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MICROBIOLOGY

Prospection of strains of *Bacillus* sporogenes in the digestive tract of native crustaceans and characterization of the probiotic potential

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Abstract: The cultivation of marine shrimp is one of the fastest growing activities in the world. However, the emergence of diseases has resulted in a decrease in production and losses for the sector. Probiotics emerged as an option to the use of antibiotics to control these pathogens. The efficiency of applying this technology depends on the characteristics of the bacterial agents and their bioavailability in the shrimp intestine. The objective is to evaluate the viability and efficiency of bacteria isolated from the digestive tract of healthy crustaceans as probiotic agents in the cultivation of shrimp *Litopenaeus vannamei*. Eighteen strains of the genus *Bacillus* belonging to the following species were tested: *Bacillus* sp., *B. cereus*, *B. thuringiensis*, *B. circulans*, *B. megaterium*, *B. subtilis* and *B. agaridevorans*. Bacterial isolates were subjected to characterization as potential probiotics. The test results were considered satisfactory; thus, the tested strains have potential for use as probiotics in shrimp culture. Treatments that used of the genus *Bacillus* had reduced growth of the genus *Vibrio* after infection, both in the intestinal contents and in the intestine. With the results obtained, it can be suggested that further research be carried out on the probiotic potential of *Bacillus* sp.

Key words: Diseases, pathogens, shrimp, *Vibrio*.

INTRODUCTION

Shrimp farming is an important economic activity with satisfactory financial returns, in addition to generating jobs and being responsible for a product of great economic value (Igarashi 2022). These animals receive balanced feed to ensure accelerated and healthy growth. Water quality is a factor that contributes to the health and zootechnical performance of farmed animals. Preventing the emergence of diseases (Boyd 2017).

The manipulation of antibiotics in the treatment of diseases in shrimp farming is ordinary, but the indiscriminate use leads to the emergence of resistant bacterial strains, this has stimulated the study of more effective therapeutic and prophylactic measures, environmentally correct and that do not bring harm to human health, such as use of plants, nanoparticles, and probiotics, which cause low side effects (Aghamohammad & Rohani 2023).

Between the bacteria, *Vibrio* is Gram-negative genus, naturally found in marine environments, some microorganisms belonging to this genus are considered pathogens of the marine shrimp *Litopenaeus vannamei*, so they are responsible for diseases in shrimp farms (Ji et al. 2020). Outbreaks of vibriosis are commonly noticed in cultivation, caused by imbalance in the population of these microorganisms. The species *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* are frequently

associated with diseases in shrimp farming (Yin et al. 2022).

The use of beneficial bacteria has been recognized as a possibility to abandon the use of antibiotics in cultures, that is why, the use of these microbial products has grown more and more as a disease management strategy (Gatesoupe 1999). The same author defined probiotics as live microorganisms that, when administered, colonize the intestinal tract of the host, improving the health of cultured animals. Benefits include modifying the host's bacterial community or environment, as well as enhancing the host's immune response against disease (Verschuere et al. 2000).

The use of probiotics is a promising prevention method developed to oppose diseases, based on the principle of exclusion by competition and/or acting as immunostimulants (Fuller 1992). New products are routinely formulated. Probiotic strains are adapted, through natural processes, for use in aquaculture, reaching the product reach the desired effect (Kesarcodi-Watson et al. 2008). The use of probiotics during cultivation is useful to reduce the negative impacts caused by chemicals and improve water quality (Hlordzi et al. 2020).

However, bacteria of the genus *Bacillus* belong to the Bacillaceae family. They are effective in producing enzymes that degrade many organic substrates (Madigan et al. 2002). They are the main Gram-positive bacteria tested for use in aquaculture as probiotics (Rengpipat et al. 1998). Being able to produce antibiotic substances during sporulation, we can highlight bacitracins, polymyxin, tyrocidin, gramicidin and circulin (Madigan et al. 2004). Species of this genus are widely used as probiotics in shrimp farming and in commercial formulations (Vieira et al. 2013). These products have been very effective in preventing disease using a variable number of *Bacillus* species.

The main objective of this study was to investigate the viability and efficiency of bacteria isolated from the digestive tract of healthy crustaceans as probiotic agents in the cultivation of shrimp *Penaeus vannamei*.

MATERIALS AND METHODS

Origin of strains

The isolation of *Bacillus* spp. was made from the digestive tract (DT) of wild crustaceans (soft crab, crab, and shrimp) collected in the estuary of the Pacoti River (Ceará). The animals were visually inspected for signs of disease, and soon after, they were stunned in an ice bath and sacrificed for intestinal dissection. The organs of each animal were processed separately, homogenized, and diluted in 0.85% (w/v) saline solution. The samples were conditioned at a temperature of 70ºC for 1h and then plated on Nutrient Agar medium containing 1.5% (w/v) sodium chloride and incubated in an oven at 30ºC for 48 h. The characteristic *Bacillus* colonies were isolated, and the form and purity of the cultures confirmed by microscopy, after the Gram staining technique.

Extraction, amplification and sequencing of cultures

The total DNA of the strains was extracted using the Wizard® Genomic Purification Kit (Promega) and following the protocol recommended by the manufacturer. Amplification of the 16S rRNA gene was performed using universal primers fD1 [5'-CAACAGAGT TTGATCCTGGCTCAG-3'] and rD1 [5'-GCTTAAGGAGGTGATCCAGCC-3'] (Weisburg et al. 1991) and amplification kit (INVITROGEN®), as recommended. Sequencing of the 16S rRNA gene was performed bidirectionally, using the modified *Sanger method* (Hillis et al. 1996). PCR products were purified with the Wizard®SV Gel and PCR Clean-Up Systems kit (PROMEGA®). The

series produced were examined from alignment with previously published sequences, available in the GenBank database of the National Center for Biotechnology Information. For alignment, the Clustal X program, version 1.83, was used.

Characterization of potential as probiotics in shrimp farming

Cultures were subjected tests to determine tolerance to different media conditions: temperatures (35, 40, 45 and 50°C); pH in the medium (3.0, 5.0, 7.0 and 9.0) (Cai et al. 1999); sodium chloride concentration: <0.5, 3, 5 and 10% (w/v) NaCl (Vieira et al. 2013). The cultures were characterized according to the susceptibility profile to the antimicrobials commonly used in aquaculture: Chloramphenicol 30 µg (CLO), Oxytetracycline 10 µg (OXI), Erythromycin 15 µg (ERI), Sulfazotrim 25 µg (SUT) and Ampicillin 10µg (AMP), following guidelines of Clinical & Laboratory Standards Institute (CLSI 2010). The enzymatic profiles of cultures related to virulence potential were also characterized. The production of elastase exoenzymes (Rust et al. 1994); gelatinase, caseinase, phospholipase lipase (Rodrigues et al. 1993); and β-hemolysis (Furniss et al. 1979) inoculating the cultures in media with a defined specific substrate for inducing enzyme production.

Tests for antagonistic activity against *Vibrio harveyi*

Cultures were exposed to a strain of *V. harveyi* to determine the antagonistic effect against a potential pathogen of farmed marine crustaceans. The tests were carried out using the Agar Plug Diffusion method, which consists of removing a circle of agar from a plate previously added with the test strain. This agar circle is then placed on the surface of the plate inoculated with the indicator strain. The result was seen by the formation of a halo from the slot with the *Bacillus* isolate (Durand et al. 2019).

Aggregation test

Cultures were tested for their ability to adhere to surfaces. For this, they were grown in test tubes with Tryptic Soy Both 1% (w/v) NaCl culture medium, incubated for 24 h at 35°C, and after discarding the bacterial culture, the tubes were washed with distilled water, exposed to 1% (w/v) safranin solution per one minute following the protocol described by Christensen et al. 1982.

In vivo tests

Based on the results of the *in vitro* tests, cultures were chosen for the challenge in culture with animals. Characteristics related to the reduced number of exoenzymes related to virulence, antagonism against the potential pathogen and ability to adhere to surfaces were considered prerequisites for the choice. Thus, four strains were selected to carry out the challenge test. The four cultures were tested together forming a probiotic bacterial consortium and one of them was tested alone as a monoculture probiotic.

Bioencapsulation of strains

The bacterial consortium and the probiotic monoculture strain were submitted to a bioencapsulation process to be offered to the shrimp. In this procedure, a solution of 120 mL of 1% (w/v) sodium alginate was kept in a magnetic stirrer for three hours, for the dissolution of the compound. Then, 80 mL of 1% (w/v) NaCl inoculated with the *Bacillus* spp. strain was added or bacterial consortium to be tested, at a concentration of 10^8 CFU mL^{-1}. This mixture was added to a 2% (w/v) calcium chloride solution with a manual sprinkler, so that there was micropearlization of the particles. The micropearls were placed overnight in the calcium chloride solution, then filtered and washed with

distilled water. After being lyophilized, they were placed in sterilized bottles and stored at room temperature.

After one week of storage, a viability test of the micropearlized probiotic strains was performed. Thus, 0.1g of the product was diluted and inoculated on the surface of Nutrient Agar 1.5% (w/v) NaCl medium and incubated in an oven at 35°C for 24 h. The grown colonies were fished and subjected to Gram staining and taken to the microscope to verify the characteristic morphology of the genus *Bacillus*.

Challenge test conditions

The experiment was carried in Center for Studies in Coastal Aquaculture (CEAC). Adult shrimp of the species *Penaeus vannamei* weighing 10 g, from the Laboratory of Aquatic Organism Nutrition - LANOA, were required.

The experimental design was completely randomized, being arranged in four experimental groups and a control group.

- Treatment A: control (no supplementation)
- Treatment B: a bacterial strain
- Treatment C: a bacterial mix
- Treatment D: a bacterial strain bioencapsulated in sodium alginate
- Treatment E: a bacterial mix bioencapsulated in sodium alginate

In the control group, commercial feed was prepared with 35% crude protein and 2% soybean oil was added. For the other treatment groups, the introduction of the probiotic bacterial strains/mix was done by sprinkling the material diluted in saline solution on the commercial feed with 35% crude protein. The diets were placed in an oven at 50°C until complete drying, adding 2% soybean oil and then stored in closed containers and stored at room temperature.

Experimental protocol

The animals were stored individually in 4.5 L tanks, composing a biorehears system, with a daily water change of 50% of the volume to clean feed remains, feces and seedlings. The diet was offered *ad libitum* to the shrimp three times a day (8:00 am, 11:00 am and 2:00 pm) for 30 days. The animals were weighed and measured every 10 days. At the end of the cultivation cycle, an inoculum of the pathogen *Vibrio harveyi* in 1% NaCl was added to the cultivation water at a concentration of 10^8 CFU/mL. Shrimp were monitored for 24 h for morbidity and mortality. Quantitative analyzes of Total Cultivable Heterotrophic Bacteria (TCHB), *Vibrio* and sporogenic *Bacillus* in the intestine and intestinal contents of shrimp were performed on shrimp before and after exposure to the pathogen.

Quantification of bacteria

The animals were stunned by immersion in ice water, dissected and the intestines removed. The intestinal contents were separated from the tissues by scraping. The intestine tissue was externally washed with 70% alcohol and immersed in sterilized distilled water to eliminate alcohol residues and then macerated. Samples were serially diluted in 1% NaCl.

For the quantification of TCHB, aliquots of the dilutions were plated on the surface of the Nutrient Agar culture medium (1% NaCl) by spreading. Afterwards, the plates were incubated in an oven at 35°C for 48 h. and TCHB CFU counted. After counting, the plates were rinsed with sterile 1% (w/v) NaCl, the colonies disaggregated from the culture medium with the aid of a Drigalsky loop and the material collected in test tubes. They were incubated at 70°C for one hour. After this period, serial dilutions were made in 1% (w/v) NaCl saline solution and aliquots inoculated in 1.5% (w/v) NaCl Nutrient Agar medium using the pour plate

technique, and the plates were incubated in an oven at 35°C for 48 h.

The count of bacteria of the genus *Vibrio* was performed by inoculating sample aliquots on the surface of the selective medium Agar Thiosulfate-citrate-bile-sucrose (TCBS), using the Spread Plate technique. The inoculated plates were incubated in an oven at 35°C for 24 h. All analyzes were performed in duplicate and counts were performed using a counter (model PHOENIX EC550A).

Statistical analysis

The results were statistically evaluated using analysis of variance (ANOVA) for completely randomized experiments. When there was a statistically significant difference ($p<0.05$) between treatments, their means were compared two by two, using Tukey's test. The significance level adopted was 5%. For the statistical analysis, the following software was used: BioEstat 5.0® and Excel 2010 (Microsoft).

RESULTS AND DISCUSSION

The eighteen (18) strains isolated from the digestive tracts (DT) of native crustaceans are: eight (8) from crabs, six (6) from shrimps and four (4) from soft crabs. All isolates were confirmed as belonging to the genus *Bacillus*, of which eight (8) *Bacillus* spp.; four (4) *B. cereus*; two (2) *B. thuringiensis*; one (1) *Bacillus subtilis*; one (1) *B. megaterium*; one (1) *B. circulans* and one (1) *B. agaridevorans* (Table I). For a probiotic consumed in aquaculture to be efficient, it is necessary that the microorganism in question comes from the animal in question (Balcazar et al. 2006), as in this research, in which the strains used as probiotics for shrimp farming were isolated from marine crustaceans.

Table I. Molecular identification of *Bacillus* spp. isolated from soft crab, crab and shrimp samples.

Origin	Code. Strain	molecular identification	Max. ID	Temperature				pH				NaCl			
				35°	40°	45°	50°	3	5	7	9	0%	3%	5%	10%
Soft Crab	09	*Bacillus* sp.	99.0%	+	+	+	+	-	+	+	+	+	+	+	-
	12	*Bacillus* sp.	94.1%	+	+	+	+	-	+	+	-	+	+	+	-
	13	*B. subtilis*	98.5%	+	+	+	+	-	+	+	-	+	+	+	-
	14	*Bacillus* sp.	99.0%	+	+	+	+	-	+	+	-	+	+	+	+
Crab	20	*Bacillus* sp.	92.6%	+	+	+	+	-	-	+	+	+	+	+	-
	25	*B.* cereus	97.7%	+	+	+	+	-	+	+	+	+	+	+	-
	26	*Bacillus* sp.	99.9%	+	+	+	+	-	+	+	+	+	+	+	-
	27	*B. megaterium*	95.8%	+	+	+	+	-	-	+	+	+	+	+	-
	28	*Bacillus* sp.	99.0%	+	+	+	+	+	+	+	+	+	+	+	-
	29	*Bacillus cereus*	98.9%	+	+	+	+	-	+	+	+	+	+	+	+
	30	*Bacillus* sp.	99.2%	+	+	+	+	-	+	-	-	+	+	+	-
	31	*Bacillus* sp.	98.3%	+	+	+	+	-	+	+	+	+	+	+	-
Shrimp	38	*B. cereus*	99.0%	+	+	+	+	-	+	+	+	+	+	+	-
	39	*B. thuringiensis*	99.6%	+	+	+	+	-	+	+	+	+	+	+	-
	40	*B. thuringiensis*	97.9%	+	+	+	-	-	+	+	+	+	+	+	-
	41	*B. cereus*	94.1%	+	+	-	-	-	+	+	+	+	+	+	+
	45	*B. circulans*	99.3%	+	+	+	-	-	+	+	-	+	+	+	-
	46	*Paenibacillus agaridevorans*	97.5%	+	+	+	-	-	+	+	+	+	+	+	-

Bacillus bacteria are identified in an abundance of environments, with environmental, clinical, and industrial significance. The strains found in this study are easily detected in the digestive tract of crustaceans, this happens due to the ingestion of food containing this microorganism. There are several studies of bacterial species of the genus *Bacillus* being isolated from fish and crustaceans (Gatesoupe 1999, Wang et al. 2022, Kim et al. 2020). This genus is widely used as a probiotic in aquaculture, as it improves growth, immune response and is stronger against diseases (Kuebutornye et al. 2019, Ji et al. 2022). A species widely used as a probiotic in aquaculture is *Bacillus subtilis*, which was also identified in this research (Hai 2015, He et al. 2023).

The species *Penaeus vannamei* is cultivated in a tropical climate (Kir et al. 2023), the temperature is high throughout the year, with occasional changes. Feed production can occur at high temperatures, if the probiotic bacteria are insert at this time, it must be able to survive the process. The result of the temperature analysis (Table I) reveals that most of the strains showed to be tolerant to the variation of this parameter. At the temperature of 35°C and 40°C, there was growth in all strains, after these values the bacterial growth began to be impaired. At 45°C one strain did not grow and at 50°C four strains did not grow.

Another parameter that deserves attention is the pH, in shrimp cultures it is common to oscillate the pH in the water, this change can happen for several reasons, from the excretion of the cultivated organisms to the variety of microalgae in the nurseries (Huang et al. 2022). It is important to mention that for a bacterium to be considered probiotic, it must be able to adapt to different circumstances (Wang et al. 2019), including pH changes that occur during digestion. At pH 3 only one strain grew, at pH 5 growth was obtained in 16 strains, at pH 7 there was growth in 17 strains, while at pH 9 growth decreased to 13 strains. With these results, it can be concluded that some of these strains tolerate a pH variation favorable to probiotic formulation (Fernandes et al. 2019).

Other indispensable element for shrimp farming is salinity, as the shrimp *Penaeus vannamei* is naturally marine, and because of its osmoregulatory capacity tolerates salinity differences, the ideal is the bacteria probiotic have this characteristic. In commercial cultives, variations in salinity may occur, this will depend on the region in which the cultivation is being grown, the time of year, rainfall and evaporation rate. The development of *Bacillus* species in concentrations of 0%, 3%, 5% and 10% of NaCl was investigated. At 0%, 3% and 5% NaCl levels all tested strains achieved growth. The concentration of 10% NaCl was unhealthy to the growth of probiotic bacteria. Because of this result, it can be observed that these bacteria support large variations in salinity, a desired characteristic for production of probiotics for marine shrimp (Fernandes et al. 2019).

The results of antagonism against *V. harveyi*, aggregation test, antimicrobial susceptibility profile and enzyme profile are mentioned in Table II. For a probiotic strain to be considered safe, it is necessary to know the genus and species, perform *in vitro* tests, such as antimicrobial activity against pathogenic bacteria, adhesion capacity and in vivo studies that evaluate the action of probiotics (Chaudhari et al. 2022). Among the tests carried out *in vitro* are the pathogen inhibition tests, also known as the antagonism test and the aggregation test.

The inhibition factor of bacteria of the genus *Bacillus* was tested against *Vibrio harveyi*, which is a common pathogen found in shrimp farming. For the test of antagonism performed through the agar plug technique, the following

Table II. Intrinsic characteristics of probiotic candidate bacterial isolates.

				Antibiogram			enzyme profile
Origin	**Strains**	***Antagonism against V. harveyi***	**Aggregation**	**R**	**I**	**susceptible**	**Enzymes**
Soft Crab	9	-	- - -	SUT		ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
	12	+	- - -	SUT		ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
	13	+	- - -	SUT		ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
	14	-	- - -		SUT	ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
Crab	20	-	- - -			ERI, SUT, CLO, AMP, OXI	Cas, Phosph, β-Hem
	25	+	- - -	SUT	CLO	ERI, AMP, OXI	Cas, Phosph, β-Hem
	26	-	+ + -			ERI, SUT, CLO, AMP, OXI	Cas, Phosph, β-Hem
	27	-	+ + +			ERI, SUT, CLO, AMP, OXI	Gel, Cas, Phosph, β-Hem
	28	+	- - -	SUT		ERI, CLO, AMP, OXI	Gel, Cas, Phosph, β-Hem
	29	+	+ + +	SUT		ERI, CLO, AMP, OXI	Gel, Cas, Phosph, β-Hem
	30	+	- - -			ERI, SUT, CLO, AMP, OXI	Gel, Cas, Phosph, β-Hem
	31	+	+ + +		SUT	ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
Shrimp	38	+	+ + +		SUT	ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
	39	+	- - -	SUT		ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
	40	+	+ + +		SUT	ERI, CLO, AMP, OXI	Cas, Phosph, β-Hem
	41	-	- - -			ERI, SUT, CLO, AMP, OXI	Cas, Phosph, β-Hem
	45	-	+ + +			ERI, SUT, CLO, AMP, OXI	Cas, Phosph, β-Hem
	46	-	- - -		SUT	ERI, CLO, AMP, OXI	Elas, Cas, Phosph, β-Hem

***ERI: erythromycin; CLO: chloramphenicol; SUT:Sulfazotrim; AMP: ampicillin; OXI: oxytetracycline.**
***Cas: caseinase; Phosph: phospholipase; β-Hem: β-hemolysis; Gel: gelatinase; Elas: elastase.**

results were found: ten strains of *Bacillus* sp. showed the ability to prevent the growth of the pathogen through the appearance of inhibition halos, against eight strains that do not have this ability.

Studies carried *in vitro*, through inhibition halos, report the ability of the genus *Bacillus* to inhibit pathogenic species of the genus *Vibrio*. Girija et al. 2018, demonstrated that *Bacillus licheniformisis* able to inhibit the growth of *V. parahaemolyticus*. Another research carried out with *B. safensis*, proved strong antagonistic activity of the probiotic bacteria against *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus* and *V. alginolyticus*, with good adaptation to different environmental conditions and safety for potential use (Huynh-Phuoc et al. 2022).

In vitro antagonism against specific pathogens is an important tool for selecting probiotic candidates for use in aquaculture, the antimicrobial compounds of probiotic bacteria are responsible for this antagonistic action (Wang et al. 2019).

Biofilm verification, through the glass aggregation test, of bacteria of the genus *Bacillus* demonstrates their ability to adhere to the intestine of crustaceans. This feature is very useful, it also indicates the ability of microorganisms to survive in different types of environments (Chaudhari et al. 2022). Of the eighteen strains tested, seven were positive for the aggregation test, demonstrating possible colonization of the intestinal tract of crustaceans and their potential as probiotics.

The determination of antimicrobial susceptibility profiles of the *Bacillus* strains that were tested revealed that for the antimicrobials: erythromycin, ampicillin and oxytetracycline, 100% of the strains were sensitive. Using chloramphenicol, 94.44% of the strains were sensitive and 5.56% of the strains had an intermediate result. The results for the antimicrobial sulfazotrim were the most diverse: 38.89% resistant, 33.34% sensitive and 27.77% intermediate.

In a study carried out with 114 strains of *Bacillus*, resistance to antimicrobials was found: penicillin (98), ampicillin (91), sulfazotrim (10), clindamycin (8), erythromycin (2) and tetracycline (1) (Zhai et al. 2022). Because due to the accelerated bacterial growth, the acquisition of random genetic mutations is favored, which results in resistance to an antimicrobial and a competitive advantage. By testing antimicrobial susceptibility profiles, it is possible to screen and identify antibiotic-resistant strains so that this resistance gene is not transmitted and this resistance to pathogens does not spread. Environments that facilitate the spread of resistant bacteria also allow the dissemination of opportunistic pathogens (Bengtsson-Palme et al. 2017).

The results of the discovery of potential virulence factors among *Bacillus* strains isolated from the digestive tract of crustaceans, prove that in terms of occurrence, 100% of *Bacillus* sp. analyzed have caseinase and β-hemolysis enzyme activity, followed by 94.44% for phospholipase, 22.23% for gelatinase, 5.56% for elastase and none of the strains in the study in question have lipase enzyme activity. The number of strains that have exoenzyme activity is worrying, as these are indications of potential pathogenicity and a possible increase in the chances of infection by the microorganism (Silva et al. 2018).

In a study carried out using *B. licheniformis* and *B. subtilis* with probiotic activity against the pathogens *Aeromonas hydrophila* and *A. veronii*, it was possible to conclude that probiotic strains need to have good antibacterial activity and sensitivity to antibiotics, good tolerance to high temperatures, pH, gastric juices, intestinal juices and bile salts, ability to aggregate and adhere to the host's intestine and inhibition against pathogenic bacteria, to be used in the prevention of diseases caused by aquatic pathogens (Wang et al. 2022). Results similar to those found in this study.

After carrying out the *in vitro* characterization tests, followed by *in vivo* testing. Cultivation started with animals weighing around 10 g (initial weights statistically equal between treatments). After 30 days of cultivation, the shrimp achieved weight gain growth as shown in Table III.

Analyzing the results there was no statistically significant difference between treatments in terms of final weight. In treatment C, which used a bacterial sprinkling mix, there was no statistically significant difference between initial weight and final weight. The lowest survival rate was found in treatment E, which also used a microencapsulated bacterial mix. It is possible to conclude that the strains chosen for the bacterial consortium do not show efficiency for weight gain and survival when used together.

In treatment D, the highest survival and good zootechnical performance were found, these results suggest that microencapsulation is responsible for this benefit. Through the microencapsulation of probiotics, it is possible to protect the probiotic microorganisms during the stages of production, preservation and in the digestive tract, giving it the expected effectiveness (Vivek et al. 2023).

Just as this study proves the genus *Bacillus* with probiotic potential, other research confirms,

Table III. Initial weight, final weight and survival of shrimp fed with ration supplemented with probiotic.

Treatments	Initial Weight (g)	Final Weight (g)	Survival (%)
A	10.00±0.27 [a]	12.05±0.26 [b]	84.62
B	10.16±0.14 [a]	12.08±0.09 [b]	80.00
C	10.01±0.18 [aA]	11.82±0.24 [bA]	80.00
D	10.05±0.20 [a]	12.04±0.23 [b]	86.67
E	10.08±0.25 [a]	11.94±0.18 [b]	60.00

Equal letters mean they are statistically equal.

as in this study of probiotic supplementation using the species *B. coagulans*, confirmed that the concentration of 1 x 10^8 CFU.g^{-1} can promote benefits for the health of the shrimp, improves the intestinal microbiota, immune response, and digestive enzyme activity (Amoah et al. 2019). Shao et al. 2023 concluded that adding *B. cereus* LS2 strains to the diet can improve growth performance, improve innate immunity, optimize gut microbiota structure, and increase resistance to infection pathogens by *A. japonicus*.

In another study, in which strains of *Bacillus subtilis* were isolated from wild *Penaeus indicus*, it was observed that these probiotic bacteria could produce secondary metabolites with antibacterial activity against several pathogenic bacteria. And *when Penaeus indicus* was fed a probiotic diet, it increased the growth rate of the shrimp, explained by the fact that the probiotic bacteria are involved in the synthesis of various enzymes and increase the digestibility of the shrimp feed (Kim et al. 2020).

The genus in question can also benefit to fish against other pathogens, as in this study carried out with the fish *Labeo rohita* confirmed that the use of *Bacillus amyloliquefaciens* against the pathogen *Aeromonas hydrophila*, positively modulated immuno-biochemical responses and immune-related gene expression after 30 days of supplementation (Khan et al. 2022). Another study, indicates that immersing shrimp with 3.4 × 10^6 CFU/mL of *B. velezensis* for 15 days can increase its resistance to *A. salmonicida*, causing a protective effect against infection.

In Figure 1, data on Standard Plate Counts of *Vibrio* and *Bacillus* in the intestine and intestinal contents, respectively, of shrimp after being fed for thirty days with a diet supplemented with *Bacillus* sp., probiotic bacteria. The count was performed before and after an infection in the culture system with the shrimp pathogen *Vibrio harveyi*.

The concentration of bacteria of the genus *Bacillus* before infection was higher than the concentration of bacteria of the genus *Vibrio*, this calculation was expected, since the treatments (except the control) received supplementation of *Bacillus* sp., probiotic bacteria in the feed. Test treatments demonstrate *Bacillus* sp. superior to the control, in which there was no supplementation with the probiotic bacteria. In treatment C, the highest count of Bacillus sp. and the lowest *Vibrio* count.

Before infection, the highest number of *Vibrio* was found in treatment E. Treatments C and E received the same strains, however, administered differently. Probably, this way of administering the strains in the feed contributed to the result. The period of action of the treatments that used aspersion was faster than the treatments that used sodium alginate, this was concluded by comparing treatments B and C with treatments D and E, the bacteria of the genus *Vibrio* developed faster than *Bacillus* sp.

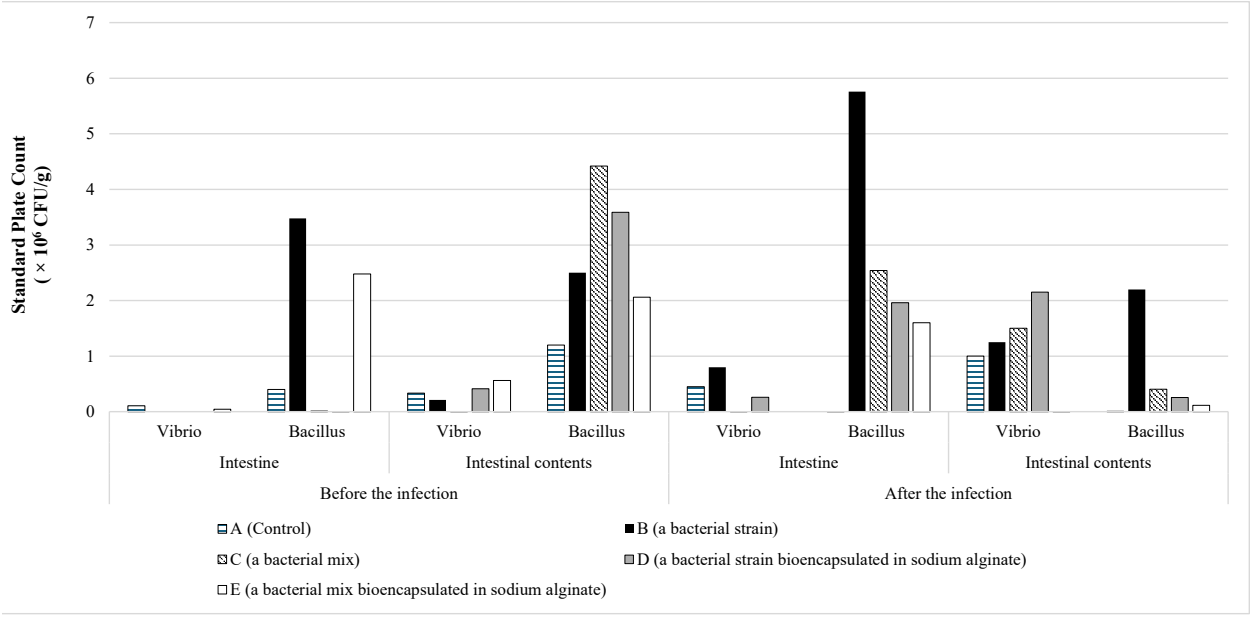

Figure 1. Prevalence, antimicrobial susceptibility, and antibiotic resistance gene transfer of *Bacillus* strains isolated from pasteurized milk Standard Plate Counts of Vibrio and Bacillus in the intestine and intestinal contents.

After the infection, the *Vibrio* count increased in the treatments, because to the inoculation of *Vibrio harveyi*. On the other hand, there was also a significant increase in *Bacillus* sp. adhered to the intestine, in all treatments, with emphasis on treatment B.

The main function of the gastrointestinal system is to digest food, but it is responsible for other important functions, such as creating a protective barrier by constructing physical, chemical, microbial, and immune activities to guard the pathophysiological homeostasis of the body (Chelakkot et al. 2018). Therefore, intestinal health is linked to the balance of the intestinal microbiota.

Probiotic and pathogenic bacteria compete for nutrients and adhesion sites on the intestine of cultured organisms, but probiotics can prevent the attachment and survival of pathogens (Acunha et al. 2023). Adhesion of probiotics to the intestine produces defensive substances against pathogens (Araujo et al. 2017). In this study, the antagonistic activity of probiotic strains against Gram-negative bacteria can be verified. This inhibition occurs mainly through the production of compounds by probiotic bacteria capable of inhibiting the growth of Gram-negative bacteria.

Probiotics can be used preventively to reduce the impacts caused by vibriosis, as they act to improve growth, antioxidant status, immunity and disease resistance in fish and shrimp. These products will modulate shrimp immunity to increase resistance to pathogens (Abdel-Latif et al. 2022). The probiotic efficacy of *B. subtilis* can be improved through encapsulation in alginate and coating with chitosan, in relation to the non-encapsulated probiotic or only encapsulated with alginate. Maintaining the probiotic viability of microorganisms under conditions like the digestive tract. Furthermore, it increases the shelf life of the probiotic relative to non-encapsulated probiotic (Adilah et al. 2022).

Excessive and incorrect use of antibiotics can generate bacteria resistant to these chemical compounds, in addition to causing

an imbalance in the natural microbiota of the water and intestine of farmed animals, which can increase the likelihood of the host being infected in the future (Banerjee et al. 2017).

Although probiotics are used as an alternative to the use of antibiotics, care must be taken with the production of antibiotic substances due to the risk of transferring antibiotic resistance. For safety, probiotic candidates should be screened for evaluation in aquatic animal models (Wang et al. 2019). With the outcome found in this study, it can be suggested that complementary research be carried out on the probiotic potential of *Bacillus* sp. used in this experiment to control *Vibrio*, reducing the effects of this pathogen in shrimp farming.

CONCLUSION

It can be concluded that the strains found in this research have probiotic activity, according to the *in vitro* results of temperature, pH, salinity, antagonistic activity and aggregation test. In the *in vivo* test, treatments that used microorganisms of the *Bacillus* genus, there was a reduction in the growth of pathogenic bacteria after infection with *V. harveyi*, in the intestinal contents and intestine. And finally, treatments that used probiotic bacteria of the genus *Bacillus* bioencapsulated in sodium alginate, achieved less adhesion of pathogenic bacteria of the genus *Vibrio* in the intestine, with satisfactory zootechnical performance.

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