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LACTIC-ACID BACTERIA INCREASE THE SURVIVAL OF MARINE SHRIMP, *Litopenaeus vannamei*, AFTER INFECTION WITH *Vibrio harveyi*

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ABSTRACT

This study evaluated the survival, post-larvae quality, and the population of bacteria in *Litopenaeus vannamei* after the addition of two strains of lactic-acid bacteria (2 and B6) experimentally infected by *Vibrio harveyi*. Fifteen hundred nauplii were distributed in 20 L capacity tanks with four replicates. The survival of control animals was lower (21%) than that of animals fed with the strains B6 (50%) and 2 (44%). Total bacterial population in the water and larvae, as well as of the *Vibrio* ssp. in water was not different among the treatments. No difference was observed in the population of *Vibrio* ssp. between the control larvae (5.5±0.5 log UFC/mL) and that fed with strain 2 (5.4±0.1 log UFC/mL). Shrimp from control and fed with strain 2 showed significantly higher bacterial population than those fed with strain B6 (1.2±0.2 log UFC/mL). It was detected the lower load of *Vibrio* ssp. bacteria with potential of pathogenicity after feeding with strain B6.Moreover, these larvae showed more active behavior and low number of necrosis in relation to the control group and to that fed with strain 2.

RESUMO

Este trabalho avaliou a adição de duas cepas de bactérias lácticas (2 e B6) na sobrevivência, qualidade de pós-larva e na população de bactérias na larvicultura de *Litopenaeus vannamei* experimentalmente infectado por *Vibrio harveyi*. Mil e quinhentos náuplios foram distribuídos em tanques de 20 L com quatro repetições. A sobrevivência dos animais controle foi menor (21%) do que a dos alimentados com as cepas B6 (50%) e 2 (44%). Sobrevivência de misis após desafio com *V. harveyi* foi maior em B6 do que nos outros tratamentos. A população total de bactérias na água e nas larvas, bem como de *Vibrio* ssp. na água não foi diferente entre os tratamentos. Não houve diferença, também, entre a população de *Vibrio* ssp. em larvas do grupo controle (5,5±0,5 log UFC/mL) e larvas alimentadas com a cepa 2 (5,4±0,1 log UFC/mL). Camarões do grupo controle e alimentados com cepa 2 apresentaram maior população de bactérias do que os alimentados com cepa B6 (1,2±0,2 log UFC/mL). Foi comprovada a menor presença de bactérias entéricas com potencial de patogenicidade nos animais alimentados com a cepa B6, apresentando também comportamento mais ativo e menor número de necroses em relação ao controle e cepa 2.

Descriptors: *Litopenaeus vannamei*, Shrimp, Probiotic, Bactéria, Experimental infection, *Vibrio harveyi.*
Descritores: Litopenaeus vannamei, Camarão, Probiótico, Bactéria, Infecção experimental, *Vibrio harveyi.*

INTRODUCTION

The development of diseases in the cultivation of shrimp is not only due to the increase in the intensity of shrimp farming but also due to microbial problems, environmental disturbances, pollution and nutritional imbalances (KAUTSKY; RÖNNBACK; TEDENGREN et al., 2000). Pathogenic bacteria are common in seawater by causing ecological changes. In the water used in

aquaculture (SKJERMO; VADSTEIN, 1999). Among the pathogenic agents, the genus *Vibrio* is the most important responsible for shrimp mortality in hatcheries of different countries (ABRAHAM; PALANIAPPANB, 2004).

For disease control, the use of antibiotic is a good strategy in shrimp farming (SKJERMO; VADSTEIN, 1999). However, the massive use of antibiotics tends to select resistant bacteria strains (HOLMSTRÖM; GRÄSLUND; WAHSTRÖM et al., 2003). Uddin and Kader (2006) reported that 25 to 50% f shrimp hatcheries in Bangladesh used five types of antibiotic (including the banned chloranphenicol) to control vibriosis in the period 2002 to 2003. Not only the development of resistant bacteria, but also antibiotic powder skin exposition and inhalation may cause diseases to workers (UDDIN; KADER, 2006). An alternative to antibiotic therapy is the addition of probiotic bacteria to control bacteria with pathogenic potential (GOMEZ-GIL; ROQUE; TURNBULL, 2000). Probiotic is a cultured or live microbial feed supplement which benefits the host by improving the host-associated microorganisms or environment microbial community (IRIANTO; AUSTIN, 2002) while it does no harm to the animals cultivated or to the environment. (BOYDE; MASSAUT, 1999).

Several studies have demonstrated the beneficial effect of the use of probiotic bacteria in shrimp farming (RENGPIPAT; RUKPRATANPORN; PIYATIRATITIVORAKUL; 2000; GULLIAN; THOMPSON; RODRIGUEZ, 2004). However, few investigations have been carried out on the beneficial effect of the use of probiotics during all larvae stages of the *Litopenaeus vannamei* hatchery.

The present study aimed to evaluate the addition of two strains of possible probiotic bacteria from the digestive tract of marine shrimp, by feeding *L. vannamei* larvae, over its survival, its resistance against *Vibrio harveyi* (BMG 2343) infection challenge and over water/larvae microbial ecology.

MATERIAL AND METHODS

Isolation and Selection of Probiotic Bacteria Strains

The bacterial strains were isolated from juvenile shrimp (12.0±2.0g) *L. vannamei* from the Laboratory of Marine Shrimps at the Federal University of Santa Catarina, Brazil. For this purpose , the digestive tract of shrimps was macerated in saline solution 2%, plated in De Man, Rogosa and Sharp culture medium MRS (DE MAN; ROGOSA; SHARPE, 1960, Difco, France) and incubated at 35^{o}C for 48 h, according to Ramirez, Cifonni, Pancheniak et al. (2006). After incubation, the colonies were identified morphologically by the Gram method. The selected colonies showed coconut shape, bacillus and cocobacillus gram positive. Then, the colonies were cultivated in MRS agar plates.

These strains were tested for their inhibitory properties against *V. harveyi*, according to Ramirez,Cifonni, Pancheniak et al. (2006). The isolated bacteria strains were plated in MRS agar and incubated for 24 h at 35^{o}C, and 1 cm diameter wells were taken from these plates. *V. harveyi* pathogenic strain was then plated in Marine Agar (Difco, France) and the wells were inoculated on the agar surface, followed by incubation for 24 h at 30^{o}C. Antibacterial activity was defined by the diameter of the clear inhibitory zone formed around the well. Two strains (2 and B6) that presented the biggest inhibitory zone against the *V. harveyi* were selected to be tested in the larval culture.

Experimental Conditions

Three treatments with four replicates were performed in plastic containers (20 L capacity) stocked with 1500 nauplii cultivated until the stage of post-larvae 1: Treatment C2: larvae fed with liquid diet (Oceandrops®-Prilabsa) supplemented with strain 2 in the concentration of 10^8 Colonies Forming Unit/mL (CFU/mL); Treatment B6: larvae fed with liquid diet (Oceandrops®-Prilabsa) supplemented with B6 strain at 10^8 CFU/mL; Treatment control: larvae fed only with liquid diet (Oceandrops®-Prilabsa).

Forty-eight hours before the experiment, the tanks water was treated with formalin solution (5ppt). Tanks water was then inoculated with the microseaweed *Chaetoceros calcitrans* at 5 x 10^4 cells/mL density to improve the water quality and larval feeding. Every day during the experiment the microseaweed density was measured in the water and a new microseaweed added to keep the 5 x 10^4 cells/mL concentration. The tank water was renewed just once during the experiment (50%) when the larvae reached the mysis 1 stage.

Larval Quality and Survival

Daily, a sample of 10 larvae from each tank was removed for larval quality evaluation under the optic microscope. The symptoms observed were necrosis presence, chromatophore expansion, full or empty digestive tract, appendix deformation, and epibionts presence. The larval survival (in percentage) was calculated by the larval stage changing from protozooea 3 to mysis 1 and from mysis 3 to post-larvae 1.

Microbiological Evaluation

When the larvae reached stages mysis 1 and post-larvae 1, water and larvae samples were removed

for microbiological evaluation. The larvae samples were sanitized with alcohol 70%, rinsed with sterile distilled water, weighed and macerated. Later, five serial dilutions (1/10) with the larvae and water samples were carried out separately, and plated on agar Marine culture medium (Difco, for total counting of CFU), agar Thiosulfate citrate bile salts sucrose agar (TCBS, Difco, France, for *Vibrio* ssp.) and MRS (for lactic-acid bacteria) and incubated at 30°C for 24 h. After this period, the colonies were counted to estimate the concentration of each bacteria group present in the water and in the larvae.

Experimental Infection with *Vibrio harveyi*

At the stage of mysis 2, four samples of 100 larvae were removed from treatments C2 and B6 and eight from control. Each sample was stocked separately in 1 L containers. The water of the treatments fed with probiotic and control was inoculated with 1mL *V. harveyi* at 10^9 CFU/mL. Four samples of larvae were kept without *V. harveyi* infection, and used as control samples. Treatments survival was evaluated 48 h after infection.

Statistical Analyses

Variance analysis to verify statistical differences between the treatments ($p<0.05$) and the Student Newmam Keuls (ZAR, 1984) multiple range test at 95% confidence level was used. Values of bacterial counts were transformed and are expressed in log for statistical analysis.

RESULTS

Isolation and Inhibition in Vitro of *Vibrio harveyi* by Probiotic Bacteria

From the digestive tract of the shrimps, 14 strains of acid-lactic bacteria were isolated, 12 of which inhibited *Vibrio harveyi in vitro*. Of these, strains 2 and B6 were selected for the *in vivo* experiment, as they presented the largest diameters of inhibition halos (Table 1).

Table 1. Inhibitory activity (mm ± standard deviation) of lactic-acid bacteria against *Vibrio harvey*i.

Strain	Zone of inhibition	Strain	Zone of inhibition
C2	8.0 ± 0.5	8	2.6 ± 0.6
B6	6.0 ± 0.6	9	2.3 ± 0.6
3	5.3 ± 0.6	10	1.7 ± 1.1
4	5.3 ± 0.5	11	1.6 ± 0.5
5	4.0 ± 1.0	12	1.3 ± 1.1
6	3.3 ± 1.1	13	0 ± 0
7	3.3 ± 1.1	14	0 ± 0

Larval Quality and Survival

No difference in larval quality was noted from the stages of protozooea to mysis ($p\geq0.05$) (Table 2). Survival from the stage of mysis to post-larvae of the C2 and B6 treatments was higher than that observed in control ($p<0.05$) (Table 2). Larvae from B6 treatment showed more active behavior, lower presence of necrosis and full digestive tract when compared to those of the other treatments. No larval deformation was observed.

Table 2. Survival (%) of *Litopenaeus vannamei* after feeding with strain B6, 2 and control in the stages mysis 1 and pos-larvae 1.

Treatment	Survival after changing from protozoea 3 to mysis 1	Survival after changing from mysis 3 to pos-larvae 1
Strain B6	63.0 ± 5 a	50.0 ± 2,8 a
Strain C2	68.0 ± 11 a	44.0 ± 4,3 a
Control	61.0 ± 10 a	21.0 ± 11,3 b

Data expressed in means ± standard deviation, means followed by the same letters are not significantly different according to SNK test (5%).
CV for mysis = 14%; CV for pos-larvae = 15%

Microbiological Analysis

The presence of lactic-acid bacteria (MRS medium culture) was observed only in the C2 e B6 treatments, both in the water and larvae macerate. The population of total bacteria did not vary significantly in the two evaluated stages in the water and larvae (Table 3). During the mysis 1 stage the population of *Vibrio* ssp. showed no significant difference between treatments C2 and B6 ($p\geq0.05$). However, in the stage of post-larvae 1 the B6 treatment revealed lower population of *Vibrio* ssp. than the other treatments ($p<0.05$) as shown in Table 3.

Experimental Infection with Vibrio harveyi

The survival rate of B6 (79.8%) and of the group without inoculation (73.3%) with *V. haveyi* was higher than that observed in C2 (48.0%) and control animals (49.0%) (Table 4). No difference was noted between B6 and the group without inoculation and between C2 and control infected shrimp.

Table 3. Bacterial population in tank water and shrimp after feeding with probiotic B6, C2 and control.

Treatment	Stage	Bacterial population					
		Water in log (UFC/mL) ± SD			Shrimp in log (UFC/g) ± SD		
		TCBS Agar	Marine Agar	MRS	TCBS Agar	Marine Agar	**MRS**
Strain B6	M1	2.0 ± 0.06 a*	2.9 ± 0.2 a	1.5 ± 0.1a	0.8 ± 0.05 b	3.1 ± 0.02 a	1.3 ± 0.3 a
Strain C2	M1	1.4 ± 0.24 a	3.1 ± 0.1 a	1.6 ± 0.2 a	0.7 ± 0.2 b	2.7 ± 0.35 a	1.2 ± 0.5 a
Control	M1	1.5 ± 0.14 a	3.1 ± 0.03 a	0 ± 0 b	0.8 ± 0.04 a	3.0 ± 0.15 a	0.0 ± 0 b
Strain B6	PL1	1.1 ± 0.3 a	5.9 ± 0.3 a	1.8 ± 0.3 a	1.2 ± 0.2 a	5.8 ± 0.5 a	3.7 ± 0.2 a
Strain C2	PL1	2.4 ± 0.4 b	6.1 ± 0.4 a	1.7 ± 0.2 a	5.4 ± 0.1 b	6.1 ± 0.5 a	3.7 ± 0.5 a
Control	PL1	3.5 ± 0.5 b	6.3 ± 0.2 a	0 ± 0 b	5.5 ± 0.5 b	5.7 ± 0.4 a	0 ± 0 b

Data expressed in means ± standard deviation, means followed by same letters are not significantly different according to SNK test (5%).
M1 - Larvae stage mysis 1; PL1 – post-larvae stage 1

Table 4. Effect of probiotic on the survival (%) of shrimp mysis (*Litopenaeus vannamei*) challenged with *Vibrio harveyi* 48h after infection.

Treatment	Survival
Strain B6 infected with *V. harveyi*	79.8 ± 18 a
Without *V. harveyi*	73.3 ± 3 a
Strain C2 infected with *V. harveyi*	48.0 ± 9 b
Control infected with *V. harveyi*	49.0 ± 4 b

Data expressed in means ± standard deviation, means followed by the same letters are not significantly different according to SNK test (5%).
cv = 25%

DISCUSSION

This study has demonstrated that the use of the lactic-acid bacteria strains B6 and C2 can improve the zootechnical performance in larval culture of marine shrimp by reducing the mortality rates. The control of pathogenics *Vibrio sp*. in aquaculture by using non-pathogenic bacteria (probiotic) has received especial attention in the last decade (SUGITA, HIROSE, MATSUE et al., 1998). The benefit of probiotic bacteria is associated to improvement of microorganisms inhabiting the larval digestive tract (GOMEZ-GIL, ROQUE and TURNBULL, 2000).

When larval marine shrimp reaches the nauplii stage, its nutrition is only from the vitelo, without exogenous feeding. In this case, there is a small number of bacterial colonization in the larval anal pore according to the observations of Gomez-Gil, Roque and Turnbull (2000). The transformation of microorganisms in the digestive tract occurs at the stage of protozoea, in which when it begins to ingest exogenous feeding. The probiotic-supplemented diet can favor the intestinal microbial community in this important moment of shrimp life cycle. This study evidenced that the probiotic bacteria had colonized the shrimp digestive tract (Table 4) and improved the larval survival until the stage of post-larvae 1 (Table 2).

The strains C2 and B6 showed a great capacity to inhibit *in vitro* the growth of *V. harveyi* (Table 1). However, only bacterial strain B6 proved to be efficient in inhibiting the *Vibrio* ssp. growth *in vivo*. A low population of *Vibrio* ssp. on post-larvae 1 fed the probiotic B6 supplemented diet (Table 3) has been confirmed. This can explain the reduced incidence of necrosis and more active larvae in the treatment with strain B6. The properties of probiotic bacteria competing for space in the digestive tract in protozoea, mysis and post-larvae of *Fenneropenaeus indicus* and in post-larvae of Penaeus monodon were reported by Ziaei-Nejad; Rezaei, Takami et al. (2006) and Rengpipat; Phianphak; Piyatiratitivorakul et al. (1998), respectively. Contrary to the observations by RENGPIPAT; PHIANPHAK; PIYATIRATITIVORAKUL et al. (1998), we were able to confirm the efficacy of the use of probiotic in the various stages of protozoea 3 to mysis 1 and in post-larvae 1. This study corroborated the findings of Ziaei-Nejad; Rezaei; Takami et al. (2006).

The strain B6 also revealed the most efficient action in the larval survival during mysis 1 infected by *V. harveyi*. The improvement of survival rates for shrimp fed with probiotic was already discussed for other shrimp species such as *P. monodon* (RENGPIPAT; PHIANPHAK; PIYATIRATITIVORAKUL et al., 1998) and *Penaeus chinensis* (LI; TAN; MAI et al., 2006).

Many researchers have demonstrated that the use of probiotic bacteria can improve the shrimp immunology (RENGPIPAT; RUKPRATANPORN;

PIYATIRATITIVORAKUL et al., 2000). As recommended by Skjermo and Vadstein (1999) probiotic bacteria should be isolated from healthy shrimp and fed to early larval stages, as followed in this study. It must be added that the strain B6 can be safely used as a probiotic for *L. vannamei* and to prevent *Vibrio* ssp. infection. However, further studies must be carried out to verify if the B6 bacteria strain might act in the shrimp immune system.

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