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Research Article

Isolation, characterization, application, and effect of bacteria with probiotic potential from the prawn *Macrobrachium tenellum* in Guasave, Sinaloa, Mexico

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ABSTRACT. The prawn *Macrobrachium tenellum* is a species with high expectations for farming. However, like all cultivated species, it is exposed to bacterial infections caused by captivity and cultivation conditions. In this sense, probiotics in aquaculture have been used as an alternative against the excessive use of antibiotics in farming, thus preventing diseases. In the present work, 106 strains with probiotic potential were isolated, of which 77 are bacilli, and 29 are lactic acid bacteria (LAB) [(intestine (43), stomach (42) and hepatopancreas (21)] from the shrimp *M. tenellum* collected from a freshwater environment in Guasave, Sinaloa. Biochemical characterization of the isolated bacterial strains and molecular identification of the 16S ribosomal gene were performed. According to the hemolytic activity, the isolates MT4H2 (bacilli) and MT1E2 (LAB) showed gamma hemolysis (ɣ), and the LAB strain presented catalase-negative. Both were Gram (+). They presented high hydrophobicity values (99.93 and 73.21%) in autoaggregation (90%) and coaggregation (99%). Regarding biofilm formation, both presented moderate capacity. On salinity tolerance, they grew in concentrations of 0 to 9% NaCl and a pH tolerance within a range of 5 to 9. Regarding enzyme activity, they exhibited activity in both proteases and lipases. Both presented resistance to some of the antibiotics tested. Only the strain of bacillus exhibited antagonistic activity against *Vibrio parahaemolyticus*. They were identified as *Pontibacillus* sp. and *Pediococcus pentosaceus* at a molecular level.

Keywords: *Macrobrachium tenellum*; prawn; probiotic bacteria; bacillus; lactic acid bacteria

INTRODUCTION

Animal production systems have various challenges associated with bacterial and viral diseases; one way to solve them is by effectively managing factors such as water quality, soil, food, and environmental parameters (Ringø et al. 2014). The prawn *Macrobrachium tenellum* is a species with very good expectations of farming. However, like all cultivated species, it is exposed to bacterial infections caused by its captivity and cultivation conditions (Guzmán-Arroyo 1987, Ponce-Palafox et al. 2006). Nowadays, diseases caused by bacteria occur more frequently, increasing their incidence and impact, leading to global economic losses in production (Bradley-Dunlop et al. 2004, Aguirre-Guzmán et al. 2005). They are traditionally controlled by the application of antibiotics in the feeding diets; however, this practice produces adverse

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effects on the environment and human health, and over time the bacteria causing the disease may generate resistance to the antibiotic (Hansen & Olafsen 1999, Lambert et al. 1999, Alabi 2000). In this sense, probiotics in aquaculture are applied as an alternative against the excessive use of antibiotics in the culture, thus preventing diseases. Interest has been developed in the application of probiotics due to the beneficial effects obtained by their utilization, reflected both in the state of health and growth rate of the organisms, as well as in the quality of the water and the biological control of infectious diseases (Rodríguez et al. 2007). Probiotic bacteria are living microorganisms that benefit the organism to which they are supplied, causing modifications in the gastrointestinal microbiota and generating effects such as disease resistance (Luis-Villaseñor et al. 2011).

Among the bacteria mainly used as probiotics in aquaculture are lactic acid bacteria and bacillus. However, there are other genera, such as *Aeromonas*, *Alteromonas*, *Arthrobacter*, *Bifidobacterium*, *Clostridium*, *Paenibacillus*, *Phaeobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Rhodosporidium*, *Vibrio* and yeast (Ringø 2020).

One of the most important properties of probiotic bacteria is the ability to cross the digestive barrier, allowing them to multiply and colonize the intestine and possess antimicrobial activity to produce a barrier against pathogens. Another characteristic is adhesion, an important factor in colonization (Gómez & Balcázar 2008). According to the above, it is important to know those bacteria that meet the mentioned criteria, so this study aimed to isolate and characterize, at a biochemical and molecular level, the probiotic bacteria of the prawn *M. tenellum* in brackish and freshwater environments in Guasave, Sinaloa, Mexico and its possible application in aquaculture, evaluating the effect on the growth and survival of *M. tenellum*.

MATERIALS AND METHODS

Dissection of organisms

A dissection of the intestine, stomach, and hepatopancreas of prawns *M. tenellum* was performed. Samples were removed aseptically, placed in Eppendorf tubes of 1.5 mL with 400 µL of sterile distilled water, and homogenized with a sterile pestle for 5 min. The volume was then adjusted to 1 mL.

Presumptive isolations of bacilli and lactic acid bacteria

A total of 100 µL of bacilli were taken from each homogenized mix, heated to 80ºC for 10 min, and inoculated by spreading 100 µL on plates with trypticase soy agar (TSA agar, BD, Bioxon™, Sparks, MD, USA). The lactic acid bacteria (LAB) was inoculated in plates with de Man, Rogosa, and Sharpe agar (MRS; BD Difco™, Sparks, MD, USA) and incubated at 32°C for 24 and 48 h, respectively. Each of the resulting colonies was purified by the cross-streak method and were inoculated again by spreading them in Petri dishes with TSA agar (bacilli) and MRS agar (LAB) and incubated at 32°C for 24 and 48 h. Bacteria were harvested and placed in Eppendorf tubes with 1 mL of TSA and MRS broth with 15% (v/v) of glycerol. The isolates were kept at -80°C (stock) for further characterization.

Biochemical characterization of presumptive isolates

Hemolysis test

Each isolate was cultivated in TSA broth (bacilli at 32°C for 24 h) and MRS broth (LAB, at 32°C for 48 h) and was centrifuged at 10,000 *g* for 20 min. The pH of each bacillus and LAB supernatant was adjusted within a range of 6.5 to 7 with NaOH (1 M) to avoid false positive halos (Balcázar et al. 2008), 50 µL of supernatant were inoculated in pots of 8 mm in diameter in Petri plates with blood agar (BA, BD Bioxon™, Estado de México, Mexico) in duplicate. The plates were incubated at 32°C for 24 h. The lysis of the halo was observed to determine the type of hemolysis (β, α y ɤ), and those isolates with gamma hemolysis (γ) were used for the characterization.

Catalase test

From each pure colony of each presumptive LAB isolate, one drop of H_2O_2 (3%) was added to a slide. Those colonies that did not show bubble formation (negative result) were used in the characterization as possible probiotics.

Morphology, cellular arrangement, and Gram staining

The cellular morphology of the isolates was conducted with a commercial kit (Golden Bell™, Zapopan, Jalisco, Mexico). The isolates' shape and arrangement were determined using a compound microscope (Carl Zeiss™) with a 100x lens.

Microbial adhesions to solvents

Microbial adhesion to solvents (ϐ-Xylene and ethyl acetate) was measured according to the method proposed by Rosenberg et al. (1980), with modifications by Crow & Gopal (1995). Bacteria were harvested during the stationary growth phase by

centrifugation at 5000 *g* for 20 min. The biomass was washed out twice and resuspended in PBS buffer (NaCl, 137 Mm; KCl, 2.7 Mm; Na_2HPO_4, o Na_2HPO_4 Mm; pH, 7.4) with approximately 1×10^6 CFU mL^{-1}. The absorbance of cell suspension was measured at 600 nm (A0) with a spectrophotometer (PerkinElmer UV/VIS Spectrometer Lambda 25™), and for each bacterial suspension added, 1mL of β-Xylene and 3 mL of ethyl acetate. The solution was incubated for 10 min at room temperature, and a two-phase system was obtained, which was mixed in a vortex (Vortex-Genie 2™, Scientific Industries) for 2 min. After 20 min of incubation at room temperature, the aqueous phase was removed, and the absorbance was measured at 600 nm (A1). The percentage of bacterial adhesion was calculated with the equation (1 - A1 / A0) × 100. The isolates with low adhesion to β-Xylene were discarded as possible probiotics.

Autoaggregation and coaggregation

Both selected isolates in the adhesion of solvents were tested for the capacity of autoaggregation and coaggregation according to the methodologies of Handley et al. (1987), Del Re et al. (2000), and Kos et al. (2003). The isolates were inoculated in TS and MRS broth at 32°C for 24 and 48 h. Bacteria were centrifuged at 3900 *g* for 20 min; the cell drop obtained was washed twice and resuspended in PBS (NaCl, 137 Mm; KCl, 2.7 Mm; Na_2HPO_4, o Na_2HPO_4 Mm; pH, 7.4) to get 10^6 CFU mL^{-1}. The bacterial suspension (4 mL) was mixed with a vortex for 10 s and was incubated at room temperature for 5 h. Each hour a total of 0.1 mL of the bacterial suspension was transferred to a tube with 3.9 mL of PBS, and the absorbance (A) was measured with a spectrophotometer (PerkinElmer UV/VIS Spectrometer Lambda 25™) at 600 nm. PBS was used as blank. The percentage of autoaggregation was expressed as (1 - (At/A0) × 100), where At represented the absorbance in time t = 1, 2, 5 h and A0 the absorbance in t = 0.

Strains were inoculated in TSA and MRS medium. They were incubated at 32°C for 24 and 48 h to determine coaggregation. The pathogenic strain of *Vibrio parahaemolyticus* IPNGS16 (López-León et al. 2016) was inoculated in TSA broth, incubated for 18 h at 30°C and was carried out in the same way as the technique of autoaggregation.

Biofilm formation

Biofilm was determined for both isolates according to Knobloch et al. (2001) and Mandhi et al. (2010). The isolates were cultivated in TS and MRS broth and were incubated at 32°C for 24 and 48 h. The bacterial absorbance was measured at 595 nm with a spectrophotometer (PerkinElmer UV/VIS Spectrometer Lambda 25™). A dilution of 1:100 of glucose at 2% was made from the bacterial culture that grew during the night in TSA and MRS; 200 µL were placed on microtitration plates with 6 U-bottom wells, and 20 µL from the previous suspension were inoculated. Each strain was made in triplicate. The plates were incubated aerobically at 32ºC for 24 h. The culture medium was eliminated, and the wells were washed out twice with PBS (NaCl, 137 Mm; KCl, 2.7 Mm; Na_2HPO_4 Mm; pH, 7.4) to eliminate the non-adherent cells and were dried inverted. The adherent bacteria were fixed with ethanol at 95% and were stained with 100 µL of crystal violet (Golden Bell™, Zapopan, Jalisco, Mexico) for 5 min. Wells were washed out thrice with 300 µL of sterile distilled water and air-dried. The absorbance was measured at 595 nm. The capacity of adhesion was measured by Optical Density (OD ≥ 1), as high, (0.1 ≤ OD595 < 1) moderate or (OD595 < 0.1) low.

Kinetics of bacterial growth

To determine the logarithmic phase in the growth of bacteria, 20 µL of stock from each isolate was inoculated in 50 mL of TSA medium (bacilli) and MRS medium (LAB), incubating at 32°C. The absorbance of the cultures with respect to control (TSA and MRS medium) was read at 580 nm with a spectrophotometer (Perkin Elmer UV/VIS Spectrometer Lambda 25™) to register growth. The readings were done at 0, 3, 6, 9, 12, 24, 48, 72, and 96 h.

Count of CFU mL^{-1}

The decimal serial dilution method was used to count the bacteria. Each bacterial isolate grew according to the result of the kinetic bacterial growth. Each culture was centrifuged at 13,000 *g* for 20 min. The capsule was washed out twice with 1 mL of sterile distilled water, and the absorbance was read at 580 nm with the spectrophotometer. It was adjusted to 1, by adding a higher amount of cells or by diluting it in sterile distilled water. A total of 100 µL were taken and inoculated by spreading them in Petri dishes with TS and MRS, and the inoculation was made in triplicate.

Salinity tolerance

Different concentrations of NaCl (0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12%) were placed in falcon tubes (15 mL) with 10 mL of TSA and MRS broth. A total of 20 µL of bacillus and LAB isolates were inoculated and incubated at 32ºC for 24 and 48 h, and 200 µL of each culture were placed in plates with 96 U-bottom wells.

TSA and MRS medium were used as blank and was made in triplicate. Absorbance was measured at 550 nm with a spectrophotometer Multiskan GO™ with microplates (Thermo Scientific™, North Carolina, USA).

pH tolerance

Isolates grew in TS and MRS broths with pH of 5, 6, 7, 8 and 9, and the medium was adjusted by adding HCl or NaOH (1 mol L^{-1}). The bacterial cultures were incubated for 24 h at 32ºC, and 200 µL of each sample were placed in sterile plates with 96 U-bottom, and a sterile medium was used as blank. Absorbance was measured at 550 nm in the spectrophotometer.

Extracellular enzyme activity

The determination of extracellular enzyme activity of protease and lipase in isolates was made according to León et al. (2000). Bacteria were inoculated in TS and MRS broth, and the supernatants were obtained. Petri boxes were prepared with a basal medium (agar 1.5% and yeast extract 0.5%) and the additional enzyme substrate (skim milk 2%, (Fluka, Sigma-Aldrich™, Switzerland) gelatin 1% (Knox) for proteolytic activity and Tween 80 1% (Sigma-Aldrich™, Steinheim, Germany, for lipolytic activity), perforations (6 mm in diameter) were made homogeneously with a sterile drill. From each culture, 1 mL was taken and centrifuged at 10,000 *g* for 20 min; 50 µL of supernatant was obtained and inoculated in the wells. The plates were incubated at 32°C for 24 h.

Antibiotic resistance

The disk diffusion method analyzed antibiotic response and resistance (Bauer et al. 1996). The antibiotics tested were oxytetracycline (30 U), florfenicol (30 U), enrofloxacin (5 U), streptomycin (10 U), sulfamethoxazole (23.75 µg) and gentamycin (10 U).

Antagonism *vs. Vibrio parahaemolyticus*

The isolates were cultivated and incubated for 24 h at 30°C. The bacteria inoculated was *V. parahaemolyticus* IPNGS16 (Lopez-León et al. 2016), and it was incubated at 30°C for 18 h. The bacteria were subsequently adjusted at 1.0 absorbance, and 30 µL were taken from the *Vibrio* strain for mass spreading in TSA boxes, which were left to dry at room temperature. After this, 6 mm in diameter perforations were drilled, in which 60 µL of each possible isolated probiotic strain were inoculated (Fig. 1).

Molecular characterization (identification) 16S ribosomal gene

Bacterial DNA was extracted using the cetyltrimethylammonium bromide method (CTAB). Amplification of the 16S ribosomal DNA was made by PCR using the primers 16SF (5' AGAGTTTGATC CTGGCTCAG'3) and 16SR (5' CGGGAACGTATTC ACCG'3) 16SR (Edwards et al. 2002), which amplify a genome fragment of 1300 bp. PCR products were purified with a QIAquick PCR purification kit (Invitrogen™, Carlsbad, CA, USA). PCR-purified products were sent to LANGEBIO of CINVESTAV (Irapuato, Mexico) to be sequenced. The sequence analysis was made with the database GenBank from the National Center for Biotechnology Information (NCBI) with the BLAST program (Basic Local Alignment Search Tool) (Escamilla-Montes et al. 2015) (Fig. 1).

Experimental design

Preparation of freshwater bacteria strains for the microencapsulation process

The strain of *Pontibacillus* sp. was cultivated in a TSB broth (broth of TSB BD, Bioxon™, Mexico) for 24 h at 32°C; it was adjusted to an optical density of 1.0 (595 nm) in a spectrophotometer (Spectrophotometer PerkinElmer™ UV/VIS Lambda 25). Aliquots of 100 µL were taken to perform serial dilutions, of which they were cultivated in a Petri dish with TSA agar (TSA agar, BD, Bioxon™, Mexico) to determine the colony-forming units (CFU) per mL.

Microencapsulation of probiotics by ionic gelling technique

The microcapsules were prepared using the ionic gelation method with modifications to the protocol established by Calero et al. (2008) to formulate empty particles. An aqueous sodium 2% p/v solution was kept in high magnetic agitation until a dispersed solution was obtained. Soybean vegetable oil was added to the above solution until a final concentration of 1.5% v/v (98.5% sol. sodium alginate and 1.5% vegetable oil) was reached. The sodium alginate solution + the vegetable oil was emulsified by sonication (ultrasound) at an amplitude of 60% for 1:30 min, and previously cultivated bacteria (12×10^6 CFU mL^{-1}) were added to integrate with the above solution for 30 s of emulsification. During the sonication process, the samples were kept in ice to prevent bacterial degradation by heat.

A solution of sodium alginate + vegetable oil and bacteria was added to a 5% p/v calcium chloride solution adjusted to a pH 4 with HCl at 1M, keeping it under magnetic stirring. During this stage, calcium

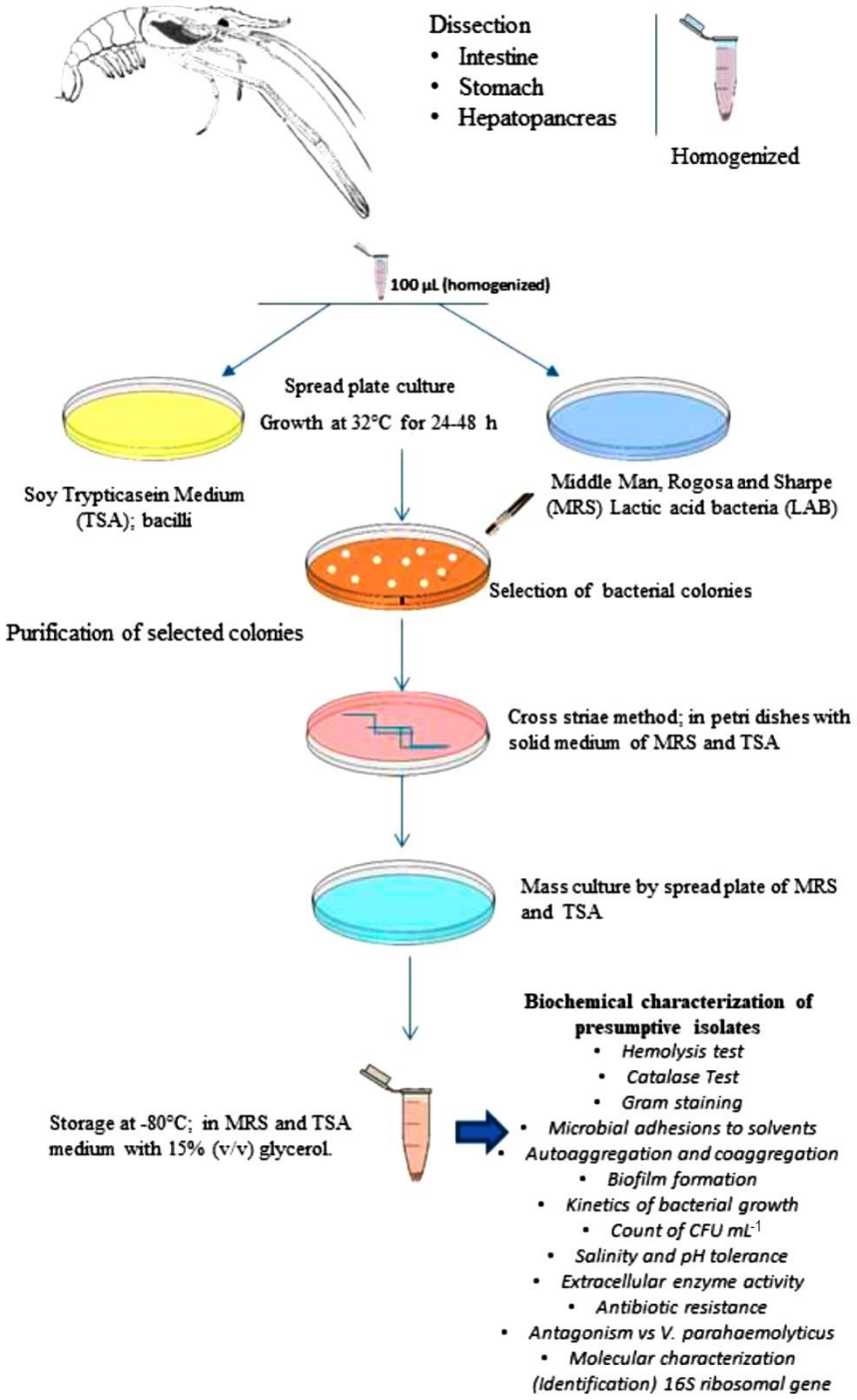

Figure 1. General scheme of the isolation and biochemical characterization of the strains of bacilli and lactic acid bacteria of the prawn *Macrobrachium tenellum.*

alginate microparticles (pearls) were formed, and the solution remained in agitation for 30 min. The moisture from the microparticles was filtered with a Whatman filter and was washed with sterile distilled water. Finally, they were allowed to dry for 24 h at 32°C.

Incorporation of additive (microencapsulated *Pontibacillus* sp. to food)

The commercial food (Purina™, 35% protein) was pulverized into an electric food processor to add the probiotic (*Pontibacillus* sp.). A paste was formed with

the mixture, adding distilled water and powdered gelatin (40 g of gelatin and 410 mL of distilled water kg^{-1} of food), and the pellets were made again and dried in an oven at 100°C for 1 h. Subsequently, they were cooled at room temperature with a fan for 24 h and stored at 4°C. For the control treatment, the additive of interest was replaced by cellulose (Trejo-Flores et al. 2016).

Bioassay of the release of the microencapsulated *Pontibacillus* sp.

Catching prawn *Macrobrachium tenellum*

Prawns *M. tenellum* were collected (with nasa traps) in July 2019 at the irrigation channel N°15 (25°34'42"N, 108°36'31"W) in Guasave, Sinaloa, Mexico. The organisms were transported to the Biochemistry and Molecular Laboratory of CIIDIR-IPN Guasave, Sinaloa, in plastic containers with water from the collection site with oxygenation (>4.0 mg L^{-1}) and ambient temperature (30°C).

Determination of the lethal mean concentration of *V. parahaemolyticus* IPNGS16

Before each infection with *V. parahaemolyticus*, a bioassay (4 days) was performed to determine CL_{50} (lethal concentration, 50%) using prawns with an average weight of 1.98 ± 0.5 g. The experimental plastic tubs contained 20 L of filtered fresh water (salinity <2, filtered at 20 µm) with constant aeration (dissolved oxygen >5 mg L^{-1}).

The bioassay consisted of six treatments, each in triplicate (30 organisms, 10 per tub). The treatments were: I) control and commercial food (CF). II) CF + *V. parahaemolyticus* (1×10^2 CFU mL^{-1}); III) CF + *V. parahaemolyticus* (1×10^3 CFU mL^{-1}); IV) CF + *V. parahaemolyticus* (1×10^4 CFU mL^{-1}); V) CF + *V. parahaemolyticus* (1×10^5 CFU mL^{-1}); VI) CF + *V. parahaemolyticus* (1×10^6 CFU mL^{-1}). The prawns were fed (food with 35% of protein) twice a day (08:00 and 16:00 h). The experiment was performed under the natural photoperiod. There was no cleaning of the tubs during the infection, and the temperature remained between 29.5 and 30.5°C to favor the infection of the freshwater shrimps. Mortality was recorded three times a day, and the final data were used to calculate the LC_{50} using Probit™ analysis, PASW Statistics 18™ (Finney 1952).

Bioassay of the release of the microencapsulated *Pontibacillus* sp.

A bioassay was performed for 54 days with 10 prawns (186 ± 0.5 g) per plastic tub (20 L) with 8 L of filtered water (salinity <3, 20 µm) under constant aeration. The bioassay consisted of four treatments: I) commercial food (CF) + *V. parahaemolyticus* (CL_{50}) positive control; II) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 1 g kg^{-1} of food + *V. parahaemolyticus* (CL_{50}) c/2 d; III) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 2 g kg^{-1} of food + *V. parahaemolyticus* (CL_{50}) c/2 d; IV) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 3 g kg^{-1} of food + *V. parahaemolyticus* (CL_{50}) c/2 d (Fig. 2). The organisms were fed (food with 35% of protein) twice a day (08:00 and 16:00 h). The experiment was performed under natural photoperiod conditions. The temperature was kept between 29.5 and 30.5°C.

Each treatment was performed in triplicate. The temperature (ºC) was determined daily, and the oxygen (mg L^{-1}) was measured with an oximeter YSI™, the salinity with a Vital refractometer SINE™ and for the pH, a HANNA™ potentiometer was used. Every three days, the tubs were cleaned by siphoning, and 50% water replacement was carried out. Biometrics were performed on prawns every seven days until day 53 to obtain the specific growth rate (SGR).

The following formula was used to get the SGR:

$$SGR\ (\%d) = \frac{(Ln\ W2 - Ln\ W1)x\ 100}{t}$$

where: W_2 is the final weight, W_1 is the initial weight, and t is the number of days of cultivation.

For the experiment with *V. parahaemolyticus*, the $CL_{50} = 4.5\times10^4$ CFU mL^{-1} was added only once to each tub on day 53.

Statistical analysis

SGR and survival values were tested for normality (Kolgomorov-Smirnov) and homoscedasticity (Bartlett). To determine the differences between treatments in the SGR and survival, a one-way ANOVA ($P < 0.05$) was performed. In case there were differences among treatments, a Tukey test was performed to identify where these differences were found ($P < 0.05$) (Zar 2010).

RESULTS

Isolation of bacilli and presumptive LAB

In the present work, 106 strains with probiotic potential were isolated, of which 77 are bacilli, and 29 are LAB [(intestine (43), stomach (42) and hepatopancreas (21)] from the prawns *M. tenellum* collected from a freshwater environment in Guasave, Sinaloa.

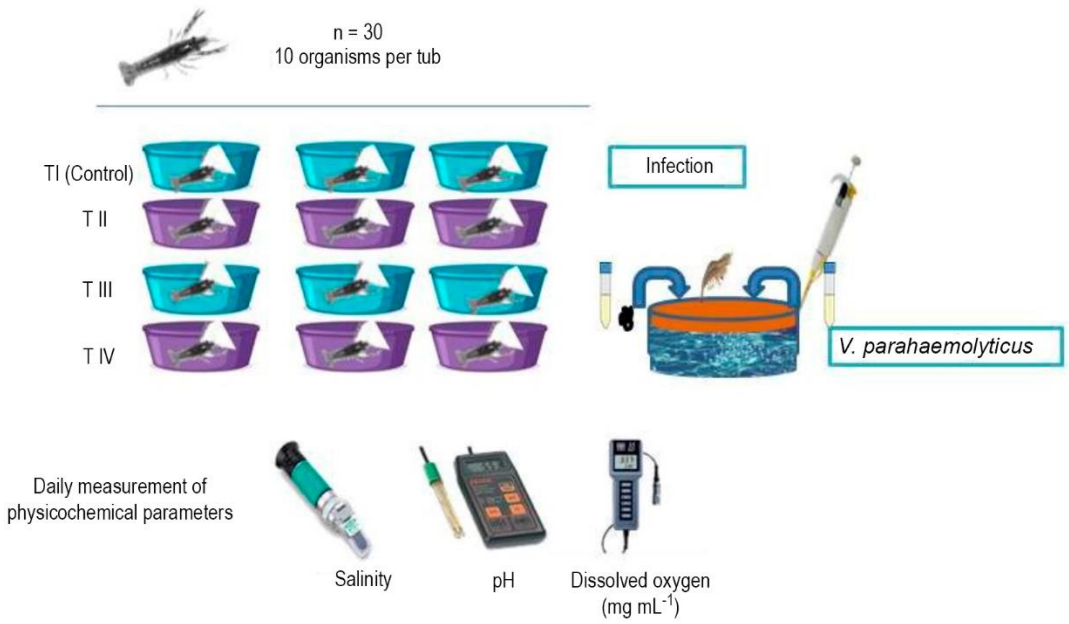

Figure 2. Experimental design bioassay of the release of the microencapsulated *Pontibacillus* sp. in the growth and survival of the prawn *Macrobrachium tenellum.*

Gram stain procedure

For bacilli, the bacterial strains were Gram (+), except for a Gram (-) isolate. All had a rod shape with spores. While in LAB, two isolates were Gram-negative, and three were Gram-positive, with spherical shape.

Hemolytic activity of bacilli and LAB

According to the hemolytic activity, 57 bacilli strains with hemolysis β, 13 with hemolysis α, and 7 with hemolysis γ were obtained. In LAB, 15 strains with hemolysis β, 7 with α, and 7 with γ were obtained. Only those isolates with hemolysis γ were used to continue with the characterization.

Catalase test

Of the seven LAB isolates with hemolysis γ, two were discarded for being catalase positive, and only five isolates were catalase-negative, selected for characterization as possible probiotics.

Microbial adhesion to solvents

Four presumptive bacilli strains with low hydrophobicity were obtained (1.54 to 27.89%) and three with moderate (68.95 and 60.25%). Most of the LAB isolates showed low hydrophobicity (5.47 to 11.15%), and only one isolate with high hydrophobicity 73.21% (MT1E2). During adherence to ethyl acetate, all bacilli isolates resulted in low hydrophobicity except for a bacillus with a high hydrophobicity of 99% (MT4H2). This test selected the isolates with a higher percentage and probiotic potential for further characterization. The selected strains were MT4H2 (bacillus) and MT1E2 (LAB) (Table 1).

Autoaggregation and coaggregation

The percentages of autoaggregation for isolates were high, with 90.9% for MT4H2 and 90.8% for MT1E2. While in coaggregation, the bacilli strains showed a value of 90% (MT4E2) and 99.97% (MT1E2) for LAB (Table 1).

Formation of biofilm

The results showed that both isolates of bacilli (0.197 ± 0.01) and LAB (0.671 ± 0.03) have a medium capacity to form biofilms (Table 1).

Kinetics of bacterial growth

The bacillus strain MT4H2 reached an exponential phase between 12 and 48 h, while the LAB strain (MT1E2) at 6 and 24 h (Table 1).

Count of CFU mL^{-1}

The CFU of the selected isolates was 62×10^{6} to 42×10^{7} CFU mL^{-1} (Table 1).

Tolerance to salinity

According to the test, five bacillus strains grew at 0.5 - 5% salinity, unlike LAB, which ranged from 0.5-9% salinity (Table 1).

Table 1. Characterization of isolates obtained from stomach and hepatopancreas of prawn *Macrobrachium tenellum*. NA: not applied.

Characteristics	Strains *Pontibacillus* sp. (MT4H2)	*Pediococcus pentosaceus* (MT1E2)
Hemolytic activity	γ	γ
Gram stain	+	+
Shape	bacilli	cocci
Catalase activity	NA	-
Hidrophobicity (%)		
• p- xylene	60.25 ± 0.05	73.21 ± 0.02
• Ethyl acetate	99.93 ± 0.03	6.97 ± 0.05
Biofilm formation	0.197 ± 0.02	0.671 ± 0.05
Autoaggregation (%)	90.9 ± 0.03	90.0 ± 0.05
Coaggregation (%) with Vibrio parahaemolyticus	90 ± 0.05	99.97 ± 0.05
Growth Phase (h)		
• Lag	3-9	3
• Log	12-48	6-24
• Stationary	72	48
• Death	96	72-96
Count (CFU mL^{-1})	62×10^{6}	42×10^{7}
pH of tolerance		
• pH 5	+	+
• pH 6-7	+	+
• pH 8-9	-	+
Salinity of tolerance (%)		
• 0.5-2	+	+
• 3-5	+	+
• 6-9	-	+
• 9-12	-	-
Extracellular activity (mm)		
• Proteases (Gelatine)	1.1 ± 0.01	1.25 ± 0.05
• Proteases (Casein)	1.1 ± 0.01	1.2 ± 0.02
• Lipases (Tween 80)	1.55 ± 0.03	-
Antibiotic resistance		
• Gentamicin	-	-
• Streptomycin	-	-
• Sulfamethoxazole	1.8 ± 0.05	-
• Oxytetracycline	-	1.4 ± 0.05
• Florfenicol	1.9 ± 0.05	1.7
• Enrofloxacin	1.7 ± 0.05	-
Antagonistic activity	+	-

pH tolerance

The isolated MT4H2 (bacillus) grew at a range of pH 5 to 7, whereas the MT1E2 strain (LAB) grew at a range of pH 5-9 (Table 1).

Extracellular enzyme activity

The MT4H2 isolate showed enzyme activity in protease and lipolytic activity in this test. In contrast, MT1E2 did not show activity in lipase in Tween 80 (Table 1).

Antibiotic resistance

The isolate MT4H2 presented resistance to three antibiotics, gentamicin (GM10), streptomycin (S10), and oxytetracycline (OT30), but was susceptible to sulfamethoxazole (1.8 ± 0.05), florfenicol (1.9 ± 0.05) and enrofloxacin (1.7 ± 0.05). The isolate MT1E2 showed resistance to almost every antibiotic except oxytetracycline (1.4 ± 0.05) and florfenicol (1.7) (Table 1).

Antagonistic activity

The bacillus strain MT4H2 showed bacterial activity against *V. parahaemolyticus* IPNGVE16, while LAB did not show this bacterial activity against *V. parahaemolyticus* IPNGVE16 (Table 1).

Molecular identification

The results from the BLAST homology indicated that the bacillus strain MT4H2 seems to be related to *Pontibacillus* sp. with 98%, while LAB MT1E2 with 98.66% to *Pediococcus pentosaceus.*

Bioassay of the release of the microencapsulated *Pontibacillus* sp.

Physical and chemical parameters

The average temperature in the bioassay was 23.5 ± 0.2°C, 7.35 ± 0.4 mg L^{-1} of dissolved oxygen, and pH 8.3 ± 0.1.

Specific growth rate (SGR)

The SGR for the treatments were: TI (3.07 ± 0.15), TII (2.56 ± 0.06), TIII (3.13 ± 0.05), TIV (3.27 ± 0.04). TIII and TIV did not show any significant differences ($P > 0.05$) concerning TI (control); it was only seen regarding TII ($P = 0.00232$) by the end of the 53 days of the bioassay (Fig. 3).

Survival

The recorded survival in the bioassay of infection with *V. parahaemolyticus* was as follows: TI (positive control) with 51.68 %, TII (60.65 %), TIII (82.50 %), and TIV (89.98 %). Significant differences were obtained among the treatments ($F_{(4,10)} = 6.8472$, $P = 0.0073$). The TI (control) had highly significant differences, with TIII ($P = 0.0082$) and TIV ($P = 0.0027$) (Fig. 4).

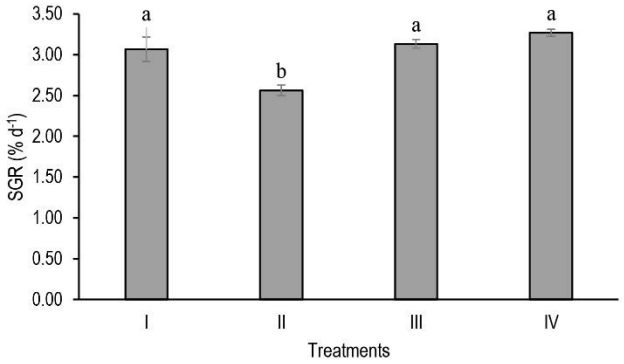

Figure 3. Specific growth rate (SGR) of *Macrobrachium tenellum*. Treatments: I) positive control, commercial food (CF), II) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 1 g kg^{-1} by food c/2 d, III) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 2 g kg^{-1} c/2 d, IV) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 3 g kg^{-1} c/2 d. The data show average ± standard error. Different letters show significate differences.

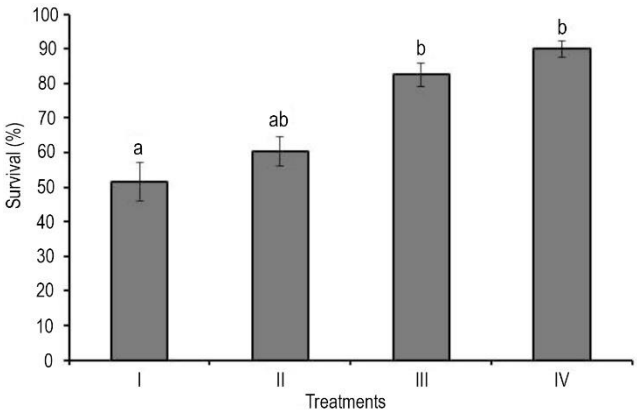

Figure 4. Survival of *Macrobrachium tenellum* after the infection with *Vibrio parahaemolyticus*. Treatments: I) positive control, commercial food (CF), II) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 1 g kg^{-1} poor food c/2 d, III) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 2 g kg^{-1} c/2 d, IV) CF + sodium alginate microcapsules of *Pontibacillus* sp. (Mt4 Hp2b) 3 g kg^{-1} c/2 d. Data show average ± standard error. Different letters indicate highly significant differences.

DISCUSSION

Currently, two basic principles must be taken into account for a microorganism to meet the characteristics of a probiotic and to be administered to animals to reverse, the first being the specificity of the host, which indicates that the most likely strains of microorganisms to be probiotic are those that come from the same or similar species, and the second implies the proximity of the ecosystem, which examines the importance that microorganisms have in their use at the site where they act on the host (Fuller 1989, Verschuere et al. 2000, Gullian et al. 2004). In this study, Gram (+) and (-) bacteria from the stomach and hepatopancreas of the prawn *M. tenellum* were isolated and captured in Guasave, Sinaloa, Mexico. These bacteria belong to the group of bacilli and LAB, which are more frequently used as probiotics in culture systems of marine and freshwater organisms (Ronsón-Paulín & Medina-Reina 2002, Escamilla-Montes et al. 2015).

In the isolates selection to be considered as probiotics, *in vitro* investigations should be made. One is the hemolytic activity, given by the hemolysin enzyme (lysing the red blood cells, which are the blood cells in charge of transporting oxygen to organs and tissues). This property is undesirable regarding selection criteria for a probiotic, as probiotic strains should not be able to lysate erythrocytes (Zamora-Rodríguez 2003). In this study, isolates presented three types of hemolysis, where those bacteria that were α-hemolysis (partial lysis of erythrocytes) and β-hemolysis (total lysis of red blood cells) were discarded, and only those microorganisms with hemolysis γ- (absence of hemolytic activity) were selected (Escamilla-Montes et al. 2015).

The catalase enzyme is present in most aerobic bacteria; its function is to eliminate those substances that are produced during bacterial metabolism, such as hydrogen peroxide, which is decomposed as gaseous oxygen, which is released as bubbles (Bertrand et al. 2002, Watine et al. 2003). High concentrations of this enzyme can act over those substances produced by other microorganisms, which has led to the proposition that negative catalase isolates could be less harmful (Bertrand et al. 2002, Watine et al. 2003). This work obtained LAB isolates with negative catalase; five out of seven strains had hemolysis γ-. This test is also used to classify bacteria; if both have the same morphology, the absence or presence of this enzyme makes it easy to distinguish between families, such as *Streptococcus*, a negative catalase, and positive *Staphylococcus*. In addition, the genus *Bacillus* is positive catalase and is differentiated from the genus *Clostridium*, which is negative, among others (Ronsón-Paulín & Medina Reyna 2002). In this work, most of the isolates were Gram (+), and only three strains were Gram (-) (bacillus: Mt1 Est1a, LAB: MT3 EST2a, and MT5 Est2).

According to their morphology, the LAB strains had a spherical shape and the bacilli a rod one with the presence of spores which are considered as capsules, allowing bacteria to resist environmental changes such as high temperatures and also, in some cases, they generate resistance to chemicals as antibiotics and disinfectants, enabling them to remain visible for a certain time until the conditions are favorable for their development (Tejera-Hernández et al. 2011, Barra-Carrasco et al. 2014).

Within the relevant properties of probiotics, there is the proliferation and colonization of the digestive tract (Senok et al. 2005). The adhesion of bacteria to the intestinal mucosa is one of the main criteria for selecting microorganisms as possible probiotics since it is a prerequisite for colonizing the intestine. All these comprise the first defense mechanism against the invasion of pathogens (Ouwehand et al. 1999). It is an important factor related to the colonization and growth of bacteria by having better access to the organic matter attached to the surface (gastrointestinal or epithelial cells) (Kos et al. 2003, Otero et al. 2004, Mandhi et al. 2010). This process in the first phase is based on the forces of attraction and repulsion, in which hydrophobicity stands out (ability to repel water), enabling the establishment of interaction with the proteins of the cell wall, thus starting the adhesion and colonization (Jabra-Risk et al. 2001, Vidotto et al. 2003). In this study, only two strains with high hydrophobicity were the bacillus (MT4E2) and LAB (MT1E2). Nevertheless, a lower hydrophobicity value does not indicate that the bacterial strains have less chance of adhering to the intestinal epithelium since the hydrophilic domains could also be implicated in the adhesion process (Savage 1992).

The ability to aggregate is a particularity used in the analysis of microbial relationships. There is an association between the ability of microorganisms to adhere to the intestinal epithelium, the aggregation activity, and surface hydrophobicity (Wadström et al. 1987). It is important to know the properties that are involved in the colonization and the capacity to adhere with other microorganisms of the same species in the case of autoaggregation, as coaggregation is defined as the union between microorganisms of different species, therefore preventing the colonization of pathogen agents (as barrier formation) (Decostere et al. 1999, Del Re et al. 2000, Yuehuei & Friedman 2000, Rickard et al. 2004). In this study, isolates presented autoaggregation values of 90%, opposite to coaggregation, where the values were higher and closer to 99%. This behavior is similar to what was reported for the strain of *Bacillus licheniformis* BCR 4-3, where a high percentage of hydrophobicity was recorded and a high capacity of forming biofilms in abiotic surfaces (Del Re et al. 2000, Escamilla-Montes et al. 2015).

Adhesion and colonization are the main defense mechanisms against pathogen agents through competition for space and nutrients (Salyers & Whitt 2002). In consequence, the formation of biofilms is a relevant aspect of the colonization of these microorganisms by the host. The biofilm is made of extracellular material (polymers) from some bacteria (Hall-Stoodley et al. 2004, Huq et al. 2008). It provides advantages such as protection against strong temperature and pH changes (Nazar 2007). In this study, the bacilli isolates (MT4E2)

and LAB (MT1E2) presented a moderate capacity to form a biofilm, which enables us to deduce that these bacteria can adhere easily to the intestinal epithelial cells and colonize it, competing with pathogens for space and nutrients, displacing them.

The growth capacity of an organism is a characteristic that has much interest at the industrial level. Those able to generate more biomass per unit of time, with low nutritional requirements, gather competitive advantages against others as they can be obtained at a lower cost. This property benefits the animal, as it incorporates it, enabling the organism to predominate over the rest of the autochthonous microbiota. Moreover, analyzing bacterial growth is a strategy that allows us to manipulate bacterial strains, which are exploited mainly for probiotic production, as they must be harvested in a certain phase for their commercialization (Jantzen et al. 2013). Bacterial strains obtained their exponential phase in a range of 6 to 24 h, coinciding with those isolated bacilli by Escamilla-Montes et al. (2015), which ranged between 6 and 24 h. Only those LAB strains from the freshwater environment reached the exponential phase at 24 h, but all isolates reached the stationary phase at 48 h.

Regarding tolerance to salinity, knowing this property is important due to the wide variety of salt concentrations in which bacteria can stand through the host and the aquatic environment. In this present study, the isolates grew within a salinity range of 6 to 9%, allowing us to consider those strains that grew in a medium with concentrations ranging from 0.5 to 12% of NaCl as weak and moderate halophilic bacteria (Kushner & Kamekura 1988).

According to FAO/WHO (2002), those microorganisms used as probiotics must be able to survive passing through the intestine and have the ability to withstand gastric fluids (Senok et al. 2005). Most bacteria prefer a neutral pH (6.5-7.5), but once they reach the host's digestive tract, they must tolerate different pH ranges (Galtsoff 1964, Tuomola et al. 2001). In this research, the isolated bacteria, the bacilli, and the LAB grew within an acid-base range between 5 and 9 pH. García-Ruiz et al. (2014) found a relationship between the pH and bacterial growth; in this sense, isolates of LAB tolerate all pH ranges and have the highest growth.

Extracellular enzymes produced by bacteria are important to decompose organic compounds, which can be lipase and protease enzymes that have been reported to aid in the nutrition of those cultivated organisms (Martínez et al. 1996, León et al. 2000, Nausch 2000, Quesada-Herrera et al. 2004, Balcázar et al. 2006, Jayachandra et al. 2012). Studies undertaken with probiotic strains suggest that these microorganisms have a beneficial effect on the digestive processes of aquatic organisms (Sakata 1990). In this research, the isolated bacilli (MT4H2) and LAB (MT1E2) showed enzyme activity both in proteases and lipases. Certain bacteria can intervene in the digestive processes of bivalves, elaborating extracellular enzymes such as proteases and lipases, besides providing necessary factors for the growth of organisms (Prieur et al. 1990).

The inappropriate use of antibiotics as prophylactic agents in disease prevention is common and contributes to the development of antibiotic resistance. Several studies have reported antibiotic residues or resistances in culture species, water columns, and sediments (González-Salas et al. 2021).

Since then, the use of antibiotics in aquaculture has been generalized, possibly affecting the development of pharmacoresistant bacteria and the exchange of resistant characteristics to nonresistant bacteria. Also, improper use of antibiotics can cause unnecessary environmental harm (Aoki 1992, Rhodes et al. 2006). The use of antibiotics is the method used to fight diseases caused by bacteria in the culture of marine organisms. To treat bacterial infections, antibiotics like oxytetracycline, enrofloxacin, sparfloxacin, and florfenicol are used (Alabi 2000, Roque et al. 2001), hence probiotics have emerged as a promising tool to control diseases in aquaculture (Verschuere et al. 2000, Balcázar et al. 2008) so it is necessary to use bacterial strains that are resistant to the antimicrobials (Coppola et al. 2005, Kim & Austin 2008). In this work, all the LAB strains of the brackish zone presented resistance to the tested probiotics. However, the LAB of the freshwater environment was only resistant to streptomycin, sulfamethoxazole, and oxytetracycline. It is important to mention that antibiotic resistance is directly related to the volume of antibiotics used (Anderson & Levin 1999). Tendencia & De la Peña (2001, 2002) observed that when antimicrobial agents, including oxytetracycline, are used often in an environment, resistant microorganisms in this site will be greater. In this work, the bacillus was resistant to antibiotics like gentamicin, streptomycin, and oxytetracycline and sensitive to sulphamethoxazole, florfenicol, and enrofloxacin. The isolate of LAB was sensitive to most antibiotics except oxytetracycline and florfenicol. Thus it can be suggested that they can be incorporated continually into the culture systems to retrieve the affected microbiota by the antibiotics and therefore contribute to the balance in the ecosystem.

Bacterial antagonism is a general manifestation in nature, where microbial relationships are important in

the equilibrium between beneficial and potentially pathogen microorganisms. Despite this, the structure of microbial communities can be modified by the culture systems and the environmental conditions that stimulate the proliferation of certain species of bacteria. The gastrointestinal microbiota of aquatic animals can be modified, so management constitutes a feasible tool to reduce or eliminate the incidence of opportunistic pathogens in organisms (Balcázar 2002). Adhesion and colonization on mucous surfaces are possible protection mechanisms against pathogens through the competition for union sites and nutrients (Salyers & Whitt 2002, Avendaño-Herrera et al. 2005, Sánchez-Ortiz et al. 2015). This work found that the MT4H2 isolate showed antagonistic activity against *V. parahaemolyticus*, while the MT1E2 did not inhibit its growth. It could be caused as the probiotic bacteria produce a variety of chemical compounds of a wide spectrum which include bacteriocins, siderophores, lysozymes, proteases, and hydrogen peroxide in the intestine of the host, creating a barrier against the proliferation of opportunistic pathogens, as well as the alteration of the intestinal pH due to the production of organic (Oppegard et al. 2007, Martín-Visscher et al. 2009, Korkea-aho et al. 2011, 2012, Pérez-Sánchez et al. 2011, Strom-Bestor & Wiklund 2011, Ahmad et al. 2021). In shrimps, the investigations have been based on the evaluation of probiotics like *Bacillus cereus*, *Paenibacillus polymyxa*, and *Pseudomonas* sp. (PS-102) as biocontrol agents against pathogens like *Vibrio* spp. (Vijayan et al. 2006, Ravi et al. 2007).

Regarding identification, the FAO/WHO (2002) suggests that the current and admissible methodology and a combination of phenotypic and genotypic methods should be used to speciate probiotic strains. However, the use of molecular identifying methods is mandatory (Morelli & Capurso 2012) as phenotypical identifications are not reliable by themselves. The molecular identification of the isolate bacillus (MT4 Hp2b) showed a high identity with *Pontibacillus* sp.; these bacteria are mildly halophilic, growing optimally in environments with 1-15% (p/v) of NaCl, being widely distributed in different marine environments. This bacterial group is interesting as it has great biotechnological potential due to the production of compatible solutes or hydrolytic enzymes (Margesin & Schinner 2001). The isolate MT1 Est2 showed a high percentage of similarity of its sequence with *Pediococcus pentosaceus*, which is bacteria that stands acids, cannot synthesize porphyrins, and possesses a strictly fermentative metabolism with lactic acid as the main final metabolic product (Garvie 1986, Axelsson 1998) and can be isolated from a variety of vegetal material and mature cheese. It also can ferment raffinose, melibiose, and sacarose, as well as produce bacteriocines (González & Kunka 1983, Daeschel & Klaenhammer 1985).

In freshwater shrimp farming, food constitutes 40 to 60% of production costs, so part of the success depends on developing diets with additives that allow good growth, food survival, and efficiency that contribute to reducing environmental pollution (Espinosa-Chaurand 2013). Natural immunostimulants derived from bacteria (probiotics) and plant extracts are considered areas with good development for cultivating marine and freshwater organisms because they are safe for the environment and human health (Ahmed et al. 2016).

Including beneficial bacteria in diets for aquatic animals can have varied effects, such as having a better health state or the growth of the animals. However, the latter may be, in part, a consequence of the former. In recent years probiotics and antimicrobial agents have been used as an alternative to antibiotics (Verschuere et al. 2000, Geiger 2001, Mateus et al. 2014, Prabu et al. 2018, Rathnakumari et al. 2018, Tran et al. 2018, Soowannayan et al. 2019); against bacterial pathogens such as *V. parahaemolyticus*, a causative agent of AHPND (Hong et al. 2016).

In this study, growth was very similar among treatments, with significant differences in the SGR. TIII and TIV showed higher growth compared to the control and TII. The addition of the sodium alginate microcapsules of *Pontibacillus* sp. with a concentration of 2 and 3 g kg^{-1} and with an application every two days in the diet compared to control showed differences, with an increase in the growth of *M. tenellum*. According to Jory (1998) and Gatesoupe (2008), one of the beneficial effects associated with probiotics could be the increase in growth rate because when they establish in the intestinal tract of the host, they contribute to the balance of the microbiota and improves the absorption of nutrients by producing digestive enzymes (proteases and amylases) and exoenzymes that break the cellulose and starch of the food, thus facilitating its assimilation.

Villamil-Díaz & Martínez-Silva (2009) and Seenivasan et al. (2013) mention that the benefits of probiotic supplementation in crustacean cultures are the increase in the survival rate during the experimental infections, presumably associated with an increase of the immune response and antimicrobial activity. In this study, we showed better survival in *M. tenellum* in TIII and TIV, with over 80% relative to the control, where survival was 50%, consistent with the results obtained.

Loh et al. (2010) mention that the synergy between live microorganisms and biological metabolites may result in better protective qualities.

The isolates MT4H2 (*Pontibacillus* sp.) and MT1E2 (*P. pentosaceus*) present ideal characteristics of a probiotic (Gamma hemolysis, high ability to form biofilm, high level of hydrophobicity, antibiotic resistance, and antagonistic activity) therefore they can be considered as presumptive probiotics. The addition of the sodium alginate microcapsules of *Pontibacillus* sp., with a concentration of 2 and 3 g kg^{-1} and with an application every two days in the diet showed differences, with an increase in the growth of *M. tenellum*, indicating that adding a probiotic to the diet of *M. tenellum* shrimp was reflected in growth and survival. This research is the first contribution to potentially probiotic bacteria isolated from the prawn *M. tenellum* in the northwest region of Mexico (Guasave, Sinaloa).

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