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Isolation and Characterization of Potential Probiotic Bacteria Suitable for Mollusk Larvae Cultures

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Abstract

Guts of *Crassostrea gigas*, *C. corteziensis*, and *Atrina maura* were screened for bacilli and lactic acid bacteria with probiotic properties for bivalve mollusks. Characterization of the isolates included Gram stain, morphology, cell arrangement, catalase and hemolytic activity, hydrophobicity, autoaggregation, coaggregation against *Vibrio sinaloensis* VHPC23, biofilm formation, counting of colony forming units (CFU), NaCl and pH tolerance, enzymatic activity, resistance to antibiotics, antagonism against *Vibrio sinaloensis* VHPC23, and molecular identification. Three isolates (BCR 4-3, BALCR 5-2, and BALOR 2-3) showed no hemolytic activity, high tolerance to pH and salinity, high adhesion potential, high autoaggregation and coaggregation, biofilm formation, well growth, and some resistance to antibiotics. The isolates were identified as *Bacillus licheniformis* (BCR 4-3) and *Leuconostoc mesenteroides* (BALCR 5-2 and BALOR 2-3). The strains showed basic characteristics to be used as probiotics in mollusk larvae cultures.

Keywords: bacilli, lactic acid bacteria, larvae, mollusks, probiotics

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Introduction

Aquaculture industry is growing fast as a result of increasing demand for seafood. In 2010, world aquaculture production (plants not included) was 59.9 million tons. Fish (43.1 million tons) was the main group of cultured animals, followed by mollusks (14.4 million tons), crustaceans (5.7 million tons), and other aquatic animals (814,300 tons). Infection disease outbreaks affect production in aquaculture (Kesarkodi-Watson et al., 2008; FAO, 2012). To prevent production losses, farmers rely on basic practices of good management, vaccination, and chemotherapy (Subasinghe and Barg, 1998). In the last years, the use of natural additives (probiotics, prebiotics, immunostimulants, and medicinal plants) has emerged to prevent bacterial and viral infections in cultured animals (Partida-Arangure et al., 2013). Microbials are very important in aquaculture production, and effective treatments with potential probiotics may provide broad spectrum and greater nonspecific disease protection (Pandiyan et al., 2013).

Live microorganisms with a beneficial effect on the cultured animals are called probiotics and they have been applied in aquaculture for disease control, replacing the use of antibiotics, improving water quality, providing nutrients and enzymes, and enhancing immune capacity (Verschuere et al., 2000; Balcázar et al., 2006). The probiotics used in aquaculture belong to *Vibrio* sp., *Lactobacillus* sp., *Pediococcus* sp., *Bifidobacterium* sp., *Saccharomyces* sp., *Enterococcus* sp., and *Bacillus* sp. (Panigrahia et al., 2005; Kumar et al., 2006; Leyva-Madrigal et al., 2012; Luna-González et al., 2013). Nowadays, there are several probiotic formulations in the market that have been introduced to fish, shrimp, and mollusk farming as feed additives, or incorporated in pond water (Verschuere et al., 2000; Wang et al., 2005; Wang and Xu, 2006; Wang, 2007).

Selection of potential probiotics is very important to avoid undesirable effects in the host. Selection steps need to be adapted for different host species and environments and it is essential to understand the mechanisms of microbial action to select potential probiotics (Pandiyan et al., 2013). Microbial action may include the production of inhibitory substances, competitive exclusion of pathogens, competition for nutrients and adhesion sites, production of enzymes and nutrients for the host, and immunomodulatory capacity (Verschuere et al., 2000; Balcázar et al., 2006; Gómez and Balcázar, 2008). When selecting probiotic microorganisms, many criteria have to be met, but one of the most important is the ability to adhere to intestinal surfaces (Jöborn et al., 1997; Nikoskelainen et al., 2001).

In the present study, the isolation and characterization of potential probiotic bacteria were done to be applied in mollusk larvae cultures.

Materials and Methods

***Animals and gut samples*:** *Crassostrea gigas* (n = 10), *C. corteziensis* (n = 10), and *Atrina maura* (n = 10) were collected in Topolobampo (Ahome, Sinaloa, Mexico) and La Pitaya (Guasave, Sinaloa, Mexico). Intestinal tracts of the organisms were aseptically removed, placed in Eppendorf tubes with 400 µL of sterile saline solution (2% NaCl), and homogenized with a pestle for 5 min.

Isolation of presumptive lactic acid bacteria (LAB): One hundred microliters of each homogenate was spread on plates with MRS agar (BD Difco, Sparks, MD, USA) supplemented with 2% NaCl and 200 mg/L aniline blue (Sigma-Aldrich, St Louis, MO, USA). The plates were incubated at 30ºC for 24 h. Each selected growing colony was spread on the same MRS plates by the cross-streak method and incubated as before. Each blue isolate was grown in MRS agar and stored at -70ºC in MRS broth with 15% (v/v) glycerol.

***Isolation of presumptive bacilli*:** One hundred microliters of each intestinal homogenate was incubated at 80ºC for 10 min to favor spore-forming bacteria (bacilli) and then it was spread on plates with trypticase soy agar (TS agar, BD, Bioxon, Sparks, MD, USA) supplemented with 2% NaCl and incubated at 37ºC for 24 h. Each selected growing colony was spread on the same TS agar plates by the cross-streak method and incubated as before. Each isolate was grown in TS agar and stored at -70ºC in trypticase soy broth (TS broth) with 15% (v/v) glycerol.

Characterization of bacterial isolates

Gram stain: Gram staining of the isolates was performed using a commercial kit (Golden Bell, Zapopan, Jalisco, Mexico). Shape and cell arrangement were determined by observation under a microscope with 100 x.

Catalase test for LAB: A pure colony of each presumptive LAB isolate was placed on a slide, then a drop of H_2O_2 (3%) was added covering the colony to observe immediate formation of bubbles (positive result). Presumptive catalase-positive LAB were discarded as potential probionts.

Hemolytic activity: Petri plates were prepared with blood agar (BA, BD Bioxon, State of Mexico, Mexico) with 5% (v/v) human blood, making 6-mm diameter wells on the plate with a sterile puncher. The isolates were cultured in TS broth (bacilli, at 37ºC for 24 h) and MRS broth (LAB, at 30ºC for 48 h) and centrifuged at 10 000 x g for 10 min. The bacilli and LAB supernatant were adjusted to pH 6.5 with NaOH (1 M) to avoid false positives (Balcázar et al., 2008). The wells were inoculated with 50 µl of the supernatant or MRS broth and TS broth (negative control) and incubated at 37°C for 24 h. Lysis halo was observed to determine the type of hemolysis (beta, alpha, or gamma). The isolates with alpha or beta hemolysis were discarded as potential probionts.

Microbial adhesion to solvents (MATS): Microbial adhesion to solvents (*p*-xylene, ethyl acetate, and chloroform) was measured according to Rosenberg et al. (1980) with modifications of Crow and Gopal (1995). Bacteria were harvested at the stationary phase by centrifugation at 5000 x g for 15 min, washed twice, and resuspended in 0.1 M KNO3 (pH 6.2), to approximately

10^8 CFU/mL. Absorbance of the cell suspension was measured at 600 nm (A_0). One milliliter of the solvent was added to 3 ml of the cell suspension. The sample was incubated for 10 min at room temperature and then the two phase system was mixed in a vortex (2 min). The aqueous phase was removed after 20 min of incubation at room temperature and the absorbance at 600 nm (A_1) was measured in a Thermo Spectronic Genesys 2 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The percentage of bacterial adhesion to solvent was calculated as $(1-A_1/A_0) \times 100$. The isolates with low adhesion to *p*-xylene (0-30%) were discarded as potential probionts. Only three isolates (one presumptive bacillus and two presumptive LAB) with medium (30-60%) to high (60-100%) adhesion to *p*-xylene were selected for further characterization.

Autoaggregation and coaggregation assays: The three isolates selected on MATS were tested for autoaggregation and coaggregation capacity according to Del Re et al. (2000), Kos et al. (2003), and Handley et al. (1987). Bacteria were grown for 18 h at 37°C in MRS or TS broth. The cells were harvested by centrifugation at 5000 x g for 15 min, washed twice and resuspended in phosphate buffered saline (PBS) to obtain 10^8 CFU/mL. Cell suspensions (4 mL) were mixed in a vortex for 10 s and then incubated at room temperature for 5 h. Every hour, 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the absorbance (A) was read in a Thermo Spectronic Genesys 2 Spectrophotometer at 600 nm, PBS was used as blank. The autoaggregation percentage was expressed as: $1 - (A_t/ A_0) \times 100$, where A_t represents the absorbance at time t = 1, 2, 3, 4, or 5 h and A_0 the absorbance at t = 0. The method for preparing the cell suspensions for coaggregation was the same as that for autoaggregation assay. *Vibrio sinaloensis* (VHpC23) was grown in TS broth as in bacilli. Cell suspensions (2 mL) of each isolate were mixed with the same volume of *V. sinaloensis* in a vortex for 10 s. Falcon tubes containing 4 mL of each bacterial isolates or vibrios were used as controls. The absorbance was read as above.

Biofilm formation: The three isolates were tested for biofilm formation according to Mandhi et al. (2010) and Knobloch et al. (2001). Briefly, following overnight incubation at 37°C, the optical density of bacteria at 595 nm was measured using a spectrophotometer (Multiskan™ GO Microplate, Thermo Scientific, North Caroline, USA). An overnight culture grown in TSB and MRS at 37°C was diluted to 1:100 in TSB and MRS was diluted to 1:100 in TSB and MRS with 2% (w/v) glucose. At a total of 200 µL these cell suspensions were transferred to a U-bottom 96-well microtitre plates. Each strain was tested in triplicate. The plates were incubated aerobically at 37°C for 24 h. The cultures were removed and the microtitre wells were washed twice with PBS (7 mmol/L Na_2HPO_4, 3 mmol/L NaH_2HPO_4 and 130 mmol/L NaCl at pH 7.4) to remove non-adherent cells and were dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µL of cristal violet (Golden Bell, Zapopan Jal. México) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 µL of sterile distilled water. The water was then cleared and the microplates were air dried. Adhesion ability was interpreted as strong (OD ≥ 1), average (0.1≤OD595<1) or slight (OD595< 0.1).

Kinetics of bacterial growth: To identify the log phase, growth curves for each isolate were determined. The isolate stock (20 µL) was inoculated into 50 mL of TSB (bacilli) broth and MRS (LAB) broth with 2.5% NaCl. The cultures were incubated at 37°C (bacilli) or 30°C (LAB) to determine growth by reading the absorbance in a Thermo Spectronic Genesys 2 Spectrophotometer at 580 nm for 96 h. MRS or TS broth with 2% NaCl was used as a blank.

Colony forming unit (CFU): Each isolate was grown as described under the preceding kinetics of bacterial growth paragraph. Bacterial cultures were centrifuged at 12 000 x g for 20 min. The bacterial pellet was resuspended in 1 mL of sterile saline solution (2.5% NaCl). The bacterial suspension was adjusted to an optical density of 1 in a Spectrophotometer as above. Ten-fold serial dilutions were used to determine the CFU per milliliter of bacterial suspension, using Petri dishes with either MRS or TS agar.

***Salinity tolerance*:** Falcon tubes (15 mL) with 10 mL of either MRS or TS broth were supplemented with 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12% NaCl in 15-mL tubes (Falcon), inoculated with 20 µL of bacilli and LAB and incubated at 35°C for 24 h. All tests were performed in sterile flat-bottom 96-well plates. Each well was filled with 200 µL of the cultures of isolates. Absorbance was read in a spectrophotometer Multiskan™ GO Microplate (Thermo Scientific, NC, USA) at 600 nm. Sterile medium was used as blank.

***pH tolerance*:** Isolates were grown in TS and MRS broth at pH 4, 5, 6, 7, 8, 9, and 10. pH of media was adjusted by adding HCl or NaOH (1 mol/L). Bacterial cultures were incubated for 24-48 h at 35°C. Absorbance was read as in salinity tolerance.

Extracellular enzymatic activity: The isolates were tested for extracellular protease and lipase activity according to León et al. (2000). Isolates of bacilli or LAB were grown in TS or MRS broth as above to obtain the supernatants. Petri plates were prepared with basal medium (agar 1.5% and 0.5% yeast extract) supplemented with 2% skim milk (Fluka, Sigma-Aldrich, Switzerland) or 1% gelatin (Knox) for proteolytic activity (PA) or Tween 80 (1%) (Sigma-Aldrich, Steinheim, Germany,) for lipolytic activity (LA). Wells that were 6 mm in diameter were filled with 50 µL of the supernatant and incubated at 37°C for 24 h. Trypticase soy (bacilli) and MRS broth (LAB), supplemented with 2.5% NaCl, were used as negative control. A clear zone around the well was considered positive for proteolytic activity and a cloudy zone was considered positive for lipolytic activity.

Resistance to antibiotics: Antibiotic sensitivity and resistance were assayed by the disc diffusion method (Bauer et al., 1996). The tested antibiotics were:

amoxicillin (10 U), penicillin G (10 U), bacitracin (10 U), and gentamicin (10 µg).

Antagonic activity: Antagonic effect of the supernatants of selected isolates against the pathogenic *V. sinaloensis* VHPC23 (Flores-Miranda et al., 2012) was determined. LAB and bacilli were grown in MRS and TSB (broth) supplemented with 2% NaCl and incubated at 30ºC for 24 h. Vibrios were inoculated on TS broth with 3% NaCl and incubated at 37ºC for 24 h. Thereafter, 1 mL of the *Vibrio* culture was centrifuged at 13 000 x g for 20 min. The bacterial pellet was resuspended in sterile saline (3% NaCl) to an optical density of 1 (580 nm) and then it was streaked on TS agar plates with 3% NaCl. Wells that were 6 mm in diameter were filled with 50 µL of the LAB and bacilli supernatants. The remaining cultures were adjusted to a pH value of 6.5 and added to the wells as described above. Two wells were filled with MRS (pH = 4) and TSB (pH = 7) as negative controls. Petri plates were incubated at 37ºC for 24 h. Inhibition zones (anti vibrio activity) around the wells were analyzed.

Molecular identification

16S ribosomal gene: Bacterial DNA was extracted according to DNAzol kit. The amplification of the 16S ribosomal DNA was performed by single PCR using primers 27 F (5'AGAGTTTGATCMTGGCTCAG) and 1492 R (5'TACGGYTACCTTGTTACGACTT) (Lane, 1991) which amplified a genome fragment of 1500 bp. Purification of the PCR products was performed with the cleaning kit QIAquick PCR Purification Kit (Invitrogen, Carlsbad, CA, USA). Purified PCR products were sent for sequencing to CINVESTAV (Irapuato, Mexico). Sequence similarity was searched for in the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLAST program (Basic Local Alignment Search Tool).

Phylogenetic analyses: Phylogenetic analyses were performed with the Molecular Evolutionary Genetics Analysis software (MEGA 5 Beta) (Tamura et al., 2011). The neighbor-joining method (NJ) was used to infer evolutionary relationships among sequences (Satoui and Nei, 1987; Tamura et al., 2004). Robustness of the NJ topology was evaluated by bootstrap test using 1000 replicates. The species *Thermotoga marina* was used as out-group (Zhang et al., 2000).

Results

Presumptive LAB and bacilli isolation: In this study we isolated 63 presumptive bacilli and 22 presumptive LAB from the gut of *A. maura*, *C. gigas*, and *C. corteziensis*.

Gram stain: All isolates were Gram positive, rod (presumptive bacilli) and cocci (presumptive LAB) shaped.

Catalase test for LAB: Eleven isolates were discarded because they were catalase positive, only catalase negative isolates (11) were selected.

Hemolytic activity of bacilli and LAB: Thirty-one presumptive bacilli showed β-hemolysis, α-hemolysis (four), and γ- hemolysis (28). Eleven presumptive LAB showed β-hemolysis and eleven more depicted γ-hemolysis. Only isolates with γ-hemolysis were further characterized.

Microbial adhesion to solvents (MATS): The bacterial adhesion to *p*-xylene (hydrophobicity) showed 23 presumptive bacilli with low hydrophobicity (0.20-25.70%), four with medium hydrophobicity (40.18-56.63%), and one with high hydrophobicity (62.46%). Five LAB isolates showed low hydrophobicity (0.83-7.03%), one showed medium hydrophobicity (34.01 %), and ~~two~~ showed high hydrophobicity (69.69-78.71%). The adhesion of bacilli to chloroform and ethyl acetate was from 44.10 to 93.99% and from 1.6 to 26.741%, respectively. The adhesion of LAB to chloroform and ethyl acetate was from 7.39 to 62.35% and from 28.63 to 50.68%, respectively.

From this test, the bacilli and LAB isolates with the highest probiotic potential were chosen for further characterization The selected isolates were BCR 4-3 (bacilli), BALOR 2-3 (LAB), and BALCR 5-2 (LAB) (Table 1).

Autoaggregation and coaggregation: The autoaggregation capacity of isolates was 64.18% (BCR 4-3), 99.37% (BALOR 2-3), and 99.45% (BALCR 5-2), whereas the coaggregation of isolates with *V. sinaloensis* was 66.6% (BCR 4-3), 89.8% (BALOR 2-3), and 95.7% (BALCR 5-2) (Table 1).

Biofilm formation: Results showed that bacillus BCR 4-3 showed a strong ability to form biofilm (1.01 ± 0.1), whereas BALCR 5-2 (0.1427 ± 0.01) and BALOR 2-3 (0.1325 ± 0.02) showed a medium capacity (Table 1).

Kinetics of bacterial growth: The log phase of BCR 4-3 was found to be between 6 and 24 h, whereas for LAB isolates (BALOR 2-3 and BALCR 5-2) it was between 9 and 48 h (Table 1).

Colony forming unit (CFU): The colony forming units of selected isolates were between 47×10^6 to 10×10^7 per milliliter (Table 1).

Salinity tolerance: As shown in Table 1, all isolates grew well at concentrations of NaCl from 0.5 to 9%.

pH tolerance: The bacillus and LAB did not grow at pH 4. Growth was observed from pH 6 to 10, although they grew better at intervals of pH 6-7 (BCR 4-3) and pH 6-8 (LAB) (Table 1).

Extracellular enzymatic activity: The isolate BCR 4-3 showed no enzyme activity. BALOR 2-3 and BALCR 5-2 showed only proteolytic activity (13 ± 0.01 and 18 ± 0.02 mm, respectively) (Table 1).

Resistance to antibiotics: The ~~I~~solate BCR 4-3 showed resistance to bacitracin but it was susceptible to penicillin (26 ± 0.01 mm), amoxicillin (28 ± 0.02 mm), and gentamicin (30 ± 0.01 mm). The LAB isolates

(BALOR 2-3 and BALCR 5-2) were resistant to all antibiotics (Table 1).

Antagonistic activity: The three isolates did not show bactericidal activity against *V. sinaloensis* VHPC23 (Table 1).

Molecular identification: Results of BLAST homology searches revealed that BCR 4-3 seemed to be related (98%) to *Bacillus licheniformis* NC006322.1, whereas BALCR 5-2 and BALOR 2-3 were related (99%) to *Leuconostoc mesenteroides* NC008531.1. The phylogenetic tree clearly grouped the isolates with the mentioned species (Fig 1).

Table 1 Characterization of isolates obtained from the gut of *Crassostrea gigas*, *C. corteziensis* and *Atrina maura*

	Strains		
Characteristics	*Bacillus licheniformis* (BCR 4-3)	*Leuconostoc mesenteroides* (BALOR 2-3)	*Leuconostoc mesenteroides* (BALCR 5-2)
Gram stain	+	+	+
Shape	bacilli	cocci	cocci
Catalase activity	NA	-	-
Hemolytic activity	γ	γ	γ
Hydrophobicity (%):			
• *p-xylene*	62.46 ± 0.05	69.69 ± 0.01	78.71 ± 0.01
• Chloroform	69.63 ± 0.03	24.30 ± 0.02	62.35 ± 0.02
• Ethyl acetate	61.14 ± 0.02	7.40 ± 0.01	50.69 ± 0.02
Autoaggregation (%)	64.18 ± 0.02	99.37 ± 0.02	99.45 ± 0.03
Coaggregation with *Vibrio sinaloensis* (%)	66.66 ± 0.01	89.8 ± 0.02	95.7 ± 0.01
Biofilm formation	1.01 ± 0.1	0.1472 ± 0.01	0.1325 ±0.02
Growth Phase (h):			
• Lag	0- 6	0-9	0-9
• Log	9-24	12-48	12-48
• Stationary	48-72	48-96	48-96
Count (CFU/ml)	47 x 10^6	96 x 10^6	10 x 10^7
NaCl tolerance (%)			
• 0.5-2	+	+	+
• 3-5	+	+	+
• 6-9	+	+	+
• 9-12	-	-	-
pH tolerance			
• pH 4	-	-	-
• pH 5-7	+	+	+
• pH 8-10	+	+	+
Enzyme activity (mm)			
• Proteases (casein)	-	13 ± 0.01	18 ± 0.02
• Proteases (gelatin)	-	-	-
• Lipases (Tween 80)	-	-	-
Resistence to antibiotics (mm)			
• Penicillin	26 ± 0.01	-	-
• Amoxicillin	28 ± 0.02	-	-
• Gentamicin	30 ± 0.01	-	-
• Bacitracin	-	-	-
Antagonism vs *Vibrio sinaloensis*	-	-	-

NA = Not applied

Discussion

The use of antibiotics in the rearing of mollusks is inadequate because it gives rise to antibiotic resistance of pathogens (Kesarkodi-Watson et al., 2008). Therefore, probiotics have emerged as a promising tool for disease control in aquaculture (Verschuere et al., 2000; Balcázar et al., 2008). Probiotics must be isolated preferably from the same organism in which they are going to be tested and should be from the same region (Verschuere et al., 2000). Moreover, according to Sica et al. (2012), the different tests performed to select potential probiotics should be complementary but not restrictive.

In this work, Gram positive bacteria belonging to the bacilli and LAB groups were preferred because they have been used as probiotics in aquaculture (Castex et al., 2009; Kumar et al., 2006; Leyva-Madrigal et al., 2011) and have species generally recognized as safe (GRAS) (Tompkins et al., 2008; Yang et al., 2012).

Hemolysis analysis is very important as a safety prerequisite since many organisms are able to synthesize exotoxins that induce partial or total lysis of human or animal cells (FAO/WHO, 2002; Zamora-Rodríguez, 2003). In this study, the bacilli and LAB with alpha or beta hemolysis (hemolytic activity) were discarded and only those with gamma hemolysis (lacking hemolytic activity) were selected. Likewise, Leyva Madrigal et al. (2012), Allaneh et al. (2012),

among others, discarded hemolytic isolates to avoid injuries in tissues of white shrimp (*Litopenaeus vannamei*) and fish (*Channa striatus*).

The most important criteria for the selection of probiotic strains is the ability to adhere to epithelial cells and mucosal surfaces (Kos et al., 2003; Nikoskelainen et al., 2003). However, the adherence capacity is a feature that is exploited in the same manner by probiotic and pathogenic bacteria (Nikoskelainen et al., 2003). Although the adherence capacity of probiotics is very important to exert their beneficial effect, it is important to remark that some probiotics without true capacity to colonize the intestine can provide beneficial effects (Ohland and MacNaughton, 2010). In this study, only the isolates with high hydrophobicity (>50%) were further characterized because in some bacteria a correlation between hydrophobicity and adhesion ability has been observed (Del Re et al., 2000; Giaouris et al., 2009; Kotzamanidis et al., 2010). Previous studies have shown that glycoproteins at the microbial cell wall show high hydrophobicity, whereas polysaccharides are associated with hydrophilic surfaces (Rojas and Conway, 1996; Pelletier et al., 1997).

Autoaggregation capacity of probiotic strains is necessary for adhesion to intestinal epithelial cells and coaggregation abilities may form a barrier that prevents colonization of epithelial cells by pathogens (Del Re et al., 2000). The hydrophobicity of the cell surface may affect autoaggregation and adhesion of bacteria to different surfaces (Pérez et al., 1998; Del Re et al., 2000). In this work, only the isolates with high autoaggregation and coaggregation against *V. sinaloensis* VHPC23 were selected. Spencer and Chenson (1994) suggested that in the urogenital and gastrointestinal tract of animals, coaggregation of LAB with pathogens is an important host defense mechanism.

The adhesion of microorganisms to abiotic materials is determined by both solid and cell surface properties (Mandhi et al., 2010). It has been reported that the genus *Bacillus* can adhere to abiotic surfaces as observed in *Bacillus cereus* that formed a biofilm on stainless steel surfaces (Peng et al., 2001). Presumptive bacillus BCR 4-3 showed high potential to adhere to abiotic surfaces, whereas the LAB isolates showed a medium capacity.

Bacterial growth kinetic analysis is useful because it allows microbial manipulation strategies. In this sense, it is known that *Bacillus* species typically are Gram (+) (95% to 100%) early in their growth phase in broth cultures but become Gram (-) (40% to 50%) in the late growth phase and 90% to 95% Gram (-) in the stationary phase (Beveridge, 1990). On the other hand, probiotics (in broth cultures) to be used in the water of culture systems can be harvested in the late growth phase because bacterial cells divide at a constant speed and have an increase in the total number of viable cells. However, bacteria for industrial processes, like encapsulation by spray drying, need to be in the stationary phase because this phase is characterized by a reduced proliferation rate, less physiological activity, and increased resistance to stress (Jantzen et al., 2013).

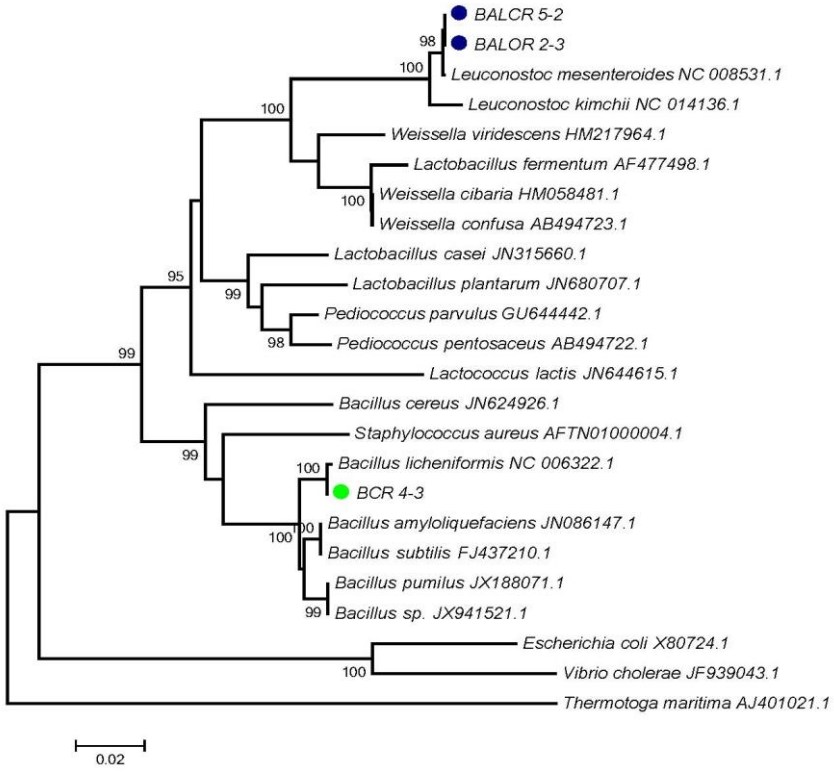

Figure 1 Phylogenetic tree constructed with the neighbor-joining method from 16S rDNA gene sequences of the three isolates (BCR 4-3, BALCR 5-2, and BALOR 2-3) and reference strains (accession numbers are indicated). Species used as the outgroup was *Thermotoga maritima*. Scale bar shows nucleotide substitution rate per site. Bootstrap probabilities as determined for 1000 replicates are given as percentage.

In the culture of larvae and juvenile mollusks, salinity is not a major issue due to low evaporation and water exchange. However, the knowledge of salinity tolerance of potential probiotics is needed. Marine bacteria are considered as slight halophiles because they grow best in media with 1% to 3% NaCl (Kushner and Kamekura, 1988). In this study, the selected isolates (BCR 4-3 [bacillus], and BALOR 2-3 and BALCR 5-2 [LAB]) grew in media with up to 9% NaCl. According to Kushner and Kamekura (1988), the isolates are considered as moderate halophiles, growing best in media with 3% to 15% NaCl.

In the search of probiotic characteristics, the bacterial physiology is very important. In this sense, pH is involved in the control of ion transport systems and is closely related to cellular membrane permeability (Booth, 1985). Most bacteria prefer neutral pH (6.5-7.5) (Tuomola et al., 2001). In mollusks, probiotics cultured in broth media are added to the water of the culture systems, but it is expected that they adhere to the tegument and to the digestive tract. Therefore, pH tolerance of potential probiotics is needed. According to Galtsoff (1964), in *Crassostrea virginica*, the pH in the stomach varies from 5.4 to 5.6, in the midgut from 5.5 to 6.0, and in the rectum from 5.8 to 6.3. In this work, the selected isolates (BCR 4-3, BALOR 2-3, and BALCR 5-2) were capable of growing at a pH range from 5 to 10 with optimal growth range at 6 to 7 (BCR 4-3) and 6-8 (BALOR 2-3 and BALCR 5-2).

Extracellular enzymes produced by bacteria are important in the degradation of organic compounds and for providing food substrates to bacteria (Nausch, 2000; Martínez et al., 1996). The extracellular enzymes produced by bacteria include amylases, glucoamylases, glucose isomerases, pectinases, agarases, chitinases, alginases, lipases, DNases, proteases, and esterases (Leon et al., 2000; Jayachandra et al., 2012). In this study, only the LAB isolates (BALOR 2-3, BALCR 5-2) showed extracellular proteolytic activity (casein degradation). In aquaculture, it has been suggested that extracellular enzymes such as proteases and lipases help the nutrition of cultured animals (Balcázar et al., 2006; Farzanfar, 2006). However, Quesada-Herrera et al. (2004) reported that production of these enzymes by pathogens is a virulence factor.

The LAB isolates (BALOR 2-3 and BALCR 5-2) were resistant to all antibiotics tested in accord with Halami et al. (2000) and Coppola et al. (2005), who mentioned that lactic acid bacteria were normally resistant to penicillin, ampicillin, vancomycin, and ~~cloramphenicol~~. Conversely, the bacillus (BCR 4-3) isolate was resistant to bacitracin but sensitive to penicillin, amoxicillin, and gentamicin. Kim and Austin (2008) emphasized that antibiotics and other antimicrobials were still applied in aquaculture. Therefore, probiotic strains that are resistant to antibiotics are preferred ~~and~~ for long term colonization of the intestine.

In nature, the equilibrium between beneficial and pathogenic microorganisms occurs due to antagonism between them (Balcázar et al., 2006). In this work, the selected isolates did not show antagonistic activity against *V. sinaloensis* VHPC23. Competitive exclusion of pathogenic bacteria is recommended for selecting beneficial microorganisms. However, the lack of antagonism against pathogenic bacteria can be reinforced with pH changes in the digestive tract, adhesion inhibitors, production of hydrogen peroxide, or competition for nutrients (Bjorn et al., 2003; Farzanfar, 2006).

The molecular identification of LAB isolates (BALOR 2-3, BALCR 5-2) showed high identity with *Leuconostoc mesenteroides*, which is a LAB found in milk, dairy products, seafood products, plants, and the gastrointestinal tract of endothermic animals (Cuervo et al., 2010). Strains of *L. mesenteroides* have been used as probiotic in humans (Kekkonen et al., 2008) and chickens (Seo et al., 2012). In aquaculture, *L. mesenteroides* has shown promising probiotic characteristics in snakehead fish (*Channa striatus*) (Allameh et al., 2012). The isolate BCR 4-3 showed high identity with *Bacillus licheniformis*, which is a species widely distributed in soil and many other environments, including milk and food (Logan and De Vos, 2009). Strains of *B. licheniformis* have been used as probiotic to equilibrate microflora in the intestine of humans (Sorokulova et al., 2008) and pigs (Alexopoulos et al., 2004). In aquaculture, *B. licheniformis* has been used as probiotic in Persian sturgeon (*Acipencer persicus*) (Faramarzi et al., 2011), shrimp (*Penaeus japonicus*) (Zhang et al., 2011), and giant freshwater prawn (*Macrobrachium rosenbergii*) (Kumar et al., 2012).

In conclusion, the results of this study showed that ~~B~~. *licheniformis* (BCR 4-3) and *L. mesenteroides* (BALCR 5-3 and BALOR 2-3) showed basic characteristics to be used as probiotics in mollusk larvae cultures.

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บทคัดย่อ

การแยกและจำแนกลักษณะของแบคทีเรียที่มีศักยภาพเป็นโปรไบโอติกและเหมาะสมต่อการเพาะเลี้ยงตัวอ่อนของหอย

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ทางเดินอาหารของ *Crassostrea gigas*, *C. corteziensis* และ *Atrina maura* ได้ถูกคัดกรองเพื่อหาแบคทีเรียรูปแท่ง และแบคทีเรียที่สร้างกรดแลคติกที่มีคุณสมบัติเป็นโปรไบโอติกสำหรับหอยสองฝา การจำแนกลักษณะของเชื้อที่แยกได้ ได้แก่ ย้อมด้วยสีแกรม รูปร่างลักษณะ การเรียงตัวของเซลล์ การทำงานของเอนไซม์คาตาเลสและการทำให้เม็ดเลือดแตก ความไม่เข้ากับน้ำ การจับกลุ่มกันเอง การจับกลุ่มร่วมกันต้าน *Vibrio sinaloensis* VHPC23 การสร้างไบโอฟิล์ม การนับ colony forming units (CFU) ความทนทานต่อ NaCl และความเป็นกรด-ด่าง ความสามารถในด้านเป็นเอนไซม์ ความต้านทานต่อยาปฏิชีวนะ การเป็นปรปักษ์ต่อ *Vibrio sinaloensis* VHPC23 และการจำแนกชนิดทางอณูชีววิทยา พบเชื้อที่แยกได้ 3 isolate (BCR 4-3, BALCR 5-2, and BALOR 2-3) ที่ไม่แสดงคุณสมบัติทำให้เม็ดเลือดแตก มีความทนทานสูงต่อความเป็นกรด-ด่าง และความเค็ม มีศักยภาพในการยึดเกาะสูง มีการจับกลุ่มกันเองและร่วมกันได้สูง มีการสร้างไบโอฟิล์ม เจริญเติบโตดี และมีความต้านทานต่อยาปฏิชีวนะบ้าง Isolate เหล่านี้ได้ถูกระบุชนิดเป็น *Bacillus licheniformis* (BCR 4-3) และ *Leuconostoc mesenteroides* (BALCR 5-2 and BALOR 2-3) สายพันธุ์เหล่านี้แสดงคุณลักษณะพื้นฐานที่สามารถนำไปใช้เป็นโปรไบโอติกในการเพาะเลี้ยงตัวอ่อนของหอยได้

คำสำคัญ: แบคทีเรียรูปแท่ง แบคทีเรียที่สร้างกรดแลคติก ตัวอ่อน หอย โปรไบโอติก

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