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PROBIOTIC POTENTIAL OF *Bacillus cereus* AGAINST *Vibrio* spp. IN POST-LARVAE SHRIMPS[1]

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ABSTRACT – *Bacillus* spp. have been used against diseases caused by bacteria that affect cultured shrimp, providing beneficial effects on the host shrimps by altering their microbial community, and improving zootechnical indexes. The objective of this work was to evaluate the effects of a diet supplemented with *Bacillus cereus*—a bacterium with probiotic potential—on post-larvae *Litopenaeus vannamei* shrimps grown in laboratory. The experiment lasted for fifteen days and consisted of six treatments—control (T_1), probiotic (T_2), *Vibrio parahaemolyticus* (VP) (T_3), probiotic and VP (T_4), *V. alginolyticus* (VA) (T_5), and probiotic and VA (T_6). The survival rate, weight gain, colonization capacity of the probiotic bacteria, pathogen count, and histopathological lesions were evaluated. There was no significant difference ($p \geq 0.05$) in survival between treatments. The groups with pathogens and without probiotics presented lower weight gain. The result of the *Bacillus cereus* count in the treatments T_2, T_4 and T_6 were significantly different ($p<0.05$), the probiotic bacteria were more aggressive in competing for space and nutrients when compared to *V. parahaemolyticus* than when compared to *V. alginolyticus*. Animals fed with the probiotic presented lower counts of these pathogens than those fed without the probiotic ($p<0.05$). No histopathological lesions were found in the organs and tissues of the shrimps. *Bacillus cereus* showed a high colonizing capacity in post-larvae shrimps, causing a significant reduction of pathogens, probably by secreting antimicrobial substances and the competitive exclusion, which justifies their use as probiotic bacteria.

Keywords: *Litopenaeus vannamei*. Antimicrobial. *Bacillus cereus*. Vibriosis.

AVALIAÇÃO DE POTENCIAL PROBIÓTICO FRENTE À INFECÇÃO EXPERIMENTAL POR VÍBRIOS EM PÓS-LARVAS DE CAMARÃO MARINHO

RESUMO – *Bacillus* spp. têm sido utilizados contra bacterioses que acometem camarão marinho cultivado, proporcionando efeito benéfico sobre o hospedeiro, alterando a comunidade microbiana intestinal e melhorando índices zootécnicos. O objetivo deste trabalho foi avaliar os efeitos de uma dieta suplementada com *Bacillus cereus,* bactéria com potencial probiótico em pós-larvas de *Litopenaeus vannamei* cultivados em laboratório. O experimento teve duração de quinze dias e consistiu de seis tratamentos: T_1 controle; T_2 somente com probiótico; T_3 com *Vibrio parahaemolyticus* (VP); T_4 com probiótico e com VP; T_5 com *V. alginolyticus* (VA); e T_6 com probiótico e com VA. Foram avaliados a sobrevivência, o ganho de peso, a capacidade de colonização das bactérias probióticas, contagem de patógenos e lesões histopatológicas. Não foi verificada diferença significativa ($P \geq 0,05$) entre os tratamentos na sobrevivência. Os grupos desafiados com patógenos e sem uso de probióticos foram os que apresentaram menor ganho de peso. Na contagem de *Bacillus cereus*, houve diferença significativa ($P<0,05$) entre os tratamentos T_2, T_4 e T_6, observando-se que o probiótico competiu melhor por espaço e nutrientes quando confrontado com *V. parahaemolyticus* do que com *V. alginolyticus*. Os animais alimentados com ração suplementada de probiótico, apresentaram contagem inferior daqueles alimentados sem o uso ($P<0,05$). Não foram observadas lesões histopatológicas nos órgãos e tecidos dos animais. O *Bacillus cereus* demonstrou uma alta capacidade de colonizar pós-larvas de camarão, causando uma diminuição significativa de patógenos, provavelmente pela secreção de substâncias antimicrobianas e/ou por exclusão competitiva, justificando seu uso como uma bactéria probiótica.

Palavras-chave: *Litopenaeus vannamei*. *Bacillus cereus*. Vibrioses.

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INTRODUCTION

Diseases that affect cultured shrimps are triggered by imbalances in the environmental conditions of the nursery, the health status of the shrimps, and potentially pathogenic agents (MERCIER et al., 2006). *Vibrio* spp. are among the bacteria that frequently cause diseases in cultured shrimps; they can infect them in all stages of life, and are responsible for mass mortalities in shrimp farms and, consequently, economic losses (AGUIRRE-GUZMÁN et al., 2010).

These pathogens have been controlled with the use of antibiotics, which brought negative consequences, especially bacterial resistance (THUY; NGA; LOAN, 2011). In the search for safe prophylactics, the use of probiotics emerged as an alternative to the use of antibiotics (BALCÁZAR et al., 2006).

Probiotics in aquaculture are cultured products or living microbial supplements that have beneficial effects on the hosts by altering their intestinal microbial community, improving their responses to diseases (VERSCHUERE et al., 2000), and increasing their appetite, promoting a better growth and feed conversion (NEWAJ-FYZUL; HARBI; AUSTIN, 2014). Live probiotics offered as supplements in diets must to be able to survive and pass through the animal's intestinal tract (NAVINCHANDRAN et al., 2014).

Results of pathogen antagonism tests, adhesion capacity and, consequently, colonization in the intestinal tract must be considered in the selection of probiotics (LUIS-VILLASEÑOR et al., 2011), as well as their ability in inhibiting or reducing the colonization of *Vibrio* bacteria (CHABRILLÓN et al., 2005). Moreover, the microorganism must be non-pathogenic, biochemically, and physiologically well characterized, and preferably isolated from the intestine of juveniles of the species itself, so that the microorganism will be more adaptable to its conditions (BALCÁZAR et al., 2006).

Gram-negative and gram-positive bacteria have been identified as potential probiotics for aquaculture, with effects against various pathogens (BRUNT; NEWAJ-FYZUL; AUSTIN, 2007). Gram-negative, spore-forming bacteria such as *Bacillus* spp. have shown considerable success as probiotics (HONG; DUC; CUTTING, 2005). The species *B. subtilis, B. clausii, B. cereus, B. coagulans* and *B. licheniformis* are the most studied. These species form spores, thus, they are heat stable and can be stored at room temperature as dehydrated products without any deleterious effect affecting their viability. Therefore, these probiotics are widely used in humans as dietary supplements and in aquaculture to increase growth and disease resistance in cultured shrimps (CUTTING, 2011).

Several researches have been conducted using *Bacillus* spp. in shrimp farming, some of them include *Litopenaeus vannamei*, Boone, 1931 (LUIS-VILLASEÑOR et al., 2011; NIMRAT; BOONTHAI; VUTHIPHANDCHAI, 2011; LIU et al., 2010) and *Penaeus monodon*, Fabricius, 1798 under the effect of *Bacillus cereus* (NAVINCHANDRAN et al., 2014).

The objective of this work was to evaluate the effects of a diet supplemented with *Bacillus cereus* as a probiotic on the post-larvae shrimps (*Litopenaeus vannamei*) survival, weight gain, intestinal microbiota, and histological conditions, while challenged with pathogens of the *Vibrio* genus.

MATERIAL AND METHODS

Ten-day-old post-larvae shrimps were obtained from a commercial laboratory in the state of Rio Grande do Norte, Brazil. The animals were kept for 14 days in a nursery, in a 1.0-m^3 water box, at a density of 24 post-larvae per liter (PL L^{-1}), constant aeration, temperature of 28.0±0.3 °C, and salinity of 28 mg L^{-1}. They were fed six times a day with a commercial feed consisted of 56% crude protein, according to the observed consumption. They were treated with a medicated feed with oxytetracycline (purity of 50%) at dose of 0.4 g kg^{-1} to ensure that the animals were free of pathogens during this period. The drug was incorporated into the diet following the method described by Brock and Main (1994). The medicated feed was offered in two of the daily meals for seven days. Subsequently, the post-larvae shrimps remained in the nursery for another five days, which was the time required for the starting of the experiment due to the antibiotic application.

Experimental design

The experiment was conducted in a completely randomized design, with six treatments and three replications:

T_1 – without use of probiotic and without pathogen (CT);

T_2 – with probiotic and without pathogen (PB);

T_3 – without probiotic and with *Vibrio parahaemolyticus*, ATCC - 147 17802 (VP);

T_4 – with probiotic and with *V. parahaemolyticus*, ATCC 147 17802 (PB-VP);

T_5 – without probiotic and with *V alginolyticus,* IAL - Adolfo Lutz Institute 1957 (VA);

T_6 – with probiotic and with *V. alginolyticus,* IAL 1957 (PB-VA).

The experimental plots consisted of 5-liter plastic containers, with 4.3 L of water containing 130 shrimps—density of 30 post-larvae per liter—with a mean weight of 9 mg and age of 24 days. The water

used in the containers was previously filtered, sterilized in ultraviolet light, chlorinated at 15 ppm for 24 hours, and dechlorinated with ascorbic acid (5 ppm). A constantly mild aeration with porous stone was maintained in each experimental plot, and part of the water (10%) of each container was exchanged daily. A feed with 40% crude protein was offered six times a day in amounts depending on the observed consumption.

The treatments T_2 (PB), T_4 (PB-VP) and T_6 (PB-VA) received feed supplemented with *B. cereus,* isolated from the intestine of juvenile shrimps (*L. vannamei*). The *B. cereus* strain was added to the feed at a concentration of 1.0×10^8 CFU g^{-1}, as suggested by Vieira et al. (2007) and recommended in commercial products (CUTTING, 2011).

On the 7th day of the experiment, the T_3 (VP), T_4 (PB-VP), T_5 (VA) and T_6 (PB-VA) treatments were challenged with the pathogenic bacteria, which was added to culture water at a concentration of 1.0×10^8 CFU mL^{-1}, as recommended by Buglione et al. (2008). The experiment was completed seven days after this addition.

The water hydrological parameters, temperature (°C), pH, salinity (mg L^{-1}) and dissolved oxygen (mg L^{-1}) were measured every 12 hours using a multiparameter meter. Water samples were collected every three days to determine their alkalinity (mg L^{-1}) and concentrations of nitrogen compounds—total ammonia (µg L^{-1}), nitrite (µg L^{-1}) and nitrate (µg L^{-1})—as recommended by Rice (2012).

Survival and weight gain

Survival (%) was determined at the end of the experiment by counting the final post-larvae and divided by the final storage density—difference between the number of post-larvae stored and the number of post-larvae collected during the experiment for analysis (Eq. 1).

Weight was calculated using data from seven biometrics performed throughout the experiment, on the 1st, 4th, 7th, 9th, 11th, 13th and 15th days of culture.

The excess moisture of the shrimps was removed with an absorbent paper, the animals were weighed in a digital scale with an accuracy of 0.0001 g, and the number of individuals in a biomass of 0.1 g was counted (Eq. 2),

$$Sur\ (\%) = \frac{FNPL}{FPLD} \times 100 \quad (1)$$

$$PLW(g) = \frac{0.1\ g\ biomass}{NPL} \quad (2)$$

wherein *Sur* (%) is the survival rate, *FNPL* is the final number of post-larvae, *FPLD* is the final post-larvae density, *PLW* (g) is the mean post-larvae weight per gram, *NPL* is the number of post-larvae.

Microbiological analyzes

Microbiological analyzes were performed using the standard plate count method. The colonization capacity of the *B. cereus* in post-larvae shrimps was evaluated by quantitative determination of the *Bacillus* at the beginning and throughout the experiment—1st, 4th, 9th and 15th days of culture.

The health status of the animals was evaluated through quantitative determination of the pathogenic bacteria (*Vibrio* spp.) in the animals in the first day of experiment, and in the 9th and 15th days after adding the pathogenic bacteria. The bacteria of the *Vibrio* genus were counted in the culture water at the beginning of the experiment to ensure that it was free of pathogens.

The concentration of bacteria in the animals was estimated using a shrimp biomass sample of 0.1 g that was washed in 70% ethyl alcohol, then in sterile distilled water, and in a saline solution (3.5% NaCl) to remove possible external bacteria. The samples were macerated and diluted in 0.9 mL of a sterile saline solution (dilution 10^{-1}), and then successive dilutions were performed up to 10^{-4}. The samples were plated according to Buller (2004) in a Man-Rogosa-Sharpe agar medium to count the *Bacillus*, and in a Thiosulfate Citrate Bile Sucrose agar medium to count the *Vibrio* spp., and incubated at 35 °C for 24 to 36 hours and at 37 °C for 18 to 24 hours, respectively. The bacteria grew, the colonies were counted, and the results were expressed in Colony Forming Units per gram (CFU g^{-1}).

The *Vibrio* bacteria in the culture water were counted by performing successive decimal dilutions of the water sample followed by plating, according to the same methodology described for the analysis in the animals.

Histopathological analysis

Samples were collected from all treatments for histopathological analysis. Seven collections were carried out throughout the experiment on the 1st, 4th, 7th, 9th, 11th, 13th and 15th days of culture. The animals were fixed in a Davidson's AFA solution (HUMASON, 1972) for 24 to 48 hours, and then transferred to a 70% alcohol solution. The material was subjected to dehydration and diaphanization, embedded in paraffin, cut with a microtome at a thickness of 5 microns, and stained with hematoxylin-eosin (BEHMER et al., 2003).

Statistical analysis

The survival rates of the post-larvae shrimps

in all the treatments were subjected to analysis of variance (ANOVA) (α=0.05), after homogeneity evaluation by the K test (Cochran), and the means were subjected to the Tukey's test to assess significant differences ($p \leq 0.05$) between treatments.

The following mathematical model was used to evaluate the relationship of post-larvae weight gain with density, time of culture, and treatments:

$$Weight = \beta_0 + \beta_1 TC + \beta_2 SD + \beta_3 TC * SD + \sum_{n=1}^{n=6} \beta_{3+n}\, Treat_n + \varepsilon_i \quad (3)$$

wherein *Weight* is the average weight per post-larva sample (0.1 g), $\beta_{0,1,2,3,\ldots 9}$ are the model parameters, *TC* is the time of culture; *SD* is the storage density, $Trat_n$ is the treatment—T1 (CT), T_2 (PB), T_3 (VP), T_4 (PB-VP), T_5 (VA) and T_6 (PB-VA)—, and ε is the error with normal distribution and parameters (0, σ^2).

The Stepwise process (selection of variables) was used to verify the effect ($p<0.05$) of each variable of the model, and the robustness of the model was evaluated at the end of the process based on the Snedecor's F-statistic, probability value of F, R^2, normality of errors (Shapiro-Wilk), and number of discrepant points (outlier).

The results of *Bacillus* bacterial counts in post-larvae shrimp (*L. vannamei*) in T_2 (PB), T_4 (PB-VP) and T_6 (PB-VA), at the 4th, 9th and 15th days of culture, and the results of counts of *Vibrio* spp. in T_3 (VP), T_4 (PB-VP), T_5 (VA) and T_6 (PB-VA), at the 9th and 15th days of culture, were subjected to analysis of variance (ANOVA) after homogeneity evaluation through the K test (Cochran). Means with significant differences between treatments and between times of culture were compared by the t-Student test at 5% probability. The values of the counts were transformed to Ln (x +1) for the analyses. All statistical calculations were performed using the SysEapro (v.2) software.

RESULTS AND DISCUSSION

The means of temperature, pH, salinity, dissolved oxygen, alkalinity and concentrations of nitrogen compounds—total ammonia, nitrite and nitrate—of the treatments (Table 1) were adequate for the culture of Penaeidae shrimps, according to Boyd (1990). Thus, the water quality did not compromise the development of the shrimps during the experiment.

Table 1. Means±standard deviations, and minimum and maximum variations of the physical-chemical parameters of the culture water of post-larvae shrimps (*L. vannamei*).

Parameters	Mean±standard deviation	Variation
Temperature	26.33±0.45	26.9 – 25.66
pH	7.61±0.30	7.08 – 7.01
Salinity (g L^{-1})	28.15±0.03	28.21 – 28.10
Dissolved oxygen (mg L^{-1})	6.16±0.16	6.40 – 5.89
Ammonia (µg L^{-1})	483.07±36.08	518.17 – 423.37
Nitrate (µg L^{-1})	151.87±31.68	205.34 – 125.21
Nitrite (µg L^{-1})	153.16±25.92	205.34 – 125.21
Alkalinity (mg L^{-1})	182.34±2.34	185.78 – 115.66

Survival and weight gain

The treatment with the highest survival of post-larvae shrimps at the 15th day of experiment was T_2 (PB), with a rate of 60.52±23.71%, whereas the treatments with pathogens showed the lowest rates. However, no significant difference ($p \geq 0.05$) was found between the treatments (Table 2). Thus, the use of *B. cereus* as a probiotic did not contribute significantly to the survival of the animals during the experiment. Although, the treatments with the probiotic showed higher survival rates than the control groups without probiotic. These results confirm those found by Guo et al. (2009) for larvae of *Pennaeus monodon* fed with the probiotic *Bacillus fusiformis*; they found no significant difference in the survival rate when compared to the control group (without probiotic).

However, increased survival rates of shrimps were observed by other authors using probiotics. Balcázar and Rojas-Luna (2007) used a diet with *B. subtilis* UTM 126 and found efficacy in the survival rate and protection of *L. vannamei* against infection by *V. parahemolitycus.* Buglione et al. (2008) used *Lactobacillus platarum* and found increased survival of post-larvae of *L. vannamei* in salinity stress tests and experimental infections with *V. harveyi*. NavinChandran et al. (2014) evaluated the effect of *B. cereus* as probiotic on post-larvae of *Pennaeus monodon* and found a high survival rate in groups treated with feed supplemented with probiotic, compared to the control group.

The results for survival rate in the present study are probably due to the control of water quality in all treatments, and the adequate food conditions during the experiment, which caused no physiological stress to the animals.

Table 2. Effect of treatments without probiotic and pathogen (CT), with probiotic (PB), with *V. parahaemolyticus* (VP), with probiotic and *V. parahaemolyticus* (PB-VP), with *V. alginolyticus* (VA), and with probiotic and *V. alginolyticus* (PB-VA) on the average survival rate of post-larvae shrimps (*L. vannamei*) grown for 15 days in laboratory.

Treatment	Survival (%) Mean±Standard deviation
T_1 (CT)	53.01±8.48 [a]
T_2 (PB)	60.52±23.71[a]
T_3 (VP)	44.87±11.03[a]
T_4 (PB-VP)	51.48±16.51[a]
T_5 (VA)	47.47±10.75[a]
T_6 (PB-VA)	54.51±3.40 [a]
Coefficient of variation (%)	10.74
Probability of the Snedecor's F-statistic	0.0599

Means followed by the same letters do not differ significantly ($p \geq 0.05$) between treatments.

The time of culture positively affected ($p<0.05$) the weight gain of the post larvae shrimps (*L. vannamei*), while the storage density and the T_3 (VP) and T_5 (VA) negatively affected ($p<0.05$) their weight gain, based on the Eq. 3 that resulted in the model:

$$Weight_{(g)} = 0.00930 + 0.00252TC - 0.00002TC * SD - 0.00286T_3 - 0.00395T_5$$

wherein *Weight* $_{(g)}$ is the weight of post-larvae in grams, *TC* is the time of culture, *SD* is the storage density, T_3 is the treatment 3—without use of probiotic and challenged with *V. parahaemolyticus* (VP)—, T_5 is the treatment 5—without use of probiotic and challenged with *V. alginolyticus* (VA).

This model was considered robust, since it presented the following results: R^2 = 73.65%, Probability of the Snedecor's F-statistic <0.00001, six outliers, and normality of errors within the acceptable range by the Shapiro-Wilk, D´Agostino, and D´Agostino-Pearson tests. Thus, the estimates and comparisons of the results showed that, for a time of culture of 15 days and a mean of 78 individuals per experimental unit, the animals from the treatments with pathogens, but with probiotics and the controls did not differ significantly ($p \geq 0.05$) presenting the best weight gains. Contrastingly, T_3 (VP) and T_5 (VA)—challenged with *V. parahaemolyticus* and *V. alginolyticus*, without using probiotics, respectively—were the ones that presented the lowest weight gain, and presented significant differences ($p<0.05$) from the other treatments (Table 3).

Table 3. Effect of treatments without probiotic and pathogen (CT), with probiotic (PB), with *V. parahaemolyticus* (VP), with probiotic and *V. parahaemolyticus* (PB-VP), with *V. alginolyticus* (VA), and with probiotic and *V. alginolyticus* (PB-VA) on the weight gain of post-larvae shrimps (*L. vannamei*) grown for 15 days in laboratory.

Treatment	Post-larvae weight (g) Mean±Standard deviation
T_1 (CONTROL)	0.027±0.02[a]
T_2 (PB)	0.027±0.02[a]
T_3 (VP)	0.024±0.02[b]
T_4 (PB-VP)	0.027±0.02[a]
T_5 (VA)	0.024±0.02[b]
T_6 (PB-VA)	0.027±0.02[a]

Means followed by different letters in the column differ significantly ($p<0.05$) by the Snedecor's F-statistic.

The presence of pathogens in treatments without the probiotic negatively affected the shrimp weight gain. Balcázar, Rojas-Luna and Cunninghan (2007) also observed beneficial effects with the use of feed with probiotics isolated from the gastrointestinal tract of *L. vannamei*, against experimental infection with *V. parahaemolyticus.* Zokaeifar et al. (2014) found a greater weight gain with the use of probiotics in the culture water of *L. vannamei* challenged with *V. harveyi*. According to Navinchandran et al. (2014), probiotic bacteria produce digestive enzymes, and necessary nutrients for growth, such as vitamins and amino acids, and improve food absorption, resulting in a better growth rate of the host. This was observed in the present work, since treatments with *Vibrios* spp. and without probiotics presented the lowest weight gain.

Microbiological analyzes

The pathogenic bacteria of the *Vibrio* genus did not develop in the culture water and animals, and

the bacteria of the *Bacillus* genus did not develop in the post-larvae of *L. vannamei,* in the first day of experiment. The *Vibrio* bacteria did not grow in T_1 (CT), T_2 (PB), and the *Bacillus* bacteria did not grow in the T_1 (CT), T_3 (VP) and T_5 (VA), throughout the experiment.

The colonization of a probiotic in the gastrointestinal tract of an animal is a very important factor for its intestinal balance (ZOKAEIFAR et al., 2014). The adhesion capacity and consequent colonization of bacteria in the gastrointestinal tract, and the ability of inhibiting or reduce colonization of *Vibrio* spp. must be considered when choosing a probiotic (CHABRILLÓN et al., 2005).

The administration of feed supplemented with *B. cereus* was successful regarding its colonization, since it was possible to recover it from the animal samples from all groups treated with the probiotic.

The counts of *Bacillus* bacteria in post-larvae shrimps from treatments with supplementation of *Bacillus cereus* are presented in Table 4. The count of bacterial probiotics remained stable in T_2 (PB)—treatment with no pathogens—, with no significant difference ($p \geq 0.05$) throughout the experiment. In T_4 (PB-VP) and T_6 (PB-VA)—treatments with *V. parahaemolyticus* and *V. alginolyticus* added on the 7th day of experiment, respectively—, the count of *Bacillus* reduced on the 9th day; this was probably due to the presence of pathogens, and occurrence of competitive exclusion.

The three treatments were significantly different ($p<0.05$) on the 15th day of experiment; the *Bacillus* bacterial count in T_4 (PB-VP) (5.83±1.18 CFU mg^{-1}) was higher than in T_6 (PB-VA) (4.90±0.42 CFU mg^{-1}). This indicates that the probiotic bacteria were more aggressive in competing for space and nutrients when compared to *V. parahaemolyticus* than when compared to *V. alginolyticus* in post-larvae shrimps (*L. vannamei*), and promoted a positive substitution of harmful bacteria to the animals, such as *V. parahaemolyticus*, by beneficial bacteria, such as *B. cereus.*

Table 4. Probiotic bacteria count in post-larvae shrimp samples grown for 15 days in laboratory, under diet supplemented with *B. cereus*, and challenged with *Vibrio* spp. bacteria.

Treatment	Days of culture		
	4th day	9th day	15th day
	Bacterial counts (Ln CFU mg^{-1}) Mean±Standard deviation		
T_1 (CT)	Absent	Absent	Absent
T_2 (PB)	6.11±2.92^{aA}	7.47±1.39^{aA}	7.62±1.23^{aA}
T_3 (VP)	Absent	Absent	Absent
T_4 (PB-VP)	7.61±0.87^{aA}	5.91±2.20^{aB}	5.83±1.18^{bB}
T_5 (VA)	Absent	Absent	Absent
T_6 (PB-VA)	6.88±187^{aA}	6.08±3.37^{aAB}	4.90±0.42^{cB}

Treatments: T_1 (CT) without probiotic and pathogen, T_2 (PB) with probiotic, T_3 (VP) with *V. parahaemolyticus*, T_4 (PB-VP) with probiotic and *V. parahaemolyticus*, T_5 (VA) with *V. alginolyticus*, and T_6 (PB-VA) with probiotic and *V. alginolyticus*. Ln CFU mg^{-1} = log of Colony Forming Units (CFU) counts per milligram. Means followed by different lowercase letters in the same column and different uppercase letters on the same row differ significantly ($p<0.05$) by the t-Student test at 5% probability; the data were transformed to Ln (x+1).

The results of the counts of pathogenic bacteria in the animals are presented in Table 5. In the first bacteriological analysis for *Vibrio* in the 9th day of culture, no significant difference was found between treatments. However, differences ($p<0.05$) were found between the treatments T_3 (VP) and T_4 (PB-VP), and between T_5 (VA) and T_6 (PB-VA) at the end of the experiment (15th day).

Regarding the time of culture, the counts of bacteria in the 9th and 15th days of culture showed a significant difference ($p<0.05$) between the treatments T_4 (PB-VP) and T_6 (PB-VA). Thus, the groups of post-larvae fed with feed supplemented with probiotic had lower pathogen counts than those fed without the probiotic. This is probably connected to the antimicrobial action of the *Bacillus cereus* against *V. parahaemolyticus* and *V. alginolyticus*, confirming the findings of Vieira et al. (2007).

According to Abriouel et al. (2011), bacteria of the *Bacillus* genus produce many antimicrobial substances, including peptidic, lipopeptide antibiotics, and bacteriocins, the latter is very important, due to the broad spectrum of bacteria—gram-negative and gram-positive—, yeasts and fungi that it can inhibit. Moreover, according to Verschuere et al. (2000), *Bacillus* spp. are able to compete with pathogenic bacteria for nutrients and space, increasing their proportion in the intestinal microbiota of shrimps.

Table 5. Pathogenic bacteria count in post-larvae shrimp samples grown for 15 days in laboratory, under diet with feed supplemented and non-supplemented with *B. cereus*, and challenged with *Vibrio* spp. bacteria.

Treatment	Days of culture Bacterial count (Ln CFU.mg^{-1}) Means±Standard deviation	
	9th day	15th day
T_1 (CONTROLE)	Absent	Absent
T_2 (PB)	Absent	Absent
T_3 (VP)	9.59 ± 1.64^{aA}	8.54 ± 3.47^{Aa}
T_4 (PB-VP)	9.37 ± 1.33^{aA}	3.49 ± 2.15^{Bb}
T_5 (VA)	8.87 ± 3.75^{aA}	6.25 ± 2.74^{Aa}
T_6 (PB-VA)	9.82 ± 0.21^{aA}	2.91 ± 6.27^{abB}

Treatments: T_1 (CT) without probiotic and pathogen, T_2 (PB) with probiotic, T_3 (VP) with *V. parahaemolyticus*, T_4 (PB-VP) with probiotic and *V. parahaemolyticus*, T_5 (VA) with *V. alginolyticus*, and T_6 (PB-VA) with probiotic and *V. alginolyticus*. Ln CFU mg^{-1} = log of Colony Forming Units (CFU) counts per milligram. Means followed by different lowercase letters in the same column and different uppercase letters on the same row differ significantly (p<0.05) by the t-Student test at 5% probability; the data were transformed to Ln (x+1).

Histopathological analysis

No relevant morphological lesions were found in organs or tissues of the post-larvae shrimps in all treatments throughout the experiment when using 108 CFU mL^{-1} of pathogenic bacteria. Some specimens presented mild peripheral hemocytic infiltrations in the hepatopancreas, which could not be connected to the groups challenged with pathogens. Contrastingly, Soto-Rodriguez et al. (2015) found lesions, such as elongation and atrophy of the tubular epithelial cells, hemocytic infiltration, and necrosis in experimental infection with *V. parahaemolyticus.*

Despite the high counts of *Vibrio* spp. in the animals from the treatments challenged with *V. parahaemolyticus* and *V. alginolyticus*, no clinical signs or lesions could be connected to these *Vibrios* spp. Several factors may have caused the absence of alterations, such as the short period of exposure to the pathogen, and the maintenance of environmental conditions—pH, salinity, dissolved oxygen, nitrite, nitrate, and ammonia. According to Mugnier et al. (2013), sufficiently stressful factors external to the animal could anticipate the incubation period of the agent, showing earlier clinical signs.

CONCLUSION

B. cereus isolate from the intestine of shrimps *Litopenaeus vannamei* can colonize the intestine of post-larvae shrimps of the same species and promote a significant reduction of pathogens, with great effectiveness in reducing the pathogenic bacteria *V. parahaemolyticus* and *V. alginolyticus* in shrimps grown in laboratory.

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