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Bacillus and *Virgibacillus* strains isolated from three Mexican coasts antagonize *Staphylococcus aureus* and *Vibrio parahaemolyticus*

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One sentence summary: This is the first study to isolate *B. aerius*, *B. oryzicola*, *B.safensis*, *B. boroniphilus*, *B. altitudinis* and *V. senegalensis* from marine ecosystems in Mexico as well as the first study to report their inhibitory effects against both *S. aureus* and *V. parahaemolyticus*.

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ABSTRACT

This study identified marine microorganisms from Mexican coasts that had antimicrobial activity against *Staphylococcus aureus* and *Vibrio parahaemolyticus,* which are known worldwide to be food-poisoning agents. Representative specimens of algae, saline sediment, crustaceans and mollusks were collected. Of the 42 tested strains, 15 inhibited these pathogens. *Bacillus* and *Virgibacillus* strains were identified by 16S rRNA gene sequencing. The strains with the highest inhibitory activity against *S. aureus* were PCRS1-07 (*B. aerius*), BLCG-05 and GUO-01 (*B. pumilus*). The strains GUHC-04, BLCG-05, GUHC-03 (*B. altitudinis*) and BLBSe-05 (*B. oryzicola*) showed higher antimicrobial activity against *V. parahaemolyticus*. Biofilm production by all strains was moderate, but *B. altitudinis* produced a stronger biofilm. This is the first study to isolate *B. aerius*, *B. oryzicola*, *B. safensis, B. boroniphilus, B. altitudinis* and *V. senegalensis* from marine ecosystems in Mexico as well as the first study to report their inhibitory effects against both *S. aureus* and *V. parahaemolyticus*. Bioactivity of spent media from the antagonistic strains cultured as biofilm also demonstrated high antimicrobial activity. The active compounds of the antagonists are currently being studied and tested. Marine ecosystems have the highest bacterial diversity associated with invertebrates and seaweed; however, this bacterial diversity has not been well-studied on Mexican coasts.

Keywords: marine microorganisms; Mexican coasts; *Bacillus*; 16S rRNA gene sequences; antimicrobial activity; biofilm

INTRODUCTION

Many human, veterinary and agricultural diseases are caused primarily by emerging multidrug-resistant bacteria, which are becoming an increasingly serious threat. This resistance occurs naturally via different mechanisms including gene mutations or acquisitions by transformation or plasmid infection (Morens, Folkers and Fauci 2004). However, the misuse and overuse of antimicrobial drugs (i.e. antibiotics, antifungals, antivirals, antimalarials and anthelmintics) accelerate this process (WHO 2018), which is detrimental to public health and increases the

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mortality rates of infectious diseases (Amabile-Cuevas 2010). In hospitals, and even in the community, multidrug-resistant pathogens occur (Solórzano and Miranda 2012), and estimates suggest that antimicrobial resistance leads to ∼2050 000 cases and 23 000 deaths per year in the USA (CDC 2013). Emerging multidrug-resistant pathogenic microorganisms, such as the ESKAPE group (*Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas* and *Enterobacter*), nosocomial bacteria (*Stenotrophomonas maltophilia*, *Salmonella*) and *Vibrio* species (Pendleton, Gorman and Gilmore 2013), represent serious threats to global health (Zavala-Norzagaray *et al*. 2015).

Staphylococcus aureus causes food poisoning and other infections, but the resistant ones cause serious antibiotic-resistant hospital infections. Evidence indicates that *S. aureus* might be involved in sudden infant death syndrome (SIDS) (Zorgani *et al*. 1999), which is the predominant cause of death between one week and one year of life in industrialized countries (Blackwell and Weir 1999). There is no nationwide evaluation data available in Mexico, but a 14.9% prevalence of methicillin-resistant *S. aureus* (MRSA) was found in 659 isolates from infected patients at the Hospital de Pediatría del Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social (IMSS), between 1997 and 2003 (Velazquez-Meza *et al*. 2004). A 35% prevalence of MRSA in 483 isolates from hospitalized patients and carriers was reported from August 2009 to February 2011 at the Hospital de Pediatría, Centro Médico Nacional de Occidente, IMSS, Guadalajara, Jalisco (Villaseñor-Martinez *et al*. 2012). The appearance of vancomycin-resistant strains of this pathogen, which have already been reported in Mexico, as well as resistance to other drugs, highlights the need for new, safe and effective first-line treatments against this pathogen (de Leon-Rosales, Hernandez and Vidal 2015).

Vibrio parahaemolyticus causes human gastroenteritis worldwide, with rare cases of lethal septicemia associated with seafood consumption. It was also found to be responsible for 20–30% of food-poisoning cases in Japan and for seafood-borne diseases in many Asian countries. Pandemic *V. parahaemolyticus* strains have also been isolated in the USA, South America, Africa and Europe, with 45 000 cases annually (Nair *et al*. 2007; Xu *et al*. 2017). These bacteria have been reported scarcely in epidemiological outbreaks in Mexico. A small number of *tdh*+ or tdh (thermostable direct hemolysin or thermostable related hemolysin, respectively, which are considered major virulence factors, and which are highly cytotoxic to human gastrointestinal cells) environmental strains have been isolated from water and fish (Cabrera-Garcia, Vazquez-Salinas and Quiñones-Ramirez 2004). During September and October 2004, more than 1230 cases of gastroenteritis were reported due to strains of *V. parahaemolyticus* O3:K6 in inhabitants of southern Sinaloa: all of the cases were attributed to the consumption of raw or undercooked shrimp collected from the Huizache-Caimanero lagunary system (Cabanillas-Beltran *et al*. 2006). Antimicrobial susceptibility tests showed that most strains (93.8%) were resistant to ampicillin, but sensitive to chloramphenicol (98.8%). Multiple drug resistance has increased significantly from 8.6% (2004–10) to 22.93% (2011–13) (Hernandez-Diaz *et al*. 2015). Zavala-Norzagaray *et al*. (2015) evaluated the presence of *Vibrio* species and their resistance to antibiotics in 64 marine turtles from the states of Baja California Sur and Sinaloa. From the isolated strains, *V. parahaemolyticus* (94.1%) was resistant to at least one commonly prescribed antibiotic (mostly ampicillin).

Marine bacteria, isolated from several sea ecosystems, are an inexhaustible natural resource for novel antibacterial compounds to control human pathogen growth (Jayanth, Jeyasekaran and Shakila 2002). Although products with antimicrobial, antiviral, immunosuppressive, antitumor and anticoagulant activities have been known for a long time (Rosenfeld and Zobell 1947), the number is increasing rapidly, exceeding 18 000, with hundreds of new compounds being discovered annually from them (Pabba, Samatha and Prasad 2011). In Mexico, this subject has been scarcely addressed, although some bioactive marine strains isolated from different substrates have been reported: (i) a *Pseudoalteromonas* strain from the Campeche coasts on the Gulf of Mexico (Cetina *et al*. 2010); (ii) extracts produced by Firmicutes and Actinobacteria from seaweed from Baja California showed antitumor and antimicrobial activity against *S. aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Villarreal-Gómez *et al*. 2010); and (iii) methyl acetate extracts from *Streptomyces*, *Micromonospora* and *Salinospora* strains collected from the Gulf of California displayed antitumor properties against cervical and breast cancer cell lines (Torres-Beltrán *et al*. 2012). Among the Firmicutes, many members of the genus *Bacillus* have been described, such as *B. subtilis* and *B. pumilus*, who produce diverse biomolecules with effective antimicrobial activity (Stein 2005; Collins *et al*. 2016; Ravindran *et al*. 2016; Zidour *et al*. 2017). Thus, the present study's main goals were to isolate *Bacillus* species from seaweed and invertebrates from Mexican coasts and identify their *in vitro* antagonistic properties against *S. aureus* and *V. parahaemolyticus*, including their biofilm formation capacity; because of the interrelationships among bacterial strains that are good biofilm producers, they are also good producers of antibacterial compounds in cells attached to surfaces (like agar), and, as occurs in nature, this ability enhances the exopolymer synthesis (Yan, Boyd and Burgess 2002; Yan *et al*. 2003).

MATERIALS AND METHODS

Isolation of the marine strains

Samples were collected from three Mexican coasts to search for antagonistic bacteria in algae, saline sediment, crustaceans and mollusks (Table 1): Bahía de Lobos (27°15′42.0 N, 110°25′34.6 W) and Bahía de Guasimas (27°15′42.0 N, 110°25′34.6 W) in Sonora and Playa del Carmen (20°40′05.3 N, 87°01′40.7 W) in Quintana Roo (Fig. 1). Samples were processed as follows:

(i) Seaweed and sediments: three samples of seaweed (*Enteromorpha* sp. [Chlorophyta] and *Gracilariopsis* sp. [Rhodophyta] from Bahía de Lobos and Rhodophyta from Playa del Carmen) were collected in sterilized plastic bags and maintained at 4°C. The tissues were washed several times in sterilized seawater and ground under aseptic conditions. Serial dilutions were made and accomplished according to the method of Singh et al. (2011, 2015). Ten samples of saline sediment (50 g) were collected aseptically with a spatula from salt mines at low tidal periods in sterile bottles and transported immediately at 4°C to the laboratory. Each sample was mixed with sterile seawater (1:1) and disintegrated with mortar and pestle to homogenize them. The bacteria were released from these samples by filtration, and differential centrifugation was used to separate the microorganisms and the remaining sediment as described (Anas *et al*. 2016). Finally, the samples were serially diluted in sterile saline solution (10^{-1}–10^{-6}) and 100 μL was spread over the surface of culture media with some modifications according to previous work (Anas *et al*. 2016);

(ii) Crabs (*Callinectes arcuatus*): ten randomly sampled animals were collected and transported in sterile hermetically sealed plastic containers. These crabs were found only in Bahía de Lobos. Once at the laboratory, the crabs were submerged in 10%

Table 1. Collected samples and the number of bacterial strains isolated from different substrates.

Locality	Sample	Scientific name	Strain code[a]	Number of isolates
Bahía de Lobos	Green seaweed	*Enteromorpha* sp.	BLGS	1
	Brown seaweed	*Gracilariopsis* sp.	BLBS	4
	Brown sediment	–	BLBSe	4
	Dark sediment	–	BLDSe	4
	Wild crab	*Callinectes arcuatus*	BLCI	1
	Wild crab	*Callinectes arcuatus*	BLCG	3
	Wild crab	*Callinectes arcuatus*	BLCHP	2
	Marine snail	*Natica chemnitzii*	BLMS	1
Guasimas	Chinese clam	*Veneridae iliochione*	GUCHC	3
	White clam	*Veneridae dosinia*	GUWC	1
	Chinese pink snail	*Phyllonotus erythrostoma*	GUCHS	1
	Oyster	*Crassostrea* sp.	GUO	2
	Hermit crab	*Clibanarius panamensis*	GUHC	4
Playa del Carmen	Red seaweed	Rhodophyta	PCRS1	7
	Red seaweed	Rhodophyta	PCRS2	3

[a]The first two digits indicate the locality: BL, Bahía de Lobos; GU, Guasimas; PC, Playa del Carmen. The next part of the code corresponds to the sample: GS, green seaweed; BS, brown seaweed; BSe, brown sediment; DSe, dark sediment; CI, crab intestine; CG, crab gills; CHP, crab hepatopancreas; MS, marine snail; CHC, Chinese clam; WC, white clam; CHS, Chinese pink snail; O, oyster; HC, hermit crab; RS1, red seaweed sample 1; RS2, red seaweed sample 2.

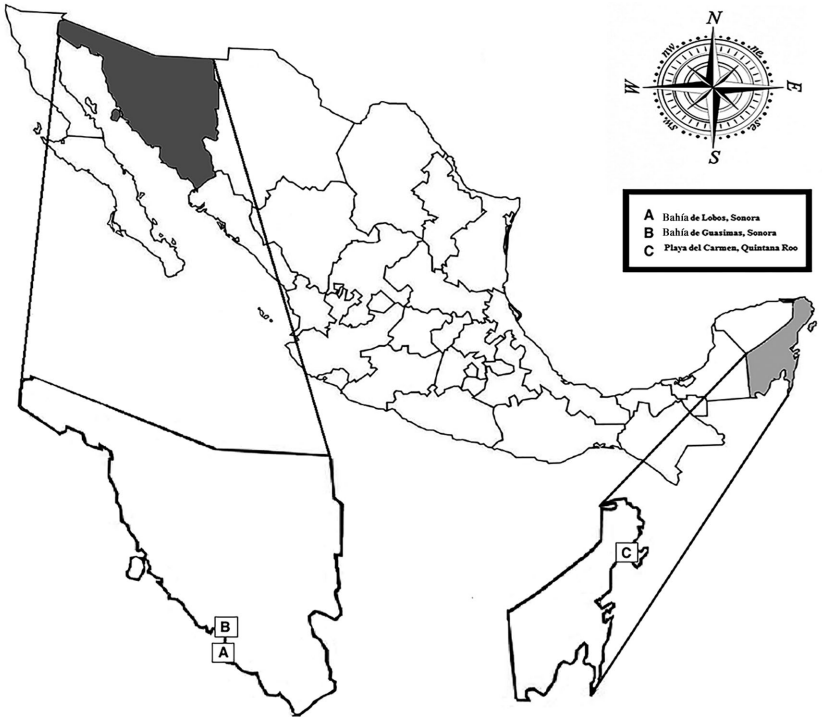

Figure 1. Study area: locations sampled from **(A)** Bahía de Lobos, **(B)** Bahía de Guasimas, Sonora and **(C)** Playa del Carmen, Quintana Roo.

formalin for 30 s, bathed with fresh water and washed in sterile demineralized water to eliminate the surface microflora (Talpur *et al.* 2011). Intestines, gills and hepatopancreas were removed with sterile dissecting materials and mixed with sterile saline solution; each organ was pulverized separately under aseptic conditions with mortar and pestle to prepare inocula by following procedures described by Talpur *et al.* (2011) and Sivasubramanian, Ravichandran and Rajan (2017);

(iii) Fifteen marine snails (*Natica chemnitzii*), Chinese pink snails (*Phyllonotus erythrostoma*) and hermit crabs (*Clibanarius panamensis*) were collected and transported using the same process described above. Specimens were immobilized at 5°C for 30 min, washed in tap water and then washed in sterile water. Afterwards, the surface was sterilized with 70% ethanol (v/v), and their shells were carefully crushed to expose the soft body; the whole gastrointestinal tracts were dissected with sterilized scissors and tweezers in aseptic conditions (Pawar *et al.* 2012; Hu *et al.* 2018) and ground in sterile saline solution. Serial dilutions and inocula were prepared as described above; and

(iv) Clam (*Veneridae iliochione, Veneridae dosinia*) and oyster (*Crassostrea* sp.): samples were collected randomly, and their surfaces were washed in tap water and sterile water; the surfaces were then sterilized with 70% ethanol. Their shells were opened with sterilized knives, and the gills, digestive glands (stomach,

gut and digestive diverticula) and residual tissues (mantle and adductor muscle) were dissected under sterile conditions. Each tissue (5 g) was mixed with 20 mL (1:4) sterile saline and homogenized in sterile grinders. Serial dilutions of the homogenate were spread onto culture media (Castro *et al*. 2002; Wang *et al*. 2014). In some cases, serial dilutions were optimized until the appropriate dilution was reached, and samples were subcultured several times by repeated streaking until pure colonies were obtained.

The dilutions were cultured in tryptic soy broth (TSB, Dibico, Mexico) with 2% NaCl and marine broth 2216 (MB, Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 33°C for 48 h. Isolates were subcultured and incubated for 24 h on tryptic soy agar (TSA, Dibico) with 2% NaCl and marine agar (MA, Becton, Dickinson and Company). The resulting bacterial colonies were selected for identification based on each strain's colonial morphology to obtain axenic cultures. Gram staining and microscopic observation were performed to differentiate the microorganisms' morphology, and oxidase and catalase tests were performed (Dunlap *et al*. 2016). Codes for each strain were determined by the sampling area: Bahía de Lobos (BL), Bahía de Guasimas (GU) and Playa del Carmen (PC). The other part of each code corresponded to the individual sample, as shown in the strain code column in Table 1.

Assessment of antimicrobial activity based on the cross streak method (CSM)

The antagonistic response against human pathogenic bacteria was evaluated using the CSM (Haber and Ilan 2014; Saha and Santra 2014). *S. aureus* and *V. parahaemolyticus* (both isolated from human patients in the Laboratory of Medical Microbiology, Universidad Autónoma de Nuevo León) were used for this experiment. Bacterial isolates from marine coasts were cultured in TSA with 2% NaCl for 18–24 h, then a sample was taken with a sterile swab, streaked longitudinally in a single middle line, dividing the plate into two halves on a fresh plate with TSA-2% NaCl and incubated for 48 h at 28–32°C, until a well-grown bacteria middle line was observed. Next, bacterial suspensions of the pathogens were adjusted to 1 on the McFarland scale (measured at 600 nm, with $\sim 3 \times 10^8$ CFU mL^{-1}) for challenge tests (Fooladi *et al*. 2014), and the pathogens were streaked at a 90° angle, crossing the well-grown bacterial isolate as described (Haber and Ilan 2014) and incubated for 24 h at 28–32°C (Fig. 2). After incubation, a vernier scale was used to measure the bacterial isolate inhibition length to determine the antagonism (Saha and Santra 2014). Three replicates were performed for each experimental set. The results were compared using one-factor ANOVA to assess differences between isolates, followed by a Tukey's HDS test using IBM SPSS v21.0 statistical software.

Amplification and sequencing of bacterial 16S ribosomal DNA (16S rDNA) of bioactive strains

Isolates positive for antagonistic activity were grown overnight in TSA with 2% NaCl at 32°C. One bacterial colony was removed using a sterile loop and re-suspended in phosphate-buffered saline (PBS). Genomic DNA was isolated using the PBS 1X + Tween 20 0.05% extraction protocol (López and Mejía 2012). DNA quality was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA purity was calculated at an absorbance of 260–280 nm, where an optical density (OD) 260/280 ratio of 1.8–2.0 indicated high purity.

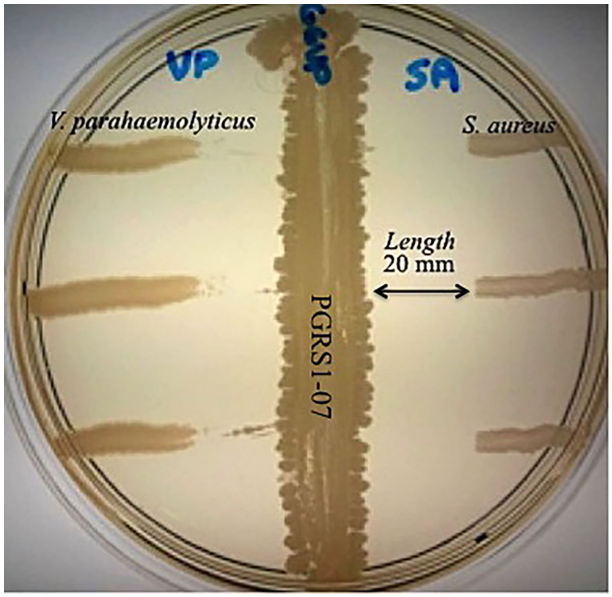

Figure 2. Antagonism test by the cross streak method. Bacterial isolates from marine coasts (i.e. PCRS1-07) were streaked longitudinally in a single middle line. After 48 h the pathogens were streaked at a 90° angle, crossing the well-grown bacterial isolate.

The DNA extracts were PCR amplification templates targeting a region in the 16S rRNA gene of the antagonist strains using the universal primers, 27F (5′-AGA GTT TGA TCM TGG CTC AG-3) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3) with MyTaq DNA polymerase kit (Bioline, MA, USA), in a MultiGene thermal cycler (Labnet International Inc., Edison, NJ, USA). PCR reactions were conducted under the following conditions (Balqadi, Salama and Satheesh 2017): initial denaturation at 94°C for 2 min followed by 35 amplification cycles at 94°C for 35 s, 55°C for 1 min and 72°C for 1 min. A final extension was performed at 72°C for 5 min. DNA extracts were also amplified and sequenced by Macrogen (Seoul, South Korea; http://foreign.macrogen.co.kr/eng/business/seq_16s_rRNA_sequencing.html) using their protocol. The Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used. PCR products were sequenced in the 3730xl automated DNA sequencing system (Applied Biosystems) using the universal primers (Lane 1991; Kim Balaraju and Jeon 2017). The nucleotide sequences of the 16S rRNA gene fragments (~600 bp) were analyzed using BLAST on the NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A homology of > 99% identity was the criterion used to identify the isolates to the species level.

Biofilm production test

The biofilm production assay was performed on microorganisms with the best antagonistic activity from the cultures incubated overnight at 30–33°C in TSB with 2% NaCl and diluted 1:100 in TSB with 2% NaCl. Diluted samples (120 μL) were transferred to a 96-well U-bottom plate, with 10 replicates per strain. The plate was incubated aerobically at 30–33°C for 24 h. The cultures were removed, and the wells washed twice with 200 μL of PBS (pH 7.4) to eliminate non-adherent cells. The plates were dried in an inverted position. The adherent bacteria were fixed

with 200 μL of ethanol (90%) for 2 min, decanted, dried at room temperature and stained with 100 μL of crystal violet. After 10 min the stain was removed and washed three times with sterile distilled water (200 μL). Finally, the water was removed, and the samples were air dried at room temperature. Absorbance was read at 595 nm (Knobloch *et al.* 2001) in a spectrophotometer (Multiscan, MS; Thermo Labsystem, MA, USA). Adhesion ability was classified as strong (OD $\geq$ 1), moderate (0.1 $\leq$ OD $<$ 1) or weak (OD $<$ 0.1) (Lopez-Leon *et al.* 2016).

Bioactivity of spent media from the strains cultured as biofilm

The antimicrobial activities of spent media were carried out as described (Yan *et al.* 2003) with some modifications. Bacteria with the best inhibitory activity were grown in marine broth at 30°C for 4 days and then they were cultivated on the surfaces of sterile semipermeable membranes of nylon discs (diameter 35 mm; Whatman, Brentford, Middlesex, UK). Each membrane was placed over the edges of petri dishes (diameter, 35 mm; height, 13 mm; Corning Glass Works, Corning, NY, USA) prefilled with TSB with 2% NaCl, allowing the membranes to be in contact with the air on the upper side and in contact with the culture media on the lower side. Next, each petri dish with the inoculated membrane was placed into a bigger petri dish (diameter, 60 mm; height 18 mm) and incubated at 30°C. After incubation for 5 days the membranes were removed and the spent media was centrifugated (6000 *g*, 10 min, 4°C) and the supernatant filtered twice (pore size 0.2 μm, Whatman). Antimicrobial activity was performed in petri dishes with TSA inoculated with pathogenic *S. aureus* or *V. parahaemolyticus*. Wells of a diameter of 8 mm were made on these cultures and filled with 100 μL of the supernatant (Aoudia *et al.* 2016). Petri dishes were incubated (30°C for 24 h) and inhibition zones were measured.

Also, cell-free supernatant of antagonist planktonic bacteria were cultivated in TSB with 2% NaCl (30°C for 24 h) to test their antimicrobial activity. Spent media of the cell-free supernatants were also centrifugated and filtrated as described. Fresh TSB was included as a negative control.

RESULTS

Isolation of the marine strains

Forty-two strains were isolated from a variety of marine samples (Table 1). These isolates were composed of three Gram-positive coccobacilli (7.14%), four Gram-positive cocci (9.5%) and 35 rods (11 Gram-negative [26.2%] and 24 Gram-positive [57.14%]). The colonial morphology was also recorded, as shown in Table 2.

Antagonistic assay using the CSM

Fifteen (35.71%) of the 42 strains had antagonistic activity against *S. aureus* or *V. parahaemolyticus* (Fig. 2). The strains GUHC-04 (an average of 16 mm of inhibition), BLCG-05 (15.7 mm), GUHC-03 (14.7 mm) and BLBSe-05 (14 mm) showed higher antimicrobial activity against *V. parahaemolyticus*. Strain PCRS1-07 had the best inhibitory activity against *S. aureus*, with an average of 20 mm of inhibition, followed by GUO-01 and BLCG-05 (18.3 mm, Fig. 3). One-way ANOVA showed a significant difference in each assay against both pathogens ($P < 0.05$), and post hoc comparisons using Tukey's HSD test indicated significant differences between the means of each subset (Fig. 3).

Bioactive strain identification by bacterial 16S ribosomal DNA

Identifying and comparing the specific ribosomal gene sequences with those deposited in GenBank (homology $>$ 99%) showed that strains belonged to species of the *Bacillus* and *Virgibacillus* genus (Table 2). Strain PCRS1-07 was identified as *B. aerius* (DDBJ/EMBL/GenBank accession number KY973966), and BLCG-05 as *B. pumilus* (KY970152), isolated from red seaweed and wild crab, respectively. The remaining *Bacillus* species were *B. oryzicola* (BLBSe-05, KY970153) isolated from brown sediment, *B. safensis* (BLBS-01, KY969737) from brown seaweed, *B. boroniphilus* (BLDSe-03, KY970154) from dark sediment, *B. paralicheniformis* (BLMS-06, KY974263) from marine snail, *B. altitudinis* isolated from oyster (GUO-02, KY969737) and hermit crab (GUHC-03, KY970156; GUHC-07, KY970088), five strains of *B. pumilus* (BLBSe-03, MH54836; BLDSe-04, KY970128; GUO-01, KY969738; GUHC-04, KY969739; PCRS1-06, KY970107) isolated from brown and dark sediment, oyster, hermit crab and red seaweed, respectively, and *V. senegalensis* (BLBS-07, MH548361) from brown seaweed.

Biofilm production test

Five strains, as indicated in Fig. 4, showed a moderate capacity to form biofilm, with an OD between 0.23 and 0.57. Strain PCRS1-07 (*B. aerius*) produced the best adherence, followed by GUHC-07 (*Bacillus altitudinis*).

Bioactivity of spent media

Inhibition zones from 15 to 30 mm were recorded with the supernatants of spent media from the antagonist strains cultured on the surfaces of sterile semipermeable membranes of nylon (Fig. 5). These results of inhibition were like those gathered with the CSM. In contrast, no inhibition zones were observed with the assays performed with the negative control and supernatants of spent media of antagonist bacteria cultured in liquid media.

DISCUSSION

The present study applied mesophilic and aerobic culture conditions to identify bacterial strains with antimicrobial activity against *S. aureus* and *V. parahaemolyticus*, two important agents of food poisoning and antibiotic resistance worldwide (de Leon-Rosales Hernandez and Vidal 2015; Xu *et al.* 2017). Rhodophyta algae were the better source of antagonistic bacteria (*B. safensis*, *B. pumilus*, *B. aerius* and *V. senegalensis*), thus increasing the list of new antagonists against both pathogens. Although *Enteromorpha* (Chlorophyta) was previously reported as the best source with the most epiphytic marine bacteria with antimicrobial properties (at 20°C and 7 days of incubation) against pathogenic bacteria of public health and/or veterinary concern (Lemos, Toranzo and Barja 1985), our findings were negative due to the stringency of the temperature and incubation period (33°C for 48 h).

Marine sediments demonstrated the same importance as sources of antagonist bacteria with four isolates: two *B. pumilus* strains, *B. boroniphilus* and *B. oryzicola*. Sediment was previously reported to contain antagonistic marine bacterium (*Staphylococcus* sp. strain MB30) isolated from deep-sea sediment that exhibited broad-spectrum antimicrobial activity against human pathogenic bacteria and *in vitro* anticancer potential (Lalitha *et al.* 2016). Anas *et al.* (2016) reported bioactive potential in 131 isolates from superficial sediments of the southeast

Table 2. Physical and morphological characteristics of microorganisms isolated from Mexican coasts. Identities of strains with antagonist activity (genus and species) were determined by bacterial 16S ribosomal DNA analyses.

Strain[a]	Gram	Morphology	Cat/Oxi[+]	Colonial morphology	Identity of the strains with antagonistic activity
BLGS-03	–	Rod	+/–	Round, big, opaque	NAA
BLBS-01	+	Rod	+/–	Flat, crater-like appearance, opaque white	*Bacillus safensis*
BLBS-07	+	Rod	+/–	Round, diffuse, crater-like appearance, cream	*Virgibacillus senegalensis*
BLBS-08	+	Coccobacilli	+/–	Creamy, round, opaque white	NAA
BLBS-09	+	Coccobacilli	+/–	Creamy, round, opaque white	NAA
BLBSe-01	+	Rod	+/–	Small, round, opaque	NAA
BLBSe-03	+	Rod	+/–	Small, round, opaque	*Bacillus pumilus*
BLBSe-05	+	Rod	+/–	Small, round, opaque, lobulated	*Bacillus oryzicola*
BLBSe-09	+	Rod	+/–	Light orange, opaque, flat growth	NAA
BLDSe-01	+	Cocci	+/–	Round, opaque white	NAA
BLDSe-02	+	Cocci	+/–	Round, small, opaque yellow	NAA
BLDSe-03	+	Rod	+/–	Flat, transparent, round	*Bacillus boroniphilus*
BLDSe-04	+	Rod	+/–	Round, opaque cream	*Bacillus pumilus*
BLCI-01	+	Cocci	+/–	Small, round, opaque white	NAA
BLCG-01	+	Cocci	+/–	Small, round, opaque white	NAA
BLCG-02	–	Rod	+/–	Creamy, indefinite growth, opaque white	NAA
BLCG-05	+	Rod	+/–	Flat, crater-like appearance, opaque white	*Bacillus pumilus*
BLCHP-01	+	Rod	+/–	Light orange, opaque, indefinite growth	NAA
BLCHP-02	+	Rod	+/–	Small, round, opaque	NAA
BLMS-06	+	Rod	+/–	Small, round, opaque	*Bacillus paralicheniformis*
GUJS-02	+	Rod	–/–	Light orange, opaque, flat growth	NAA
GUCHC-01	+	Rod	+/–	Light orange, opaque, flat growth	NAA
GUCHC-02	–	Rod	–/+	Round, big, opaque	NAA
GUCHC-03	+	Rod	–/–	Flat, transparent, round	NAA
GUWC-01	+	Rod	+/–	Crater-like, striated appearance, opaque cream	NAA
GUCHS-01	+	Rod	+/–	Round, diffuse, crater-like appearance, cream	NAA
GUO-01	+	Rod	+/–	Round, opaque cream	*Bacillus pumilus*
GUO-02	+	Rod	+/–	Small, round, opaque.	*Bacillus altitudinis*
GUHC-03	+	Rod	+/–	Round, opaque cream	*Bacillus altitudinis*
GUHC-04	+	Rod	+/–	Cream, irregular, small	*Bacillus pumilus*
GUHC-06	–	Rod	+/–	Light red, irregular, small	NAA
GUHC-07	+	Rod	+/–	Flat, crater-like appearance, opaque white	*Bacillus altitudinis*
PCRS1-01	–	Rod	+/+	Round, diffuse, crater-like appearance, cream	NAA
PCRS1-02	–	Rod	+/–	Round, diffuse, opaque orange	NAA
PCRS1-03	–	Rod	+/+	Small, round, opaque	NAA
PCRS1-04	+	Coccobacilli	+/+	Creamy, indefinite growth, opaque white	NAA
PCRS1-05	–	Rod	–/–	Round, diffuse, crater-like appearance, cream	NAA
PCRS1-06	+	Rod	+/–	Round, diffuse, crater-like appearance, cream	*Bacillus pumilus*
PCRS1-07	+	Rod	+/–	Round, diffuse, crater-like appearance, cream	*Bacillus aerius*
PCRS2-02	–	Rod	+/+	Round, diffuse, crater-like appearance, cream	NAA
PCRS2-03	–	Rod	+/+	Round, diffuse, crater-like appearance, cream	NAA
PCRS2-05	–	Rod	+/–	Round, diffuse, crater-like appearance, cream	NAA

[+]Cat/Oxi, catalase/oxidase; *NAA, no antagonistic activity.
[a]For abbreviations, see Table 1.

Arabian sea from the phyla γ-Proteobacteria (63%), Bacillales (34%) and Micrococcaceae (3%), which were bactericidal to the human pathogens, *Escherichia coli* and *Pseudomonas* sp., while 20–30% of these were bactericidal to *Vibrio* sp. and *Staphylococcus* sp. Eight isolates, belonging to *Pseudomonas* spp., *Bacillus* sp. and/or *Lysinibacillus* sp., displayed strong bactericidal/cytotoxic properties.

Marine sediment was followed by hermit crabs with three different isolates of *B. altitudinis*, and *B. pumilus* (Table 1), which appeared to be the first report of *C. panamensis* associated with antagonism against clinically important pathogens. Additionally, only one antagonistic isolate each was obtained from *Callinectes* sp. and marine snails (*B. pumilus* and *Bacillus paralicheniformis*, respectively). The current study is the first report of antagonists associated with these hosts, specifically antagonists of *S. aureus* and *V. parahaemolyticus*.

The ANOVA and Tukey's HSD test showed highly significant differences between the antagonist and pathogen challenge tests by CSM, which showed reproducibility, independent of the replicate number for both pathogens ($P < 0.05$). The CSM was chosen as the best technique for screening the microorganism's ability to produce antimicrobial compounds by providing qualitative information on marine microorganismal growth inhibition, to evaluate a large number of different bacterial strains in a short time (Brito de Asis *et al.* 2016).

In this survey, *Bacillus* and *Virgibacillus* species were the unique isolates. A higher number of species could have been identified by using different culture media, anaerobic

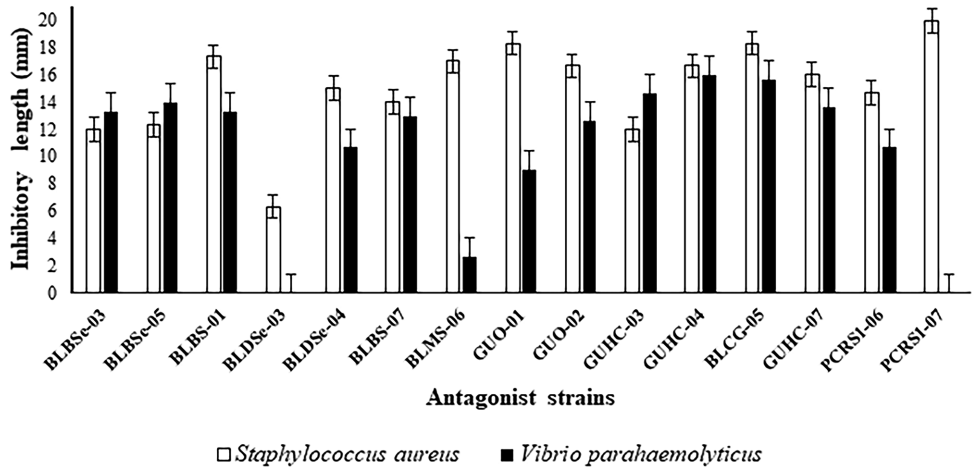

Figure 3. Results of antagonistic assay. Antagonistic activity of the microbial isolates against *Staphylococcus aureus* and *Vibrio parahaemolyticus*. Results are expressed in millimetres (mm). Data were analyzed by the Tukey´s HSD test ($P < 0.05$). Error bars at the top of each column indicate the standard deviation of the mean. For abbreviations, see Table 1.

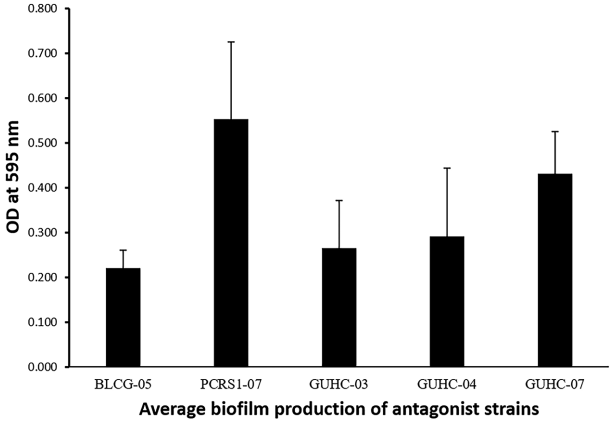

Figure 4. Biofilm production test. Average biofilm production of the five microbial isolates selected by their antagonistic activity. Antagonists' isolates showed a moderate capacity to form biofilm. Results are expressed in optical density (OD). Error bars at the top of each column indicate the standard deviation of the mean. For abbreviations, see Table 1.

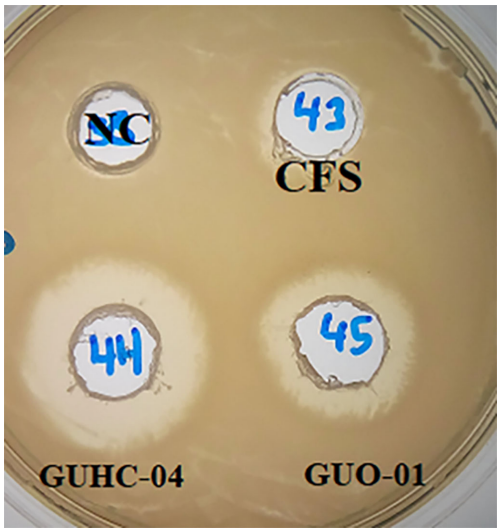

Figure 5. Representative results of bioactivity of spent media. No inhibition zone was shown by negative control (NC) or spent media of cell-free supernatants of antagonistic bacteria cultured in liquid media (CFS). Spent media from the strains cultured as biofilm (for example, GUHC-4 and GUO-01) showed inhibition zones against *Staphylococcus aureus* and *Vibrio parahaemolyticus*.

conditions, and different incubation temperatures and times, which would reveal a great diversity of microorganisms as psychrotrophic, psychrophilic or thermophilic bacterial populations (Faghri *et al.* 1984; Talpur *et al.* 2011; Anas *et al.* 2016). *Bacillus* is frequently reported to have antagonistic activity, as they produce many antimicrobial substances (Sharma, Kapoor and Neopaney 2006), but *Vigibacillus* sp. has only been reported as an antagonist of *S. aureus* (Gad 2017).

Herein we identified species with antagonistic effects against *S. aureus* (a Gram-positive) and *V. parahaemolyticus* (a Gram-negative). *Bacillus* strains have been screened for their potential antimicrobial activity against pathogenic bacteria and their ability to produce a host of enzymes, antibiotics, antimicrobial peptides, lipopeptides, iturines, polymyxins, fengycins and, mainly, bacteriocins and surfactins; the last two compounds are responsible for reducing Gram-positive and Gram-negative pathogen populations (Cole *et al.* 2006; Line *et al.* 2008; Kim *et al.* 2009; Ravindran *et al.* 2016). *Bacillus* has further beneficial applications: lipopeptides, lantipeptides and cyclical peptides with antimicrobial activities are reported to be active metabolites of *B. pumilus* and *B. paralicheniformis* (Collins *et al.* 2016). Additionally, a strain of *B. altitudinis* was found to be an excellent antagonist against the oomycete, *Phytophthora nicotianae* (Jin *et al.* 2011). *Bacillus aerius* has not been directly reported to have antagonistic activity, but some reports reveal that it produces thermophilic lipase with antitumor properties, making it a possible candidate for therapeutic applications (Saun *et al.* 2014; Saun, Mehta and Gupta 2014).

This is the first report from marine ecosystems in Mexico of *Bacillus* against bacteria of public health concern, where the highest inhibition was demonstrated by the strains PCRS1-07 and BLCG-05 (*B. aerius* and *B. pumilus*) against *S. aureus* and *V. parahaemolyticus*, respectively. Also, this is the first report of *B. aerius*, *B. altitudinis*, *B. oryzicola*, *B. safensis* and *B. boroniphilus* being isolated in Mexico. The presence of microorganisms with antimicrobial properties from Mexican marine ecosystems adds to

the scarce information available on this subject by Cetina *et al.* (2010), Villarreal-Gómez *et al.* (2010), and Torres-Beltrán *et al.* (2012).

It has been demonstrated that cells attached to agar surfaces enhanced the production of antibacterial compounds that planktonic cells could not produce. Bacterial growth on agar (like in the CSM assay) constitutes a biofilm-like growth. Bioactivity of spent media from the antagonistic strains cultured as biofilm was demonstrated with the inhibition zones observed in the assays, however spent media of bacteria cultured in liquid media did not show any activity (Yan, Boyd and Burgess 2002; Yan *et al.* 2003). Bacterial strains that are good biofilm producers may be used to produce inhibitory substances (Avendaño-Herrera, Lody and Riquelme 2005) in areas such as public health, aquaculture and agriculture, where using substrates from *Bacillus* strains represents an important approach in these practices that are currently affected by new pathogenic microorganisms (Stein 2005; D'Alvise *et al.* 2014). The biofilm production results in this study showed a potential use for these strains on a larger scale by applying the active metabolites that they produce (Yan, Boyd and Burgess 2002; Yan *et al.* 2003).

We reported five *B. pumilus* strains that had antimicrobial capability, and this is the most important species with previously reported antimicrobial properties against several pathogens (*V. parahaemolyticus, V. anguillarum, V. alginolyticus, Listeria innocua, L. monocytogenes* and *S. aureus*) (Leyton *et al.* 2012; Zidour *et al.* 2017). Thus, *Bacillus* species are excellent candidates with environmental ubiquity, persistence and stability under extreme conditions as their spores can tolerate and proliferate various environmental conditions to inhibit and compete against other potential human pathogens (Prieto, O'Sullivan and Tan 2014). In conclusion, these marine isolates may be a future research option against other clinically relevant bacteria. Using marine microorganisms to fight infectious diseases is a research field that continues to grow. These isolates can inhibit pathogenic bacterial growth, making them candidates for developing new substances that have antimicrobial activity. Their active compounds are currently being studied and tested.

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