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MICROBIOLOGY OF AQUATIC SYSTEMS

Marine *Bacillus* spp. Associated With the Egg Capsule of *Concholepas concholepas* (Common Name "Loco") Have an Inhibitory Activity Toward the Pathogen *Vibrio parahaemolyticus*

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Abstract The pandemic bacterium *Vibrio parahaemolyticus*, isolated from seawater, sediment, and marine organisms, is responsible for gastroenteric illnesses in humans and also cause diseases in aquaculture industry in Chile and other countries around the world. In this study, bacterial flora with inhibitory activity against pathogenic *V. parahaemolyticus* were collected from egg capsules of *Concholepas concholepas* and evaluated. The 16S rRNA fragment was sequenced from each isolated strain to determine its identity using the GenBank database. A phylogenetic analysis was made, and tests for the productions of antibacterial substance were performed using the double-layer method. Forty-five morphotypes of bacterial colonies were isolated, 8 of which presented an inhibitory effect on the growth of *V. parahaemolyticus*. 16S rRNA sequence and phylogenetic analysis show that these strains constitute taxa that are phylogenetically related to the *Bacillus* genus and are probably sister species or strains of the species *Bacillus pumilus*, *Bacillus licheniform,* or *Bacillus* sp. It is important to determine the nature of the antibacterial substance to evaluate their potential for use against the pathogen species *V. parahaemolyticus*.

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Introduction

The pandemic bacterium *Vibrio parahaemolyticus* is the responsible for 40% to 70% of gastroenteric illnesses caused by the consumption of molluscs, crustaceans, and uncooked fish [4, 10]. This contamination can cause pandemic episodes, human mortalities, huge economic losses, and social crises in aquaculture industrial areas where these marine resources are fished and processed. The appearance of *V. parahaemolyticus* was detected for the first time in Antofagasta, Chile, in 1998, when it affected 340 people [15]. However, in terms of epidemiology, outbreaks have decreased during recent years, even though suitable environmental conditions exist in this region for the proliferation of this pathogen (sea temperature and nutrients).

The use of antibiotics is a frequent practice for treating this kind of illness, but their excessive use has caused an evolution in the bacteria, which have become resistant to these drugs [9]. It is fundamental to isolate and cultivate bacterial strains that have the ability to inhibit the growth of *V. parahaemolyticus*, for prophylactic use against this pathogen. The antagonistic bacteria–bacteria interactions, which involve the inhibition of bacterial growth, correspond to several mechanisms: competition for nutrients, space or light, or whether it concerns the production of diverse secondary metabolites that can include antibacterial substances [22, 43]. The antagonist bacteria might be called "probiotic," a term that has been defined as "live microorganisms, which in adequate amounts confer a health benefit on the host" [31]. Several mechanisms have been postulated for how probiotics improve the health of the organisms that consume them, including the production of compounds (bacteriocin, lysozyme, protease, and hydroxide peroxide) that inhibit the growth of pathogenic bacteria. Also, bacterial metabolites can be isolated from some microorganisms and, once purified, can be used to limit the

colonization of submerged surfaces in marine environments by unwanted organisms [3].

There are few studies on marine *Bacillus* [18], and most phylogenetic studies on this genus [7] are based on terrestrial isolates, particularly because important clinical pathogens are terrestrial [36]. The genus *Bacillus* consists of about 222 accredited species (http://www.bacterio.cict.fr) distributed widely across terrestrial and aquatic environments [37], including marine sediment [23]. *Bacillus* species are known because of their ability to produce a number of antibiotics [27].

The muricid gastropod *Concholepas concholepas* (Bruguière 1789), also known as "loco," posses a wide latitudinal distribution from the central zone of Perú (12°02″S; 77°07″W) to the south of Chile (55°55″S; 67°16″W), inhabit mainly in the intermareal and submareal zone, with batimetric ranges between 0 and 50 m, where it plays an important ecologic function in the communities there [8, 20, 30, 41]. It is an important commercial species with a great economic interest for the country, where it has been exploited and overexploited during last decades [21]. In Chile, the development of *C. concholepas* culture has experienced serious problems in the larval culture stages due to high mortalities that have been attributed to microorganisms [19]. Adult *C. concholepas* produce a large number of egg capsules, which contain between 668 and 14,250 larvae (capsules of 9.7 and 30 mm, respectively) [13]. Preliminary studies in our laboratory revealed that the capsules that do not successfully finish larval development contain bacterial contamination, mostly vibrios (unpublished data). Based on this finding, the objective of the present study was to determine the presence of bacteria inside the viable capsule of *C. concholepas* and to evaluate the potential antagonist activities of these bacteria against the pathogen *V. parahaemolyticus*.

Materials and Methods

Collection and Maintaining of Capsules

Capsules of *C. concholepas* were collected in the intertidal zone of San Jorge Bay, Antofagasta, Chile, and maintained in the bioassay laboratory at the Applied Microbiology Unit (AMU) of the University of Antofagasta, in bottles of 20 l, at 20°C average, with constant aeration and daily replacement of 0.5-µm filtered seawater.

Sample Processing and Bacterial Isolation

Thirty-two capsules were analyzed microbiologically under sterile conditions in a laminar-flux chamber: each capsule was washed with 0.2-µm filtered seawater, submerged in 90% alcohol, and flamed. Intracapsular content was diluted in 1 ml 0.02-µm- filtered sterile seawater (Isopore Co, Millipore Inc, Bedford, MA, USA) cutting the upper end of the capsule (the end where the larvae hatch out). The content of each capsule was plated on three types of culture medium: Zobell (Difco 2216), ST10 [17], and ST10 with "loco" homogenized extract (20 g *C. concholepas*/1 l ST10). Then the plates were incubated at 20°C for 5 days, and colony-forming units (CFU) developed were isolated and maintained in batch cultures. The criterion used to isolate the strains was the appearance of the colonies, according to shape, pigmentation, elevation, surface, and rim criteria [35]. Part of the intracapsular content was processed to visualize the presence of bacteria by scanning electron microscopy (SEM); the intracapsular content was fixed with glutaraldehyde al 2% (Merck, Darmstadt, Germany) in sterile (0.2-µm filtered, Isopore Co, Millipore Inc, Bedford, MA, USA) seawater for 1 hour at 4°C. Then a wash was carried out repetitively to eliminate salts from seawater with different mixes of sea water and destilated water in proportions of 3:1 (SW/DW), 1:1 (SW/DW), 1:3 (SW/DW), and finally DW for 6 min. After this, samples were gradually dehydrated in ethanol series 30%, 50%, and 70% and finally with acetone 100%, dried to critical point, and shadowed with a mix of Palladio-gold. Images were obtained in a SEM, Jeol trade mark model JSM-25-SII (Tokyo, Japan) at 25 kV.

Selection and Preservation of Inhibitory Bacteria

The "double-layer" method [11] was applied to the selected bacteria that produced antibacterial substance. According to this method, 10 µl (7.1×10^4 cells/ml) of the studied strains were cultivated in the center of a plate with Muller Hinton (DifcoTM, Becton, Dickinson & Co, MD, USA) medium, and the macrocolonies formed were incubated at 20°C for 48 h. The macrocolonies were then exposed to chloroform steam for 45 min, a second layer of semirigid agar tryptone soya broth (TSB, Oxoid Ltd, Basingstoke, Hampshire, England) + 2% sodium chloride, adjusted to pH 8.0 (within the optimal pH range for *V. parahaemolyticus*) with NaOH was then added, which had been previously inoculated with the pathogenic bacterium *V. parahaemolyticus* (2.3×10^4 cells/ml). The clinic strain of *V. parahaemolyticus* used in this study was supplied for Dr. Romilio Espejo from the Institute of Nutrition and Technology of Food (INTA in spanish), University of Chile (strain PM48.5).

The culture was then incubated at 20°C for 24–48 h. The presence of a defined inhibition halo around the macrocolonies was considered as evidence of antibacterial activity. The experiment was done in triplicate, and the level of inhibition was determined by measuring the halo diameter, considering inhibitory activity with values of halo diameter more than 5 mm according to Avendaño-Herrera et al. [2]. In addition, antibiosis tests were made among the isolated bacteria to reveal autoinhibition.

Identification and Phylogenetic Analysis of Inhibitory Bacteria

The 16S rRNA fragment was sequenced to identify inhibitory bacteria, using the described method by Sambrook et al. [34]. The 16S rRNA of each strain was amplified by polymerase chain reaction, using the primer 518F/800R previously described by Amman et al. (1995) [1]. Polymerase chain reaction product was purified with the QiaGen purification kit, following the manufacturer's instructions. Finally, its identity was determined by comparison with the GenBank database.

The data for phylogenetic analysis corresponding to the sequences found in this study and others were obtained from the GenBank database (Table 1). Phylogenetic reconstruction was made using the Distance Method based on the algorithm from the closest neighbor (Neighbor-Joining, or NJ) [25], in the program PAUP* 4.0b10 [42], and the Bayesian method (BI) using the program MrBayes v3.1.2 [16]. The model with the best fit to the data was sought using the Akaike index (AIC), integrated into the JModeltest program [29]. To evaluate the support of the nodes in NJ, a bootstrapping method was used [12] with 1,000 replicates.

The Bayesian analysis began with a random tree that ran simultaneously with four Markov chains. The Monte Carlo (MCMC) analysis was run with 1,000,000 generations, sampling every 100. The first 2,500 generations were ignored following the suggestion by Felsenstein [12]. The 7,500 remainder trees were used for the construction of the consensus tree following the 50% majority rule. The values of subsequent probabilities were calculated according to the percentage of the 7,500 trees that contained the node.

Statistical Analysis

Results were analyzed with analysis of variance (ANOVA). Prior to applying ANOVA to the data, we tested the assumption of homogeneity of the variances (Bartlett's test), normal distribution of the data (Kolmogorov–Smirnov test), and normality of the residuals (Anderson–Darling test), all using MINITAB 14 statistical software. Data that did not comply with these assumptions were subjected to rank transformation. The data were analyzed with one-way ANOVA and multiple comparison tests least significant difference (LSD) with 95% significance [40, 45].

Results

Isolation and Selection of Inhibitory Bacteria

The SEM results show the presence of marine bacteria associated with egg capsules of *C. concholepas* (Fig. 1).

Table 1 Database used for phylogenetic relations between bacterial species of genus *Bacillus* and isolated bacteria from this work: inferers from the nucleotides sequences of the gene 16 S rRNA

Outside groups	
Locus AF323911	*Streptococcus gallolyticus*
Locus AF413523	*Lactobacillus pantheri*
Locus AF110272	*Sarcina ventriculi*
Locus AB045290	*Clostridium perfringens* rrnI operon
Internal groups	
Locus AB362831	*Bacillus* sp. YT0042
Locus AB362832	*Bacillus* sp. YT0315
Locus AB363738	*Bacillus simplex* strain NBRC15720
Locus AB363740	*Bacillus subtilis* strain NBRC16449
Locus AB300599	*Bacillus* sp. BAM520
Locus AB362829	*Bacillus* sp. YT0027
Locus AB363737	*Bacillus pumilus* strain NBRC12094
Locus AM900496	*Bacillus* sp. 6A-4 strain 6A-4
Locus AM900511	*Bacillus* sp. 17B-6 strain 17B-6
Locus AM900497	*Bacillus* sp. 6A-6 strain 6A-6
Locus AM900501	*Bacillus* sp. 6A-10 strain 6A-10
Locus AM900500	*Bacillus* sp. 6A-9 strain 6A-9
Locus AM900499	*Bacillus* sp. 6A-8 strain 6A-8
Locus AM900495	*Bacillus* sp. 6A-2 strain 6A-2
Locus AM900494	*Bacillus* sp. 6A-1 strain 6A-1
Locus AM900770	*Bacillus megaterium* strain SR28C
Locus EF644419	*Bacillus lincheniformis* strain BSP
Locus EF644416	*B. lincheniformis* strain RT7YC
Locus EF644418	*B. lincheniformis* strain RTPE
Locus EF644417	*B. lincheniformis* strain RTS
Locus EF644414	*B. lincheniformis* strain RT8
Locus EF644413	*B. lincheniformis* strain RT2
Locus EF644415	*B. lincheniformis* strain RT7P1
Locus EU168188	*Bacillus* sp. GW-1
Locus EU168189	*Bacillus* sp. GW-2
Locus EU170129	*Bacillus* sp. T10-2
Locus EU169603	Uncultured *Bacillus* sp. clone MZ0201A2

Forty-five bacterial isolates were obtained from a total of 32 capsules, and in 78% of the capsules, CFU development was observed. The marine agar Zobell 2216 (Difco) was the most suitable for bacterial recovery from intracapsular content.

Antibiosis Against the Pathogenic Bacterium *V. parahaemolyticus*

Eight isolated strains (17.7%) show strong antibiosis activity against the pathogenic strain of *V. parahaemolyticus*; they were selected out of the total 45. The eight strains were coded C23, C32, C36, C37, C38, C40, C47, and C51. The inhibitory activity of the selected strains varied, producing

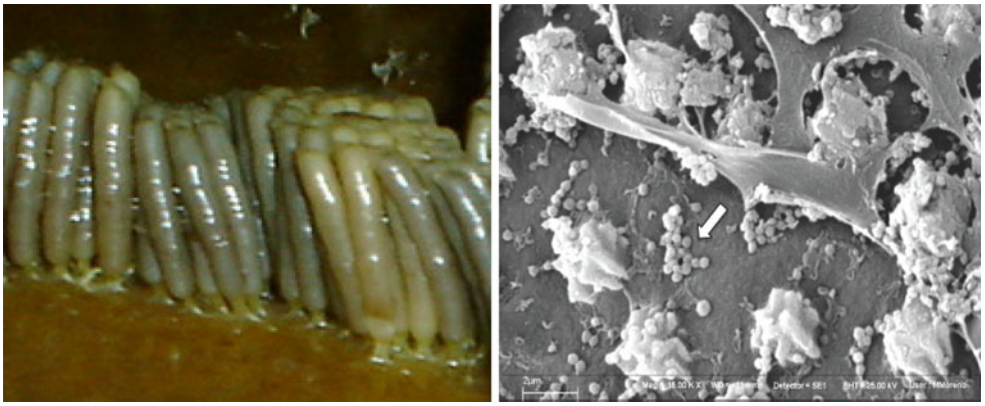

Figure 1 *Left*: Photograph of egg capsules of *C. Concholepas*. *Right*: SEM microphotograph of intracapsular bacteria

inhibition halos from 17 to 27 mm in diameter. These results were reproduced independently in the replicates analyzed, with no significant differences detected among the halos of the strains that showed inhibitory activity ($P>0.05$). The strains with the highest inhibitory activity were C23 and C32 (27 mm) (Table 2). The autoinhibition tests showed that autoinhibition did not occur in any of the isolated bacteria from the capsules.

Identification and Phylogenetic Analysis of the Inhibitory Strains

The 16S rRNA of the inhibitory bacteria was identified using the GenBank database, indicating that 100% of the bacteria with inhibitory activity corresponded to species of *Bacillus* with 99%–100% similitude.

A total of 39 sequences were analyzed, with a length of 606 bp of the 16S RNA. Four taxa were used as an external group (outgroup): *Streptococcus gallolyticus*, *Lactobasillus pantheri*, *Sarcina ventriculi,* and *Clostridium perfringens.* The sequences analyzed in this research were incorporated into the GenBank database under accession numbers GQ980252 (C23), GQ980253 (C32), GQ980254 (C36), GQ980255 (C37), GQ980256 (C38), GQ980257 (C40), GQ980258 (C47), and GQ980259 (C51).

Table 2 Analysis of inhibition of antagonist strains against pathogen bacteria *V. parahaemolyticus*

No.	Strain	Inhibition (mm)
1	C32	27
2	C36	26
3	C37	19
4	C38	21
5	C23	27
6	C40	20
7	C51	17
8	C47	26

The phylogenetic reconstruction showed that the tree topologies resulting from the two methods were similar. The phylogenetic reconstruction is shown in the cladogram results of the NJ analysis (Fig. 2). The values at the nodes corresponded to the bootstrap values for NJ (left) and the later probabilities for BI (right). It was observed that the internal group composed of strains from this research, and species of the *Bacillus* genus had strong statistical support, with bootstrap values of 98% for NJ and 97% for BI. The strains C32, C40 C23, C36, C37, and C38 form a clade with *Bacillus pumilus* and *Bacillus lichenformis*, with bootstrap values of 96% and 91% for the respective methods.

Discussion

Our results confirm the presence of bacteria of the *Bacillus* genus inside the egg capsule of *C. concholepas* that show significant inhibitory effects against the pandemic bacterium *V. parahaemolyticus*. The presence of antagonist bacteria in the capsules could be possible due to vertical transmission from the parents at the moment of copulation or when the female seals the capsules to fix them onto rocks. A similar phenomenon of vertical transmission has been reported in other mollusc of this region [33]. These bacteria could subsequently incorporate into intestinal larval microflora and thus provide with protective action against pathogens during larval development. This point relates to published research indicating the presence of bacteria with isolated inhibitory capacity from the native microflora of the digestive tract of marine or terrestrial organisms [4]. Additionally, this hypothesis is supported by preliminary data (not published) where a decrease in the bacterial concentration of the digestive tract and gonad of adult *C. concholepas* was seen to coincide during the reproductive months.

The antagonism between strains of marine bacteria and referenced strains of marine pathogens is considered to be of interest for aquaculture [4, 11, 26, 32]. The observation of a strong inhibitory effect on the pathogen *V. parahaemolyticus*

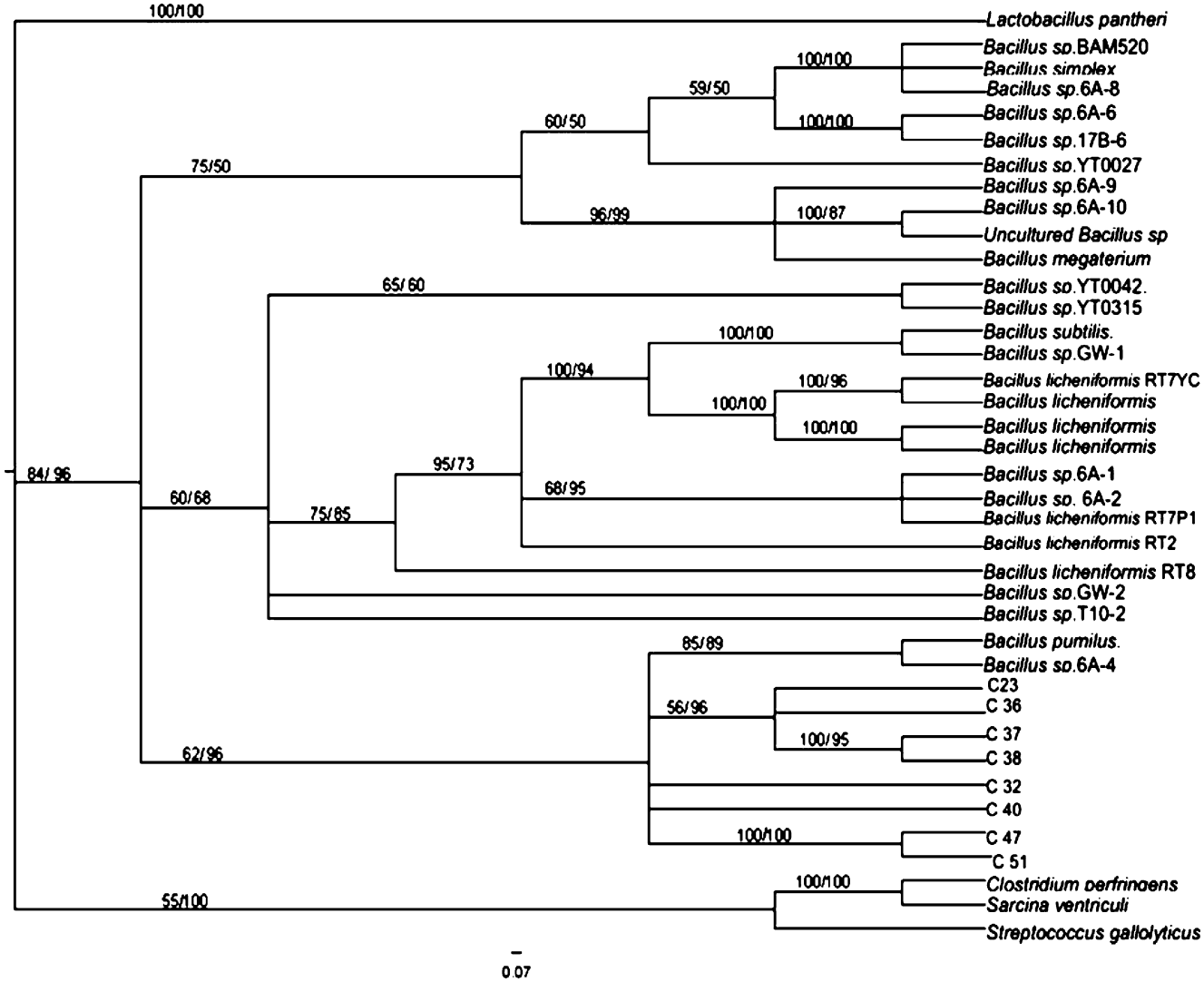

Figure 2 Phylogenetic analysis of 16S rRNA of eight studied bacterial strains by the NJ analysis

was clearly and significantly related to native strains isolated from possible pathogen hosts, as in the present case with *C. concholepas*. The inhibitory mechanism of the bacterium–bacterium interaction was not the focus of this research. However, previous research has suggested that the inhibitory effects of bacteria can possibly involve the production of antibiotics, bacteriocin, lysozyme, protease, and/or hydrogen peroxide and alter pH values due to the production of organic acids [43].

According to the phylogenetic analysis, the isolated *Bacillus* could be sister species or constitute strains from the species *B. pumilus*, *Bacillus lincheniformis,* or *Bacillus* sp. There are reports of the isolation of these species from seawater [5, 27] and from sediment [14].

At present, the amplification and sequencing of 16S rRNA have proved useful for identification and detection of bacterial species [44] like the *Bacillus* genus [18]. On the other hand, other researchers examined the 16S rRNA from 20 isolated of marine *Bacillus* and made phylogenetic comparisons with the *rpoB* gene, thus separating 13 genotypes and identifying 9 species (*B. aquaemaris*, *B. badius*, *B. cereus*, *B. firmus*, *B. halmapalus*, *B. hwajinpoensis*, *B. litoralis*, *B. sporothermodurans*, *B. vietnamensis*). These results demonstrated that the *Bacillus rpoB* gene, which coded for the subunit of DNA directly from RNA polymerase, is a potential biomarker due to the high level of conservation of 16S rRNA and provides a better means of identifying species of the *Bacillus* genus [18].

Although, there are bacteria in the *Bacillus* genus that include human pathogens, for example, *Bacillus anthracis* [6], the majority of *Bacillus* species are not harmful to mammals, including humans. Members of the Bacillales order are very important commercially because they produce important secondary metabolites like antibiotics and bioinsecticides [28]. *Bacillus* enzymes are very effective at breaking down a large variety of carbohydrates,

lipids, and proteins into smaller units. In addition, *Bacillus* species grow in an efficient way with very low-cost carbon and nitrogen sources [39]. The positive effects of the use of bacteria from the *Bacillus* genus have been tested in the culture of commercial important organisms where production is frequently affected by pathogenic vibrio contamination. For example, it was concluded that application of *Bacillus* spp. allowed a continuous culture of shrimp for 160 days, whereas in shrimp cultures where *Bacillus* sp. were not applied, it failed in less than 80 days due to *Vibrio* spp. [24]. It has also been suggested that *Bacillus subtilis* is an effective antagonist against the pathogen *V. parahaemolyticus* in cultures of infected shrimps [4]. In other research, inhibitory effects of *B. subtilis*, *B. pumilus*, and *B. lincheniformis* against pathogens *V. parahaemolyticus* and *V. alginolyticus* isolated from shrimps and brine-shrimp were evaluated, revealing a decrease from 10^8 to 10^2CFU/ml, which suggested that these bacteria could be used as protection against shrimp pathogens [5]. Additionally, other authors postulated that *B. subtilis* and *B. megaterium* have nutritive potential for shrimp cultures [26]. Otherwise, the first results on an association between a marine sponge and lipase-producing *B. pumilus* have been published [46]. The nature of this association was discussed as it resembles a symbiosis, where the bacterium can supply nutrients, give protection from incrustations or predation, and biotransform the chemical compounds eliminated by the sponge [46]. Previous studies carried out by Silva et al. [38], in which *Bacillus* sp. (B9) and *B. pumillus* (B18) were isolated from abalone *Haliotis rufescens*, show a significant antagonistic effect against *V. parahaemolyticus*. These last results are important for our research due to the phylogenetic analysis indicating that isolated *Bacillus* are probably sister species or constitute strains of the species *B. pumilus*, *B. lincheniform*, or *Bacillus* sp.

The results obtained in this study represent a contribution to the recent research on marine bacteria producing antibacterial substance and possible applications in intensive aquaculture. Commercial marine organisms could be treated with biological products that remove *V. parahaemolyticus*, benefiting the local and international markets by ensuring the production from seafood free of this pathogen. The majority of the investigations on the use of *Bacillus* as antagonists against the pathogen *V. parahaemolyticus* are concentrated on shrimp culture. No previous work has reported research on the use of *Bacillus* in *C. concholepas* culture, and the present results make it possible to consider the evaluation of *Bacillus* effects on the culture of this species.

Taking into account the favorable characteristics of bacteria from the *Bacillus* genus, the bacteria isolated in the present research deserve complementary studies but could also form a useful basis to study the nature of the antibacterial substance produced and to evaluate their potential use against the pathogen species *V. parahaemolyticus*. Additionally, this work has provided the possibility that the selected strains could be evaluated for the control of other pathogens affecting intensive cultures of marine invertebrates.

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