larvae were stocked in experimental tanks at the density of 50 larvae per litre. The experiment lasted 3 days, and there was no renewal of water during this period. The treatments consisted of addition of probiotic only in the water (P_w), only in *Artemia* (P_a), in the water and *Artemia* (P_{wa}) and a control (C). For the addition of probiotic via *Artemia*, cysts were hatched (2 g of cysts per litre of gently aerated 35‰ seawater) with 4 ppm (20.0×10^4 CFU mL^{-1}) of probiotic in the water for 18 h, then the nauplii were separated from the cysts and 4 ppm of probiotic was added again. After 10 h, the nauplii were harvested and offered to shrimp three times daily (12:00, 16:00 and 20:00 hours) at a rate of 6–7 nauplii per larvae. *Artemia* without probiotic were cultured following the same protocol used in other treatments. The microalgae *C. calcitrans* was maintained in all treatments at the concentration of 15×10^4 cells mL^{-1}.

Experiment III

The addition of probiotic was examined in the culture of the first postlarvae PL_1 to PL_{10} in experiment III. Postlarvae were stocked in experimental tanks at the density of 50 shrimp per litre. The experiment lasted 10 days and 10% of the tank water was exchanged every day. The treatments tested in this experiment were similar to those used in experiment II. *Artemia* nauplii cultured with the same methodology of experiment II was added two times daily (16:00 and 20:00 hours) at a rate of 15 nauplii per postlarvae. The microalgae *C. calcitrans* was maintained in all treatments at the concentration of 12×10^4 cells mL^{-1}.

Estimation of growth and survival

The growth and survival was recorded when all larvae attained the final stage according to each experiment. To estimate final growth of M_3 and Z_3, samples of 25 larvae per experimental tank were randomly selected at the end of each experiment to determine the total length (mm). Larval images were captured in a microscope (40X) connected to a video camera, computer digitized and analysed using the software IMAGETOOL version 2.0 for Windows (The University of Texas Health Science Center, San Antonio, TX, USA). Body wet weight (mg) was also recorded for 50 larvae sampled from each tank of the experiments I and II. The larvae were blotted dry on tissue paper and weighed together in analytical balance with precision of 0.0001 g. In experiment III, 100 postlarvae from each tank were weighed together following the same procedure described. To determine the total length (mm), 15 postlarvae from each tank were measured using a digital caliper. Final survival (%) was determined by counting the animals in each experimental tank.

Bacteriological analysis

At the end of each experiment, samples of water and shrimp were analysed to determine the presumptive *Vibrio* count (colony forming units; CFU) using Thiosulphate Citrate Bile Salts Sucrose Agar (TCBS Agar; Himedia Laboratories Corporate Office, Mumbai, India, www.himediaslab.com) (Liu, Chiu, Shiu, Cheng & Liu 2010). Water samples from each experimental tank were serially diluted (1/10) with sterilized saline solution (2.5% NaCl) until a dilution of 10^{-5} and plated (0.1 mL) in duplicate. After the incubation period of 24 h at 30°C the number of colonies on each plate was counted, considering only the plates containing

between 25 and 250 colonies (Downes & Ito 2001).

The presumptive *Vibrio* count in shrimp was performed by randomly sampling 50 larvae per tank from the experiment I and II, and 100 post-larvae per tank from experiment III. Larvae and poslarvae were washed with sterile distilled water, followed by sterile saline solution (2.5% NaCl) to remove external bacteria according Soto-Rodríguez, Simoes, Roque and Gómez Gil (2006). Then the samples were macerated, diluted and plated, following the same methodology described for the analysis of water.

Data analysis

The values of water quality factors, growth, survival and presumptive *Vibrio* in the water and shrimp were analysed by one-way analysis of variance, followed by Tukey's *post hoc* test to determine differences between treatments. All significant tests were at $P < 0.05$ levels. Survival data were arcsine transformed for analysis, but only the original values are presented. Results are presented as mean ± standard error.

Results

Water quality factors

The values of the water quality factors are shown in Table 1. There was no significant difference for all factors measured among treatments of each experiment. Temperature varied from 27.40 ± 0.18 to 28.28 ± 0.19°C, dissolved oxygen from 4.58 ± 0.05 to 5.52 ± 0.02 mg L^{-1} and pH varied from 8.15 ± 0.01 to 8.26 ± 0.01. Salinity of 34‰ was maintained in all experiments. Values of nitrite (NO_2) between experiments I and III ranged from 0.03 to 0.06 ± 0.006 mg L^{-1}, however, in experiment II, nitrite concentration was not detected in all treatments. For ammonia (NH_3), the values observed in experiment I and II ranged between 0.06 ± 0.01 and 0.12 ± 0.009 mg L^{-1}, while experiment III showed values from 0.74 ± 0.10 to 0.80 ± 0.09 mg L^{-1}.

Growth and survival of shrimp

In the first experiment, the wet weight of larvae treated with probiotic (0.22 ± 0.01 to 0.23 ± 0.01 mg) was significantly higher compared to the control (0.16 ± 0.01 mg) (Table 2). For experiment II, larvae in treatment P_w showed the highest wet weight followed by P_a which was higher than the treatments P_{wa} and C that did not differ between them. There was no significant difference in shrimp wet weight among treatments in experiment III, with values ranging from 2.31 ± 0.09 to 2.41 ± 0.08 mg. Values of total length showed no significant differences among treatments with or without probiotic in all experiments (Table 2).

The larvae survival of treatments P_w (80.6 ± 1.3%) and P_m (80.5 ± 0.3%) in experiment I

Table 1 Mean (±SE) values of temperature (°C) dissolved oxygen (mg L ^{1}) (D.O), pH, salinity (‰), NO_2 (mg L ^{1}) and NH_3 (mg L^{-1}) of *Litopenaeus vannamei* reared with and without *Bacillus* added to water and microalgae for $N_{4\text{-}5}$ to Z_3 (Experiment I), added to water and *Artemia* for M_1 to M_3 (Experiment II) and for PL_1 to PL_{10} (Experiment III)

	Treatments	Temp.	D.O	pH	Sal.	NO_2	NH_3
Experiment I	P_w	28.28 ± 0.19	4.72 ± 0.06	8.15 ± 0.01	34	0.03	0.12 ± 0.009
	P_m	28.22 ± 0.19	4.71 ± 0.05	8.16 ± 0.01	34	0.05 ± 0.006	0.06 ± 0.01
	P_{wm}	28.23 ± 0.19	4.65 ± 0.05	8.16 ± 0.01	34	0.039 ± 0.006	0.06 ± 0.02
	C	28.16 ± 0.22	4.58 ± 0.05	8.16 ± 0.01	34	0.03	0.10 ± 0.02
Experiment II	P_w	27.47 ± 0.16	5.46 ± 0.04	8.16 ± 0.01	34	ND	0.07 ± 0.01
	P_a	27.41 ± 0.17	5.50 ± 0.02	8.16 ± 0.006	34	ND	0.08 ± 0.01
	P_{wa}	27.40 ± 0.18	5.52 ± 0.02	8.17 ± 0.005	34	ND	0.09 ± 0.01
	C	27.42 ± 0.17	5.49 ± 0.02	8.16 ± 0.006	34	ND	0.10 ± 0.01
Experiment III	P_w	28.15 ± 0.19	4.98 ± 0.05	8.26 ± 0.01	34	0.03 ± 0.009	0.75 ± 0.10
	P_a	28.05 ± 0.19	4.85 ± 0.08	8.26 ± 0.01	34	0.03 ± 0.006	0.76 ± 0.07
	P_{wa}	28.08 ± 0.19	4.91 ± 0.05	8.24 ± 0.01	34	0.06 ± 0.01	0.74 ± 0.10
	C	28.19 ± 0.17	4.90 ± 0.08	8.26 ± 0.01	34	0.03 ± 0.009	0.80 ± 0.09

ND, not detected; P_w, probiotic only in the water; P_m, probiotic only in the microalgae; P_{wm}, probiotic in the water and microalgae; P_a, probiotic only in *Artemia*; P_{wa}, probiotic in the water and *Artemia*; C, control, without probiotic.

Table 2 Mean (±SE) values of final growth and survival of *Litopenaeus vannamei* reared with and without *Bacillus* added to water and microalgae for N_{4-5} to Z_3 (Experiment I), added to water and *Artemia* for M_1 to M_3 (Experiment II) and for PL_1 to PL_{10} (Experiment III)

	Treatments	Wet weight (mg)	Total length (mm)	Survival (%)
Experiment I	P_w	0.23 ± 0.01^a	2.62 ± 0.02^a	80.6 ± 1.3^a
	P_m	0.23 ± 0.01^a	2.64 ± 0.02^a	80.5 ± 0.3^a
	P_{wm}	0.22 ± 0.01^a	2.62 ± 0.02^a	76.1 ± 1.2^{ab}
	C	0.16 ± 0.01^b	2.62 ± 0.02^a	67.5 ± 4.8^b
Experiment II	P_w	1.02 ± 0.01^a	3.43 ± 0.04^a	85.6 ± 2.5^a
	P_a	0.61 ± 0.01^b	3.46 ± 0.04^a	83.4 ± 2.0^a
	P_{wa}	0.45 ± 0.01^c	3.37 ± 0.04^a	83.7 ± 2.4^a
	C	0.48 ± 0.04^c	3.34 ± 0.04^a	62.9 ± 5.4^b
Experiment III	P_w	2.31 ± 0.09^a	7.55 ± 0.10^a	77.7 ± 1.1^a
	P_a	2.41 ± 0.08^a	7.65 ± 0.11^a	73.6 ± 0.8^a
	P_{wa}	2.37 ± 0.05^a	7.42 ± 0.10^a	80.7 ± 3.1^a
	C	2.39 ± 0.02^a	7.68 ± 0.12^a	74.8 ± 1.5^a

Different superscript letters in the same column within the same experiment indicate significant differences ($P < 0.05$).

P_w, probiotic only in the water; P_m, probiotic only in the microalgae; P_{wm}, probiotic in the water and microalgae; P_a, probiotic only in *Artemia*; P_{wa}, probiotic in the water and *Artemia*; C, control, without probiotic.

were significantly higher than in the control (67.5 ± 4.8%). The survival of larvae in treatment P_{wm} (76.1 ± 1.2%) did not differ from the other treatments (Table 2). In experiment II, the treatments in which the probiotic was added showed a significantly higher survival (83.4 ± 2.0 to 85.6 ± 2.5%) compared to the control (62.9 ± 5.4%). There was no significant difference in survival among treatments with or without probiotic in experiment III, with values ranging from 73.6 ± 0.8 to 80.7 ± 3.1% (Table 2).

Bacterial development

In Z_3 stage (experiment I), presumptive *Vibrio* were not detected in the water samples of all treatments tested. However, presumptive *Vibrio* count in shrimp varied from 0.15 ± 0.01 to 0.41 ± 0.34 × 10^4 CFU larvae^{-1}, which did not differ among treatments (Table 3). In the experiment II, the presumptive *Vibrio* count in M_3 showed a significantly higher value in the control (0.75 ± 0.19 × 10^4 CFU larvae^{-1}) than in probiotic treatments (maximum of 0.19 ± 0.03 × 10^4 CFU larvae^{-1}). The development of presumptive *Vibrio* in the water, which did not differ among treatments, showed values ranging from 3.13 ± 0.28 to 5.53 ± 1.17 × 10^4 CFU mL^{-1} (Table 3).

The values found in experiment III showed that the presumptive *Vibrio* in the water and shrimp (PL_{10}) was significantly higher in the control compared to treatments with the use of probiotic (Table 3). The maximum concentration of these bacteria in the water was 2.51 ± 0.18 × 10^4 CFU mL^{-1} for the treatments with probiotic, while the control reached the concentration of 4.12 ± 0.26 × 10^4 CFU mL^{-1}. For shrimp, the presumptive *Vibrio* count reached 792 ± 327.3 × 10^4 CFU g^{-1} in the control, while the treatments with probiotic showed a maximum value of 88.6 ± 62.7 × 10^4 CFU g^{-1}.

Discussion

The use of probiotics in aquaculture can provide an improvement in water quality factors (Wang, Xu & Xia 2005). Lakshmanan and Soundarapandian (2008) investigated the effect of commercial probiotics (*Bacillus* spp.) on culture of shrimp *P. monodon* and observed that probiotics could significantly reduce the concentrations of nitrite and ammonia in pond water compared with the control. However, the present findings found no obvious effect of probiotic on the water quality, which may be explained by the good management, adequate supply of feed, low densities of culture and short time tests. Our results are in agreement with Zhou, Wang and Li (2009) that observed no significant difference between treatments with probiotic and control in the culture of larvae and postlarvae of *L. vannamei*. Similarly, the use of *Bacillus fusiformis* at a concentration of 10^5 CFU mL^{-1} in larviculture of *P. monodon* and *L. vannamei* (Z_1 to PL_1) did not affect water quality when compared with treatment without probiotic (Guo, Liu, Cheng, Chang, Lay, Hsu, Yang & Chen 2009). All water

	Stages	Treatments	Presumptive *Vibrio* counts: Water (10^4 CFU mL^{-1})	Presumptive *Vibrio* counts: Shrimp (10^4 CFU sample^{-1})*
Experiment I	$Zoea_3$	P_w	ND	0.27 ± 0.05[a]
		P_m	ND	0.41 ± 0.34[a]
		P_{wm}	ND	0.15 ± 0.01[a]
		C	ND	0.34 ± 0.21[a]
Experiment II	$Mysis_3$	P_w	4.24 ± 0.97[a]	ND[a]
		P_a	4.15 ± 0.18[a]	0.19 ± 0.03[a]
		P_{wa}	5.53 ± 1.17[a]	0.18 ± 0.02[a]
		C	3.13 ± 0.28[a]	0.75 ± 0.19[b]
Experiment III	PL_{10}	P_w	2.51 ± 0.18[a]	71.3 ± 31.4[a]
		P_a	2.35 ± 0.12[a]	82.0 ± 35.0[a]
		P_{wa}	2.04 ± 0.34[a]	88.6 ± 62.7[a]
		C	4.12 ± 0.26[b]	792.0 ± 327.3[b]

Table 3 Mean (±SE) values of presumptive *Vibrio* count in water and shrimp *Litopenaeus vannamei* reared with and without *Bacillus* added to water and microalgae for $N_{4\text{-}5}$ to Z_3 (Experiment I), added to water and *Artemia* for M_1 to M_3 (Experiment II) and for PL_1 to PL_{10} (Experiment III)

Different superscript letters in the same column (within the same ontogenic stage and experiment) indicate significant differences ($P < 0.05$). ND, not detected.
P_w, probiotic only in the water; P_m, probiotic only in the microalgae; P_{wm}, probiotic in the water and microalgae; P_a, probiotic only in *Artemia*; P_{wa}, probiotic in the water and *Artemia*; C, control, without probiotic.
*In experiments I and II, 'CFU larvae^{-1}', in experiment III, 'CFU g^{-1}'.

quality variables measured in the present study were within levels considered acceptable for shrimp culture (Menasveta, Aranyakanonda, Rungsupa & Moree 1989; Boyd 1990; Menasveta, Fast, Piyatiratitivorakul & Rungsupa 1991).

The addition of the *Bacillus* probiotic significantly improved survival for the first larvae (Zoea and Mysis) in the present study. Zhou *et al.* (2009) observed that application of probiotic *B. coagulans* SC8168 in the culture water had beneficial effects on the survival of *L. vannamei* larvae. Similarly, the application of *Bacillus* spp. to the water or via *Artemia* in the culture of *F. indicus* larvae resulted in a higher survival in treatments with probiotic compared to controls (Ziaei-Nejad *et al.* 2006). Verschuere *et al.* (2000) and Moriarty (1998) reported that *Bacillus* are able to compete with other bacteria, such as *Vibrio*, for nutrients and space, but also may exclude other bacteria by producing antibiotics, increasing their proportion in the gut flora of shrimp. This may be associated with the highest concentration of presumptive *Vibrio* found in the control ($0.75 \pm 0.19 \times 10^4$ CFU larvae $^{-1}$) compared with treatments with probiotic ($0.19 \pm 0.03 \times 10^4$ CFU larvae^{-1}).

In experiment I, the higher shrimp survival in probiotic treatments did not correspond to lower concentrations of presumptive *Vibrio* in these animals when compared to the control. This may be associated with a greater immunity acquired by the larvae ($N_{4\text{-}5}$ to Z_3) reared with *Bacillus*, which may have brought greater survival compared to control. Although we did not evaluate the immune response, Tseng, Ho, Huang, Cheng, Shiu, Chiu and Liu (2009) suggested that after *B. subtilis* E20 administration for *L. vannamei* culture there was an enhanced resistance of shrimp against *V. alginolyticus* due to phenoloxidase and phagocytic activity. The addition of *Bacillus* S11 in the culture of *P. monodon* increased survival and had positive effects on the immune response and disease resistance against the luminescent bacterium *V. harveyi* (Rengpipat *et al.* 2000). Similarly, Li, Zheng, Tian, Xi, Yuan, Zhang and Hong (2007) observed that the shrimp *L. vannamei* reared with addition of *B. licheniformis* demonstrated promising immune response stimulation. The survival results obtained for treatments of experiment I may also be related to the possible presence of non-pathogenic *Vibrio* species in the larvae. According to Gomez-Gil *et al.* (2000), there are species of *Vibrio* characterized as both beneficial and pathogenic for penaeids. These authors characterized the *Vibrio alginolyticus* as beneficial to *L. vannamei*, while Lee, Yu, Chen, Yang and Liu (1996) classified the same species as pathogenic to *P. monodon*. Some authors suggested that shrimp survival depends on the source of bacterial isolates (Karunasagar, Pai, Malathi & Karunasagar 1994; Muroga, Suzuki, Ishimaru & Nogami 1994; Austin & Zhang 2006). As an

example, a *V. harveyi* strain at a concentration of 10^2 CFU mL^{-1} caused mortality in penaeid shrimp larvae (Prayitno & Latchford 1995), while other strains of the same species did not affect survival at concentration of 10^6 CFU mL^{-1} (Abraham, Palaniappan & Dhevendaran 1997).

Previous studies reported that *Bacillus* bacteria secrete many exoenzymes, such as proteases, carbohydrolases and lipases, which are very efficient in breaking down a large variety of proteins, carbohydrates and lipids into smaller units (Moriarty 1996, 1998; Arellano & Olmos 2002; Ochoa & Olmos 2006; Ninawe & Selvin 2009). Furthermore, these authors argued that this process may contribute to enhance digestion and increase absorption of food, which in turn could improve shrimp growth. Similarly, our results showed that the body wet weight of the first larval phase increased by the addition of probiotic. Ziaei-Nejad *et al.* (2006) reported that the weight of *F. indicus* in stages N_{1-2} to Z_3 did not differ significantly when cultured with or without probiotic, however, from M_1 to PL_{1-2}, higher weight was observed in shrimp treated with probiotic. This same study showed no difference between treatments in relation to total length, which was also observed in our study within each developmental stage.

The bacteria of the genus *Bacillus* have been widely used as probiotic in shrimp culture to act as a biocontrol, reducing the concentration of *Vibrio* in the host and in the water (Skjermo & Vadstein 1999; Rengpipat *et al.* 2000). Ziaei-Nejad *et al.* (2006) added daily probiotic *Bacillus* to the water at a concentration of 7.3×10^6 CFU mL^{-1} during the culture of larvae and poslarvae of *F. indicus*, and observed a higher concentration of these bacteria in relation to total bacteria, which may include the vibrios. In contrast, our results indicated that the presumptive *Vibrio* counts in the water (Experiment II) was not influenced by the use of probiotic, which is possibly related to the lower concentration of probiotic used (5.0×10^4 CFU mL^{-1}). Additionally, presumptive *Vibrio* were not detected in the water during experiment I, which may be a result of the absence of *Artemia* in the system, as conducted in the experiments II and III. Live feeds such as *Artemia* represent a significant vector risk in the transfection of bacterial contaminants and subsequent infectious disease in larval cultures (Verdonck, Swings, Kersters, Dehasque, Sorgeloos & Leger 1994; Lopez-Torres & Lizárraga-Partida 2001). In contrast, microalgae employed in shrimp hatcheries usually have a natural bacterial load between 10^4 and 10^7 CFU mL^{-1} of heterotrophic bacteria, but rarely vibrios (Lizárraga-Partida, Montoya-Rodríguez & Gentrop-Funes 1997).

In experiment III, the action of *Bacillus* probably was responsible for the lower presumptive *Vibrio* counts in the shrimp and water in treatments with probiotic compared to control. Nevertheless, these results did not influence the zootechnical parameters (wet weight, total length and survival) among the treatments. In many cases, the vibrios are opportunists, causing disease and mortality in animals only when they are physiologically stressed (Alderman & Hastings 1998). Therefore, we must consider that similar growth performance and survival among treatments during the culture of PL_1 to PL_{10} occurred due to lack of physiological stress by the maintenance of adequate environmental conditions and food supply during the experiment.

This study also tested the effectiveness of the use of probiotic via *Artemia* and microalgae, which showed satisfactory results in the transfer of probiotic for shrimp, however, these procedures have resulted in similar growth, survival and presumptive *Vibrio* when compared to treatments with probiotic added directly in the water. Therefore, the additional labour and expense involved in these procedures could not be a cost-effective solution. According to our results, the use of probiotic *Bacillus* spp. during the culture of larvae (zoea and mysis) provides an increase in the survival and growth of animals, especially when the probiotic is added only in the water. For postlarvae phase, the probiotic did not influence on zootechnical parameters, however, there is a reduction in presumptive *Vibrio* counts both in the water and in the shrimp, providing greater security of the system.

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