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Research Articles

Isolation and characterization of potential probiotic bacteria from pustulose ark (*Anadara tuberculosa*) suitable for shrimp farming

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ABSTRACT. In aquaculture, probiotics have been tested for enhancing the immune system and promoting growth and survival rate of many marine species like shrimp and mollusks. In order to isolate bacteria with a high probiotic potential for marine shellfish aquaculture, homogenates of the gastrointestinal tract from adult mangrove cockle, *Anadara tuberculosa*, were obtained to perform *in vitro* and *in vivo* assays. Isolates were tested *in vitro* for hemolytic activity, hydrophobicity, tolerance to ammonia nitrogen, salinity and pH as well as for growth kinetics, extracellular enzymatic activity, autoaggregation, coaggregation and molecular identification. Three bacteria with high degree of hydrophobicity (>60% adherence to p-xylene) and four bacteria with medium hydrophobicity, which showed different patterns of attachment to monopolar solvents (chloroform and ethyl acetate) and a high tolerance to ammonia nitrogen (200 mg L^{-1}), were selected. Six different treatments: T1 (without addition of cultured bacteria); T2 (MAt29, *Enterococcus casseliflavus*); T3 (MAt35, *Citrobacter koseri*); T4 (GAtB1, *Bacillus subtilis subtilis*); T5 (GAt7, *Staphylococcus* sp.); and T6 (1:1:1:1 mix of strains T2, T3, T4 and T5), were used to evaluate the specific growth rate, and cellular immune response of the shrimp *Litopenaeus vannamei*. The best specific growth rate was observed for T6 and T4 treatments related to *Bacillus subtilis subtilis*. A significant difference in total hemocytes count ($P < 0.05$) was found for T4 treatment with respect to control group. Strains isolated from *A. tuberculosa* had a beneficial effect on the growth and immune response of *L. vannamei*, so they have potential use as probiotics in aquaculture of marine shellfish.

Keywords: *Anadara tuberculosa*, probiotics, bacilli, immune response, aquaculture.

Aislamiento y caracterización de bacterias de la almeja "pata de mula" (*Anadara tuberculosa*) con potencial probiótico para el cultivo de camarón

RESUMEN. En acuicultura, los probióticos han sido utilizados para incrementar respuesta inmune, crecimiento y supervivencia en especies marinas incluyendo camarones y moluscos. Con el propósito de aislar bacterias con un alto potencial probiótico para el cultivo de especies marinas, se obtuvieron homogeneizados del tracto gastrointestinal de adultos de "pata de mula" *Anadara tuberculosa* para realizar ensayos *in vitro* e *in vivo*. Las cepas se evaluaron *in vitro* para determinar actividad hemolítica, hidrofobicidad, tolerancia a nitrógeno amoniacal, salinidad y pH, cinética de crecimiento, actividad enzimática extracelular, autoagregación y coagregación, y finalmente se identificaron por métodos moleculares. Se seleccionaron siete bacterias; tres con alto grado de hidrofobicidad (>60% de adherencia a p-xileno) y cuatro con hidrofobicidad media, que mostraron diferentes patrones de adhesión a solventes monopolares (cloroformo y acetato de etilo) y alta tolerancia al nitrógeno amoniacal (200 mg L^{-1}). Se realizó un ensayo *in vivo* para evaluar la tasa de crecimiento específico y la respuesta inmune celular en camarón *Litopenaeus vannamei*, utilizando seis tratamientos: T1 (sin adición de bacterias); T2 (MAt29, *Enterococcus casseliflavus*); T3 (MAt35, *Citrobacter koseri*); T4 (GAtB1, *Bacillus subtilis subtilis*.); T5 (GAt7, *Staphylococcus* sp.); y T6 (mezcla 1:1:1:1 de T2, T3, T4 y T5). La mejor tasa de

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crecimiento específico se obtuvo con los tratamientos T6 y T4 adicionados con *Bacillus subtilis subtilis*. Se encontró una diferencia significativa en el conteo de hemocitos ($P < 0,05$) para T4 con respecto al grupo control. Las cepas aisladas de *A. tuberculosa* tuvieron un efecto benéfico sobre el crecimiento y respuesta inmune de *L. vannamei*, por lo que tienen potencial como probióticos en la acuicultura de organismos marinos.

Palabras clave: *Anadara tuberculosa*, probióticos, bacilos, respuesta inmune, acuicultura.

INTRODUCTION

In marine environment, bacteria not only carry out vital functions for the functioning of ecosystems, but also they have an important role as symbionts of marine invertebrates such as shrimp and filter feeding mollusks. In nature, there are no bacteria-free mollusks because resident and transient microbiota in the gastrointestinal tract (GIT) helps with food degradation and oxidative processes, facilitating host development in different environments (Harris, 1993).

The "pata de mula" or pustulose ark *Anadara tuberculosa* (GB Sowerby I, 1833), belonging to the *Arcidae* family, is a mollusk living among mangrove roots, a stressful environment. The high concentration of organic matter and physicochemical variations in temperature, salinity and oxygen concentration in the mangrove mud and interstitial water (Cervantes-Duarte *et al.*, 2010), suggest special characteristics for the fauna that inhabit in mangrove environment that would encourage the development of its gut microbiota. Consequently, it may be assumed that microbiota of *A. tuberculosa* could favor its survival in the mangrove mud. Therefore, this species could be a good source of bacteria with probiotic potential to be used in commercially important species, such as shrimp *Litopenaeus vannamei* and oysters *Crassostrea* spp., for bacterial and viral disease control, since disinfectants and antimicrobial drugs have limited success (Subasinghe & Arthur, 1997), and the intense use and misuse of antibiotics in aquaculture have led to the emergence and selection of multidrug resistant bacteria (Inglis, 2000; Defoirdt *et al.*, 2007). Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" (FAO, 2006). The mode of action of probiotics in their hosts has not yet been clarified with certainty; although it has been hypothesized several modes of action that still need more investigation (Harris, 1993). However, the effect of probiotic on culture systems has recently been evaluated in several studies (Schrezenmeir & de Vrese, 2001; Irianto & Austin, 2002; Ouwehand *et al*., 2002), and the use of probiotics for improving cultures has spread.

In aquaculture, probiotics have been tested in the last decades with promising results for enhancing the immune system and promoting growth and survival of marine shrimps (Rengpipat *et al.*, 2000; Lin *et al.*, 2004; Wang, 2007; Luis-Villaseñor *et al.*, 2011; Powedchagun *et al.*, 2011) and mollusks (Campa-Córdova *et al*., 2009, 2010; Aguilar-Macías *et al.*, 2010), among other marine invertebrates. The most popular genera used as probiotics are the lactic acid bacteria (LAB) *Lactobacillus* (Reid *et al.*, 1988; Jacobsen *et al.*, 1999; Ehrmann *et al.*, 2002; Reid & Burton, 2002; Venkat *et al.*, 2004; Pascual *et al.*, 2008; Yu *et al.*, 2008; Saran *et al.*, 2012).

The use of probiotics (Fuller, 1989) and other immune stimulants as dietary supplements can enhance innate defense and resistance to pathogens during periods of stress (Bricknell & Dalmo, 2005; Flegel *et al.*, 2008). Probiotics may also improve nutrient availability due to exogenous enzymes secreted into the host intestine or to endogenous enzymes available into the bacterial cells and released when they are lysed by the effect of the acidic environment of hosts' stomach. Both types of enzymes may increase the digestive activity and degradation of diet compounds of the tested animal even if not digestible by its own enzymatic machinery. In that vein, gut microbiota helps convert nutrients into energy, but can also produce some essential nutrients, such as vitamins; hindering the microbial colonization by pathogens either by competitive exclusion for space or through the production of antimicrobial metabolites (Lugioyo *et al.*, 2003) as bacteriocins produced by some *Lactobacillus* (Sanz *et al.*, 2004). Of most importance is to mention the immune modulatory capacity of the microbiota in early response, by activating the immune system of the host (Vrieze *et al*., 2010).

Shrimp and prawn cultures are one of the most important practices worldwide. According to FAO (2012) the world aquaculture production of shrimp was 4,327,520 ton. One of the main objectives in aquaculture is to decreased use of antibiotics without decreasing the shrimp production in order to avoid the usual disadvantages from the use of antibiotics mainly development of resistance and negative environmental effects (Holmström *et al*., 2003).

The aim of this study was to isolate and characterize bacteria with probiotic potential from the gut of *A. tuberculosa* to be tested in cultured white shrimp (*Litopenaeus vannamei*).

MATERIALS AND METHODS

Bacterial isolation

Mollusk collection from mangrove and GIT extraction

Adults of *A. tuberculosa* (shell height: 57 ± 5 mm) were obtained from mangrove environment. Collected animals were dissected in sterile conditions and their gastrointestinal tract (GIT) were extracted and placed in Eppendorf tubes with 300 µl of sterile saline solution (2% NaCl). Samples were homogenized using a sterile pestle and a mechanical homogenizer.

One hundred microliters of GIT homogenates were spread on Petri dishes containing solid non selective media: TSA (BIOXON 211670), LB (DIFCO 244520), Marine Broth 2216(DIFCO 279110); and selective media: MRS (FLUKA 69964) and Rogosa (DIFCO 248020). Each sample was inoculated in duplicate in each medium and incubated at 37°C. These media were also inoculated in duplicate and incubated under anaerobic conditions at 30°C. Dishes were observed at 24, 48 and 72 h and after 10 days of incubation. Each bacterium was cross-streaked in the same medium to obtain pure cultures. Each isolate was stored at -70°C in nutrient broth containing 15% (v/v) glycerol.

The isolation of lactic acid bacteria and spore forming bacilli was performed as follows: GIT was extracted and homogenized as indicated above and 100 µL of GIT homogenates were inoculated by the scattering method in MR Sagar (2.5% NaCl) prepared with Aniline Blue diammonium salt (SIGMA 415049) (200 mg L^{-1}). The rest of the homogenate was incubated at 80°C for ten minutes and subsequently 100µL were inoculated in TSA (2.5% NaCl). All dishes were incubated at 30°C and checked for microbe growth after 24, 48 and 72 h of incubation. White colonies in TSA and dark blue colonies (most probably lactic acid bacteria) in MRS with aniline blue were isolated until obtaining pure cultures. Each isolate was stored as indicated above.

Phenotypic characterization of isolates

Gram stain

Gram staining of the isolates was performed using the commercial kit from Golden Bell (Zapopan, Jalisco, Mexico, 82000).

Catalase test

A pure colony of each isolate was placed on a slide, and then a drop of oxygen peroxide (3%) was added over the colony to observe the formation of bubbles (positive result). Catalase positive isolates were discarded as potential probiotics.

Hemolysis test

Sterile core borers were used to make 6 mm diameter wells over Blood agar plates (Fluka, with 10% v/v human blood). Isolates were cultured in TSB (bacilli, at 37ºC for 24 h) and MRS (at 30ºC for 48 h), centrifuged at 10,000 g for 10 min and double washed. Supernatant were resuspended in saline solution (2%) and adjusted to pH 6.5 ± 0.2 with NaOH (1 M) to avoid false positives.

Cultures were adjusted to an optical density of 1.0 ± 0.005 in a spectrophotometer at 560 nm for standardization. Wells were inoculated with 50 µL of each strain, filling one well with MRS and TSB as negative control, and incubated at 37°C for 24 h. Strains showing no clear halos (γ-hemolytic or non-hemolytic) were selected as potential probiotics, while those having a clear hemolysis zone (β-hemolytic or completely hemolytic) or a greenish halo (α-hemolytic or partially hemolytic) were discarded.

Hydrophobicity test by adhesion to solvents

Microbial adhesion to solvents (*P*-xylene, ethyl acetate, and chloroform) was measured according to the methods of Rosenberg *et al.* (1980), and Xu *et al.* (2009). P-xylene was used because bacterial adhesion to this solvent reflects the hydrophobic or hydrophilic nature of the cell surface. In the same way, each strain was tested for adhesion to an acid polar solvent (Chloroform), and to a basic polar solvent (Ethyl acetate) to describe the properties of electron donor or acceptor at the cell surface of the bacterium. Values under 30% (<30%) were considered as "Low", values between 30 and 60% (≥30%, <60%) were referred as "Medium" and values upper 60% (≥60%) were referred as "High". Strains with low adhesion to *p*-xylene (<30%) were discarded as potential probiotics.

Kinetics of bacterial growth

The growth kinetic of each strain was performed by inoculating 20 µL (1.0 Abs 580 nm) of each isolate in 100 mL TSB or MRS supplemented with 2.5% NaCl. The absorbance (580 nm) was determined at 0, 3, 6, 9, 12, 24, 48, 72, and 96 h. The results were plotted to identify the phases, specially the log phase.

Colony forming unit count (CFU)

Isolates were grown as in kinetics of bacterial growth. Bacterial cultures were centrifuged at 12,000 *g* for 20 min to obtain the cellular pellet that was re suspended in 1 mL of sterile saline solution (2.5% NaCl). Bacterial suspension was adjusted to an optical density of 1.0 ± 0.005 as indicated above. Bacterial count (CFU mL^{-1}) was done by serial dilution method using Petri plates with culture medium (TSA or MRS).

Tolerance to ammonia nitrogen (TAN)

Strains that showed γ-hemolysis as well as medium or high hydrophobicity were tested for tolerance to ammonia nitrogen following the technique of Devaraja *et al.* (2013). Ammonia nitrogen concentrations tested were: 0.05, 0.1, 0.5, 1.0, 5.0, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 100, 120, 140,160,180 and 200 µg L^{-1}. Twenty microliters of each strain culture were inoculated in Falcon tubes with 10 mL of TSB medium with 2.5% NaCl and incubated at 35°C for 24 h. The medium was used as control. The absorbance was determined at 580 nm in a Multiskan GO microplate reader (series 1.00.40 1510-01282) with the software SkanIt RE for Multiskan GO 3.2.0.36.

Salinity and pH tolerance

Falcon tubes with 10 mL of trypticase soy broth were supplemented with 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12% NaCl, inoculated with 20 µL of each isolate and incubated at 35ºC for 24 h. All tests were performed in sterile 96-well flat-bottom plates. Each well was filled with 200 µL of each isolate culture. Absorbance was read in a spectrophotometer Multiskan™ GO Microplate (Thermo Scientific, North Caroline, USA) at 550 nm, using sterile medium as a blank.

For pH tolerance, isolates were grown in TSB and MRS broth with pH of 4, 5, 6, 7, 8, 9, and 10. The pH of media was adjusted by adding HCl or NaOH (1 mol L^{-1}). Bacterial cultures were incubated for 24-48 h at 35ºC. Absorbance was read as in salinity tolerance.

Autoaggregation and coaggregation

The isolates were tested for autoaggregation and coaggregation capacity according to Del Re *et al.* (2000), Kos *et al.* (2003), and Handley *et al.* (1987). Coaggregation was done against *Vibrio sinaloensis* VHPC23.

Extracellular enzymatic activity

Isolates were tested for extracellular protease and lipase activity according to León *et al.* (2000) using the supernatants of broth cultures.

Antagonic activity of isolates

Antagonism activity of isolates against *Vibrio sinaloensis* VHPC23 (Flores-Miranda *et al*., 2012) was determined according to Balcázar *et al.* (2007).

Resistance to antibiotics

Antibiotic resistance of isolates was assayed by the disc diffusion method (Bauer *et al*., 1966). The tested antibiotics were: amoxicillin (10 U), penicillin G (10 U), bacitracin (10 U), and gentamicin (10 µg).

Molecular identification of selected strains

Amplification of the 16S ribosomal DNA and internal transcribed spacer (ITS) sequence of strains was carried out. First, 100 ng of genomic DNA was amplified in 25 µL of a reaction mixture consisting of 2.5 µL buffer 5x, $MgCl_2$ 25 mM, dNTP 10 mM and 2 U of *Taq*DNA polymerase. The PCR primers used for amplification were forward 27f5'-AGAGTTTTGATCCTGGCT CAG-3' and reverse 1492r 5'-TACGGCTACCTTG TTACGACTT-3' (Lane, 1991; Gómez-Doñate *et al.*, 2012) and forward G1-16S5'-GAAGTCGTAACA AGC-3' and reverse L2-23S 5'-GGGTTTCCCCAT TCGGA-3' (Breidt & Fleming, 1996; Kingcha *et al*., 2012) at a final concentration of 0.2 mM. PCR conditions were as follows: pre-heating at 95°C for 5 min, the thermal profile consisted of 35 cycles of denaturation at 95°C for 15 sec, followed by an annealing step at 55°C for 1.5 min and an extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

The amplified product was checked by electrophoresis in a 1.2% agarose gel and a 400-600 bp DNA band was excised and purified using a DNA extraction kit (Qiagen, Germany). In order to increase the PCR product amount for weak amplifications, cloning was performed using pGEM-T easy vector system according to the manufacturer's instructions (Promega Corporation, Madison, WI).

PCR products and vector plasmids containing the fragment of interest were sent for sequencing services to Servicios Genómicos CINVESTAV-LANGEBIO. The obtained sequences were compared against those in data bank using a BLAST program and submitted to the Gene Bank (data base submission number 1755621).

In vivo assay

In order to test the probiotic potential of some isolates an *in vivo* assay was performed in *L. vannamei* experimental cultures.

Preparation of experimental diet with bacteria

Each one of four isolates and their mixture were sprayed on commercial feed (Purina®, Ciudad Obregón, Mexico, 35% protein) at 1×10^6 CFU g^{-1} of food according to Peraza-Gómez *et al*. (2009). The number of bacterial cells for feed inoculation was determined based on the count of CFU per milliliter of a bacterial suspension with an optical density of 1. Dry Oil (DO, Innovaciones Acuícolas, Culiacán, Mexico) was used as an adhesive and feed attractant following manufacturer's instructions. Feed was dried at room temperature for 4 h and then stored at 4°C.

Shrimp collection

"Acuícola Cuate Machado", Guasave, Sinaloa, México provided 180 juvenile shrimps. Weight of the shrimps

at the beginning of the bioassay was 1.8 ± 0.3 g. Shrimps were acclimated 24 h in sterile sea water adjusted to 36 psu at a rate of 2 psu of decreasing salinity per h.

Ten shrimps were placed in each 80 L tank filled with sterilized sea water up to three quarters of its capacity, providing constant aeration, total water changes every five days and water level recovery every three days to compensate for evaporation losses. Every treatment was performed by triplicate. Temperature, salinity and oxygen parameters were monitored and remained at 26 ± 2.0°C, 30 psu and 5.7 ± 1.0 mL L^{-1} DO, respectively. Selected strains from the GIT of *A. tuberculosa* were added to food in order to evaluate them in an experimental culture of juvenile shrimp. Bacterial cultures were spread as required to make a count of 10^6 CFU g^{-1} of food. Experimental design included six treatments by triplicate as follows: a control group (T1) without addition of cultured bacteria; T2 (strain MAt29, *Enterococcus casseliflavus*); T3 (strain MAt35, *Citrobacter koseri*); T4 (strain GAtB1, *Bacillus subtilis subtilis*); T5 (strain GAt7, *Staphylococcus* sp.); and T6 (1:1:1:1 mix of strains T2, T3, T4 and T5). Shrimps were fed three times a day, and treatments were provided every 48 h during 28 days.

Specific growth rate (SGR)

Weight was measured at the beginning and at the end of the assay. SGR was calculated as: SGR (%/day) = 100 ($\ln W_2 - \ln W_1$)/t where:W_1 = initial weight and W_2 = final weight (Ziaei-Nejad *et al*., 2006).

Hemocytes count

After 28 days of treatment, three shrimps per tank, for a total of nine shrimps per treatment were used for hemolymph sample. Approximately 200 µL of hemolymph per shrimp was extracted using syringes previously prepared with 2:1 volumes of anticoagulant (NaCl450 mM, KCl 10 mM, Hepes 10 mM + EDTA - Na_2 10 nM, pH 7.3, with a osmotic pression of 850 mOsm kg^{-1}). Hemolymph samples were placed on ice and processed immediately, then, 50 µl of each solution (hemolymph/ anticoagulant) were mixed with 150 µL of 6% formaldehyde solution (1:3) to perform cell count using a Neubauer chamber. The total hemocytes count (THC) was recorded ascel mL^{-1}.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to examine differences in SGR and hemocyte count. Values of $P < 0.05$ were considered as significantly different. When significant differences were found, Tukey's HSD test was performed to identify the source of these differences ($P < 0.05$). All statistical analysis was performed using Statistica 7.0 software (Stat Soft, Tulsa, OK, USA).

RESULTS

Initial bacterial phenotypic characterization

From a total of 161 isolates (101 from BM and 60 from BN), 36 gamma hemolytic strains were obtained, 32 of which were Gram-positive. For strains obtained by growth on MRS medium (plus aniline blue), only one gamma hemolytic and catalase negative strain was found (GAt7).

Seven strains were selected from the hydrophobicity test: three with high hydrophobicity (MAt35, MAt42, and MAt43) and four with medium hydrophobicity (MAt32, MAt100, GAtB01 and GAt7) (Fig. 1).

Preselected strains´ characterization

Despite the similarity in the percentage of hydrophobicity, selected isolates showed different patterns of bacteria surface properties given that different percentages of adhesion to chloroform and ethyl acetate were observed.

To determine the time that each strain reaches its stationary phase, a growth curve was performed in MRS broth or TSB. Most of the isolates reached their stationary phase after 48 h of culture. GAt7 showed the slowest growth. Colony forming unit counts are shown in Table 1.

Isolates showed high tolerance to ammonia. No significant growth differences at various nitrogen concentrations (0.05 to 200 mg L^{-1} of N) were found for MAt35, MAt100 and MAt32 with respect to control treatment (no $(NH_4)_2SO_4$ added). For MAt42, lower growth was observed at concentrations of 35 to 50 mg L^{-1} of N with respect to control, but significantly higher at 180 mg L^{-1} of N. This growth increase at high N concentration was also observed for MAt43 and GAtB01

The ability of the isolates to grow at different salinity concentrations was tested by inoculating each isolate in TSB medium with different salt concentrations. High bacterial tolerance to salinity was observed (up to 7-8%) but growth in all strains decreased considerably at 11-12%. In general, at pH below 5, strains growth decreased. In the case of the strain MAt42, high resistance to highly alkaline pH levels was found (Table 1).

High percentage of autoaggregation was observed in all strains, reaching their maximum value during the first hour of incubation. MAt29, MAt35 and GAt7 showed the slowest autoaggregation response, but the

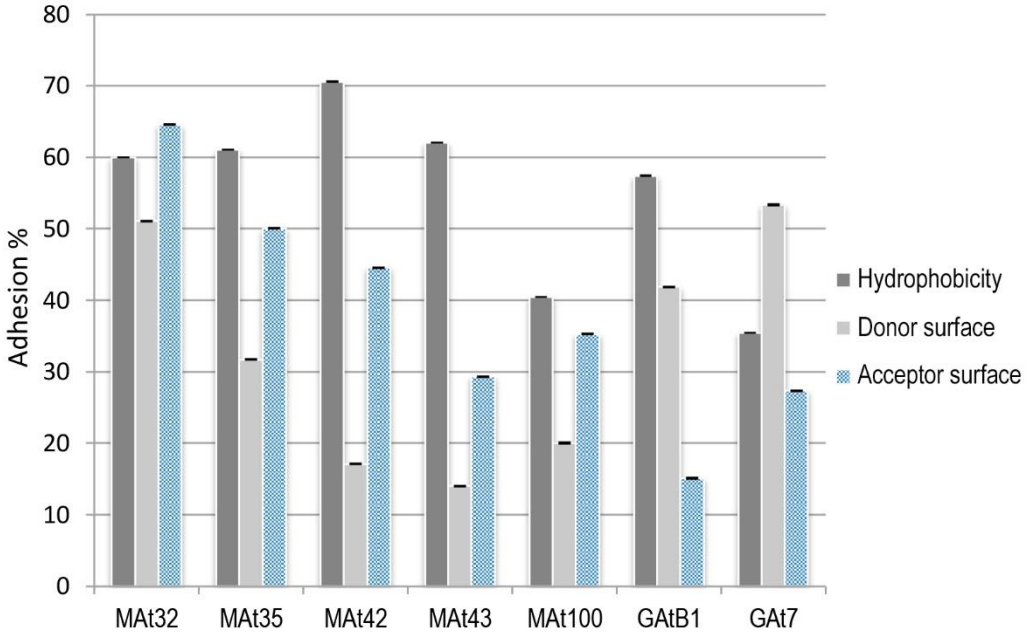

Figure 1. Selected bacterial isolates hydrophobicity and surface characteristics. Lines on bars represent standard error for three replicates.

minimal response corresponded to strain MAt29 (Fig. 2). The highest coaggregation percentage was found for GAt7 followed by strains MAt42 and GAtB1.

Strain MAt35 displayed the highest enzymatic activity although strain GAtB1 also showed a good performance. No antagonistic activity was found against *Vibrio* except for MAt42. With the exception of GAt7, all selected strains exhibited resistance to bacitracin but all of them were susceptible to gentamicin (Table 1).

In a first attempt to prove the probiotic potential of isolated strains in a *in vivo* assay, four strains were selected: strain MAt35 with high hydrophobicity, high enzymatic activity and high autoaggregation percentage; strain GAtB1 with medium hydrophobicity, high enzymatic activity and high autoaggregation and coaggregation percentages; strain MAt29 was also proven in order to compare with a strain with low hydrophobicity but a high coaggregation percentage. Strain GAt7 was also selected as catalase negative bacteria with the highest coaggregation percentage.

Experimental cultures

In the *in vivo* bioassay 100% survival was obtained for all treatments. Although no significant difference was found for total weight, a higher specific growth rate was observed in treatment T6 corresponding to the bacteria mix treatment (T6), followed by T4 treatment related to GAtB01, strain identified as *Bacillus subtilis subtilis* (Fig. 3). A significant difference in THC ($P < 0.05$) was found for T4 treatment in relation to the registered value in the control group (Fig. 4).

DISCUSSION

The large filter feeding capacity of bivalve mollusks, like *A. tuberculosa*, makes them interesting storage batteries of microbiota available in the specific aquatic environment where they live (Romanenko *et al*., 2008). Cultivable bacterial species isolated from the GIT of *A. tuberculosa* could have a potential probiotic effect and a possible application in marine invertebrates' aquaculture.

Selection of probiotics are based on desirable characteristics to provide a specific benefit to the host, so they are not restrictive (Harris, 1993). Nevertheless, certain properties has become priority in order to select safe and effective probiotics for aquaculture (Verschuere *et al.*, 2000). In this sense probiotics must: 1) be safe for the host and humans (as last consumers); 2) not present virulence resistance genes or antibiotic resistance genes; 3) show potential colonization and replication within the host; 4) reach the location where the effect is required to take place; and 5) actually work *in vivo* as opposed to *in vitro* findings (Kesarcodi-Watson *et al*., 2008).

Table 1. Summary of *in vitro* characterization, and molecular identification, of the selected bacteria. NA: not available, ND: not difference respect control, "-": negative result.

		MAt32	MAt35	MAt42	MAt43	MAt100	GAtB1	MAt29	GAt7
Medium		2216	LB	LB	LB	2216	TSA 2.5%	TSA	MRS 2.5%
Gram staining		+	+	+	+	+	+	+	+
Catalase test		NA	NA	NA	NA	NA	NA	NA	-
Hemolysis test		γ	γ	Γ	γ	γ	γ	γ	γ
Hydrophobicity % Adhesionto	*P*-Xylene	60 ± 0.1	61 ± 0.1	70 ± 0.6	62 ± 0.1	40 ± 0.5	57 ± 0.4	8±0.7	35 ± 0.4
	Chloroform	51 ± 0.1	31 ± 0.7	17 ± 0.1	14 ± 0.1	20 ± 0.2	41 ± 0.9	NA	53 ± 0.4
	Ethylacetate	64 ± 0.6	50 ± 0.1	44 ± 0.6	29 ± 0.3	35 ±0.3	15 ± 0.1	NA	27 ± 0.3
CFUml^{-1}Abs 580		7.50 x 10^7	7.50 × 10^7	4.23 × 10^{10}	5.50 × 10^7	NA	1.31 × 10^{10}	1.98 ×10^{11}	2.75 × 10^9
TAN mgl^{-1}		ND	ND	Lower 35-50; Higher 180	Higher 140 y 160	ND	Higher 120-200	-	ND
Tolerance to salinity %	Lower	11.0-12.0	11.0-12.0	11.0-12.0	8.0-12.0	11.0-12.0	9.0-12.0	-	-
	Higher	3.0-4.0	2.0-4.0	2.5-4.0	1.0-6.0	2.5-5.0	2.5-5.0	-	-
Tolerance to pH	Lower	-	5-6, 10	4, 5	4-6, 10	4, 5	4-6, 10	4-6, 10	-
	Medium	7	8, 9	6-8	7.0	6, 10	7.0	7-9	-
	Higher	-	-	9, 10	8, 9	7-9	8, 9	-	-
% Autoaggregation		99.9	99.9	86.3	99.4	99.8	99.4	99.7	76.3
% Coaggregationwith *Vibrio sp.*		17.9	17.9	80.1	8.2	17.4	75.0	69.2	83.3
Enzymatic activity (mm)	Caseine (SM)	27 ± 0.1	30±0.1	-	14.5 ± 3.5	17.5 ± 0.5	27 ± 0.1	21.5 ± 0.5	-
	Gelatine	34 ± 2.0	35±1.0	18.5 ± 0.5	33 ± 3.0	23.5 ± 1.5	29.5 ± 2.5	24.5 ± 0.5	-
	Tween 80	-	-	-	-	-	-	-	19.5 ± 5.5
Antagonism against *Vibrio*		-	-	20 ± 1.0	-	-	-	-	-
Sensitivity (mm)	Penicillin	-	-	2 ± 1.0	-	-	-	-	37.5 ± 0.5
	Amoxicillin	2 ± 1.0	20 ± 0.0	8.5 ± 0.5	-	-	-	8 ± 0.0	34 ± 3.0
	Gentamicin	10.5 ± 0.5	10 ± 0.0	11 ± 0.0	11.5 ± 0.5	13.5 ± 0.5	11 ± 0.0	8 ± 0.1	10 ± 0.1
	Bacitracin	-	-	-	-	-	-	-	35.5 ± 0.5
Specie		*Bacillus licheniformis*	*Citrobacter koseri*	*Bacillus licheniformis*	*Bacillus subtilis*	*Vibrio* sp.	*Bacillus subtilissubtilis*	*Enterococcus casseliflavus*	*Staphylococcus* sp.
% Homology		99	97	98	100	95	100	99	100

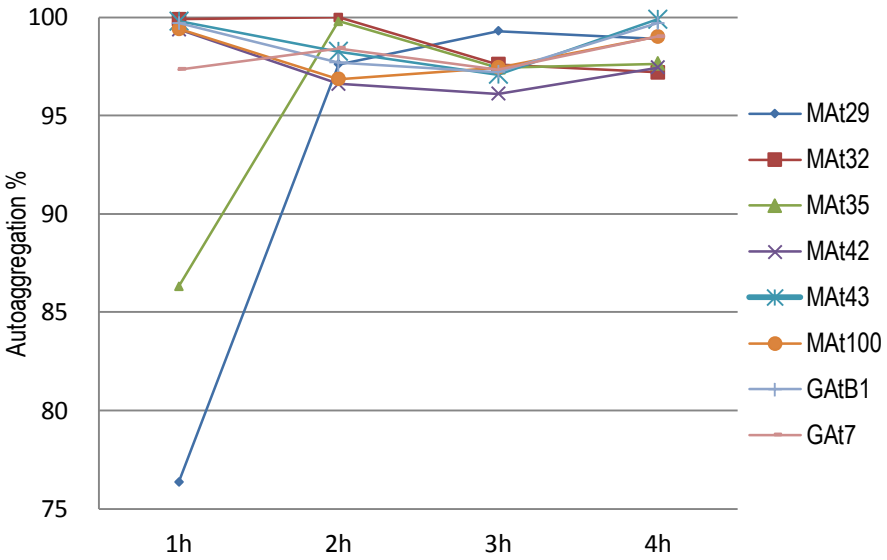

Figure 2. Autoaggregation rate of selected isolates, during four hours of incubation.

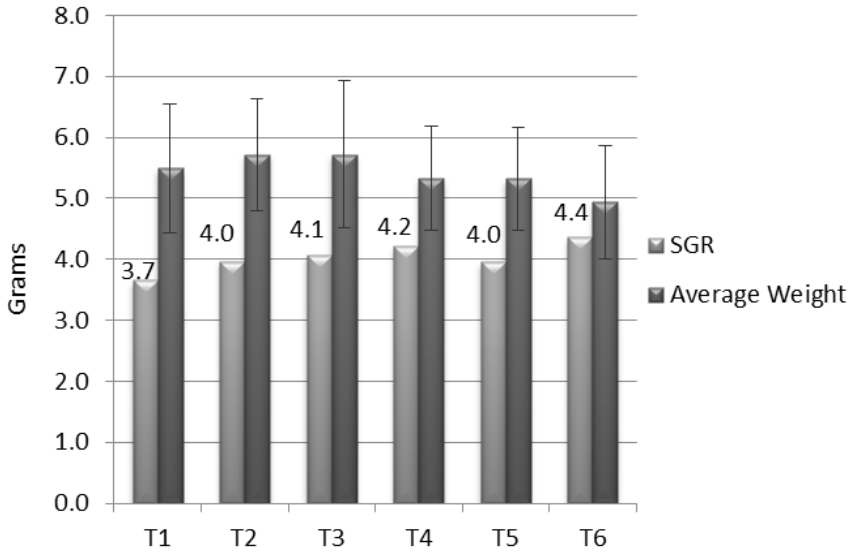

Figure 3. Average weight and specific growth rate of shrimps treated with potential probiotic bacteria for 28 days. T1: Control, T2: MAt29, T3: MAt35, T4: GAtB01, T5: GAt7, T6: mix 1:1:1:1. Lines on bars represent standard deviation n = 30.

To assess the safety of the bacterial strains isolated from the GIT of *A. tuberculosa*, *in vitro* hemolysis and catalase test were performed. The hemolysis test was based on the ability of bacteria to lyse blood cells of culture medium, so only γ-hemolytic bacteria were selected, *i.e.*, those showing no halo of degradation. Thus, it may be assumed that they will not be able of lysing host erythrocytes. One of the self-defense mechanisms that organisms present to fight bacterial infections is the production of hydrogen peroxide, to which some bacterial groups have developed the ability to produce the enzyme catalase to catalyze peroxide decomposition to water and oxygen. As a consequence of these findings, concentration of this enzyme in bacteria has been correlated with their pathogenicity. Since a very low number of strains were negative for catalase test, this test was considered exclusionary just for strains isolated in MRS medium plus aniline blue in order to eliminate possible *Staphylococcus* strains (Cowan & Steel, 1993).

Bacterial surface properties have been associated to the ability of bacteria to adhere to specific substrates provided during *in vitro* tests. Bacteria need to adhere to host tissues in order to colonize it, preventing elimi-

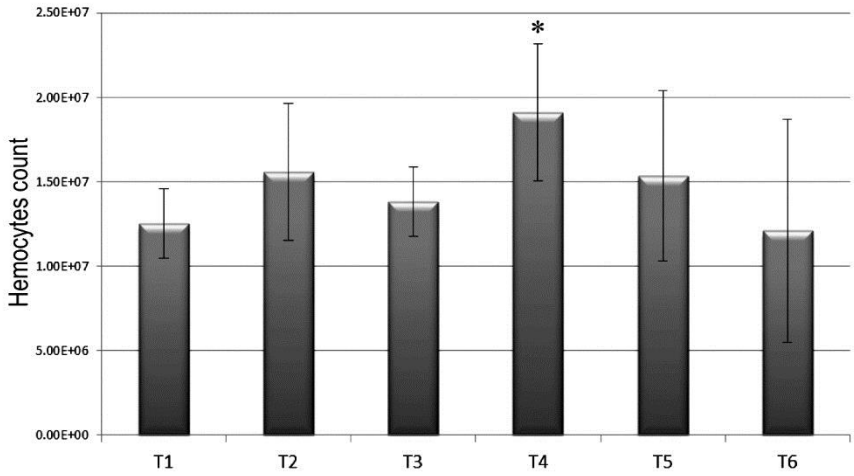

Figure 4. Hemocyte count after 28 days treatment with potential probiotic bacteria. T1: control, T2: MAt29, T3: MAt35, T4: GAtB01, T5: GAt7, T6: mix 1:1:1:1. Lines on the bars represent standard deviation n = 9. Asterisk represents significant difference ($P < 0.05$).

nation by GIT peristalsis. When adhered to host GIT, probiotic scan prevent setting of pathogens by blocking the interaction with specific cell receptors or by inhibiting their anchoring by steric interactions. After initial nonspecific contact between the bacterial surface and GIT tissue is established, specific interac-tions between specialized molecules known as adhesins among bacteria and complementary receptors on host epithelial cells, in addition to proteins of the coat-S occur (Kos *et al*., 2003; Otero *et al.*, 2004). Bacterial surface hydrophobicity can affect the ability of adhesion and autoaggregation of bacteria to different surfaces (Kos *et al.*, 2003). Hydrophobicity of the bacterial surface may be related to the growth of bacteria on hydrophobic substrates, biofilm formation and adherence to host cells, aggregation and flocculation of cells.

Microbial cell surface hydrophobicity is one determining factor in microbial adhesion to bioremediation surfaces because hydrophobic cells have shown a consistent adherence capacity absent from hydrophilic strains. In this sense, strains with high levels of cell wall hydrophobicity may have easier access to soluble materials and organic matter attached to the tank surface or to surface of uneaten shrimp feed particles. *Bacillus* sp. strains with high level of hydrophobicity had higher growth rates and higher bioremediation capabilities (Wang & Han, 2007). This behavior is consistent with presumably *Bacillus* strains found in this work (MAt32, MAt42, MAt43, GAtB1) which showed a higher growth rate and hydrophobicity than those of no *Bacillus* sp. strains with low hydrophobicity (GAt7). These four *Bacillus* sp. strains may be proved for their potential for bioremediation in aquaculture.

Adherence to hydrocarbons is a fast and simple technique to measure hydrophobicity of the bacterial surface nonspecifically (Rosenberg *et al.*, 1980), although presence of highly hydrophobic portions maybe misleading (Rosenberg, 2006). In this sense, hydrophobicity tests are divided into two types: contact angle measurements (CAM) and microbial adhesion to hydrocarbons (MATH). In the latter, hydrophobicity is measured in terms of adhesion, *i.e.*, a hydrophobic bacteria in the *in vitro* system is considered as highly capable of adhering to tissue (Otero *et al.*, 2004). Thus, isolates that showed high or medium (MAt32, MAt35, MAt42, MAt43, MAt100, GAt07 and GAtB01) hydrophobicity can be inferred that will show greater adhesiveness within the epithelium of the host.

Among organics solvents use for MATH, xylene is a nonpolar solvent, while chloroform is an acidic solvent and monopolar electron acceptor, and thus reflects the electron donating (basic) nature of the bacterial surface, ethyl acetate is a monopolar and therefore basic electron donor, and thus reflects the electron acceptor (acidic) nature of the bacterial surface (Bellon-Fontaine *et al.*, 1996). Despite sharing a high or medium hydrophobicity, bacterial surface characteristics differ considerably between isolates, giving a particular pattern for each bacterium of denoting patches or patterns of different polarities in the bacterial surface.

Total ammonia nitrogen (TAN) tolerance was tested since shrimps in high intensity aquaculture ponds incorporates about 20-25% of protein content in feed

while the rest is released to the pond as ammonium and organic nitrogen. Ammonia N transformation is done rapidly by bacteria preventing its accumulation and its related toxic effect to shrimp (Montoya *et al.*, 2002; Castillo-Soriano *et al.*, 2013). Unlike Devaraja *et al.* (2013) who reported growth inhibition of the strains at 25 mg L^{-1}, in this study, high tolerance to ammonia nitrogen (200 mg L^{-1}) was found. The ability of strains belonging to the genus *Bacillus* to produce nitrite and nitrate and employ glucose and ammonium chloride as carbon and nitrogen sources, respectively, is well documented. Ammoniacal nitrogen removal was attributed to nitrification rather than to biomass formation, and a denitrification function was inferred for *Bacillus* sp. under aerobic conditions (Yan *et al.*, 2006). Bacteria may employ one of the following two different ammonia nitrogen assimilation pathways, which also differs between Gram positive and Gram negative bacteria: 1) glutamate dehydrogenase (GDH) pathway with lower energy requirements, and 2) glutamine synthetase-glutamate synthase (GS-GOGAT) route reported as the traditional route to N_2-fixing prokaryotes (Kanamori *et al.*, 1987).

Tolerance to the high concentrations of ammonia nitrogen found in selected bacteria may provide them a potential benefit in intensive shrimp culture acting as nitrogenous waste fixers. Nonetheless, confirmation of a bioremediation effect and nitrogen assimilation pathway must still be tested for selected bacteria in order to determine nitrification, ammoniacal degradation from feeds and global contribution to regeneration rates in intensive aquaculture shrimp systems.

From the *in vitro* salinity test, all isolates showed good growth (except for GAt7) at salt concentrations ranging from 0.5 to 9%. Growth decrease at 10% is consistent to reported by Powedchagun *et al.* (2011) who found a strain, presumably identified as *Bacillus subtilis*, that was able to grow only at salt concentrations up to 8%. Salinity tolerance found in this work could be explainable and consistent with the characteristics of the mangrove species and the marine environment where these bacterial isolates came from. Salinity tolerance is a very important property of these potential probiotics due the wide range of salt concentrations that may account for their transit through the host and the aquatic environment.

Highly hydrophobic bacteria show a high capacity to autoaggregate and, this aggregation ability is directly related to the adhesion properties of the cell. Surface proteins are related to autoaggregation and adhesion of bacteria. Presence of proteinaceous material (glycol-proteins) on the cell surface results in a higher hydrophobicity, while hydrophilic surfaces are associated with the presence of polysaccharides molecules. However, both proteins and carbohydrates are involved in the adhesion process; being glycopro-teins of the S-layer which bind to lectins of the intestinal epithelial cells (Kos *et al.*, 2003). Proteins are autoaggregation mediating molecules and probably the first mediators in the adhesion process (Otero *et al.*, 2004).

The processes and mechanisms of interaction between bacteria and their host still remain unknown (Tuan *et al.*, 2013). It has been hypothesized that one of the roles they can play within the host is the production of extracellular enzymes capable of hydrolyzing the substrates present in the food available in the GIT, and thus facilitating its absorption for the benefit of the host (Ziaei-Nejad *et al.*, 2006; Wang, 2007).In this study, the capacity of isolates to degrade different protease substrates was proved.

It has been found that some bacteria are capable of producing metabolites which act as antimicrobials (Avendaño-Herrera *et al.*, 2005) inhibiting the growth of pathogenic bacteria. They can also limit the growth of pathogenic bacteria by competition for space or attachment surface or by competition for nutrients. In this study, the ability of the selected strains to compete against and inhibit the growth of a strain of *Vibrio* sp. was tested. Findings showed that selected strains were able to grow despite the presence of the pathogen thus limiting their growth by competition; although no halo of growth inhibition was found, so no strain capable of producing an inhibitory metabolite was found yet. Additional research could provide more data required for a complete evaluation.

Benefits that the use of probiotics may confers are: improved growth, feed efficiency, enhanced immune system response, increased disease resistance, and even improved water quality, but still further studies are necessary to thoroughly understand the mechanisms of probiotics, although investigations seems to show that probiotics are more effective when used in the early stages of the host development (Tuan *et al.*, 2013).

The study of the microbiota associated with *A. tuberculosa* inhabiting highly stressful environments provides important information on mangrove ecosys-tems where the accumulation of organic material generates a high bacterial load, potentially beneficial for this and other invertebrate species of commercial interest. Further studies focusing on the mangrove environment may contribute to elucidate the ecological role of these particular systems in order to develop a program of management and sustainable use of their resources.

The *in vivo* assay was a first attempt to prove the probiotic potential of selected bacteria. Our findings

match with Rengpipat *et al.* (2000) who found no significant differences in mean weight but significant differences in hemocyte counts in black tiger shrimp cultures. The increase of THC in white shrimp when GAtB1, presumably *Bacillus subtilis* s*ubtilis*, was administered, suggests a good probiotic candidate, but further studies are required. It is also recommended to test *Bacillus* sp. strains (MAt32, MAt42, MAt43, GAtB1) in larval cultures of white shrimp since the potential of *Bacillus* sp. strains to improve survival of *L. vannamei* larvae has already been proved (Luis-Villaseñor *et al.*, 2011). More specifically, *B. subtilis* (MAt43, GAtB1) and *B. licheniformis* (MAt32, MAt42) are both potentially good probiotics for shrimp farming as has been previously reported (Zhang *et al*., 2011).

This study is the first report of screening for cultivable microbiota and the probiotic potential of bacteria isolated from *A. tuberculosa* from two mangrove environments in the region of Baja California Sur and Sinaloa México, and its effect on improving performance of cultivated white shrimp *L. vannamei*.

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