Screening of *Bacillus* sp. isolated from coral *Siderastrea stellata* for antimicrobial activity against enteropathogenic strains of *Salmonella* and *Escherichia coli*

Screening de *Bacillus* sp. isolados do coral *Siderastrea stellata* para atividade antimicrobiana contra linhagens enteropatogênicas de *Salmonella* e *Escherichia coli*

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ABSTRACT
Siderastrea stellata is a coral endemic to Brazilian waters and is widely distributed throughout the coast of Bahia state. Exposure of the coral to pathogens stimulates the production of important enzymes by associated symbiont microorganisms. Besides being natural sources of bioactive compounds, these microorganisms possess characteristics that help them survive under extreme conditions. Sixty-eight bacterial strains isolated from the *S. stellata* coral were analyzed for antimicrobial activity against ten pathogenic bacteria. The cultivation of marine isolates was carried out in liquid or solid seawater. Eight isolates produced antimicrobial compounds against at least two of the ten pathogenic bacteria tested. When isolates were grown in Mueller-Hinton broth, a distinct biocidal spectrum was observed, indicating that the culture medium directly influences the production of antimicrobial compounds. Through molecular characterization of activity-positive isolates, they were identified as belonging to the genus *Bacillus*. The bioactive compounds produced by the *Bacillus stratosphericus* SS85 and SS69 strains were stable after storage time for three months. This is the first paper reporting on the antimicrobial activity of microorganisms isolated from coral *Siderastrea stellata*.

Keywords: marine water broth, marine water agar, antimicrobial compounds, marine microorganisms

RESUMO
Siderastrea stellata é um coral endêmico das águas brasileiras e amplamente distribuído por todo o litoral do estado da Bahia. A exposição do coral a patógenos estimula a produção de enzimas importantes por microrganismos simbiontes associados. Além de serem fontes naturais de compostos bioativos, esses microrganismos possuem características que os ajudam a sobreviver em condições extremas. Sessenta e oito cepas bacterianas isoladas do coral *S. stellata* foram analisadas quanto à atividade antimicrobiana contra dez bactérias patogênicas. O cultivo dos isolados marinhos foi realizado em meio água do mar líquida ou sólida. Oito isolados produziram compostos antimicrobianos contra pelo menos duas das dez bactérias patogênicas testadas. Quando os isolados foram cultivados em caldo Mueller-Hinton, um espectro biocida distinto foi observado, indicando que o meio de cultura influencia diretamente na produção de compostos antimicrobianos. Por meio da caracterização molecular de isolados com atividade positiva, eles foram identificados como pertencentes ao gênero *Bacillus*. Os compostos bioativos produzidos pelas cepas de *Bacillus stratosphericus* SS85 e SS69 permaneceram estáveis após três meses de armazenamento. Este é o primeiro artigo

relatando a atividade antimicrobiana de microrganismos isolados do coral *Siderastrea stellata*

Palavras-chave: ágar de água marinha, caldo de água marinha, compostos antimicrobianos, microrganismos marinhos

1 INTRODUÇÃO

Oceans cover most of the earth's surface, house its largest habitats, and are home to most existing life forms on the planet (Mariottini et al., 2016). Oceans have promising potential in the production of biocomposites with unique activities. Within this context, biotechnology opens up several possibilities with the main objective of rational exploration of organisms, genes, and molecules in the marine environment.

It is estimated that the marine environment hosts approximately 3.67×10^{30} microorganisms (Whitman et al., 1998), and the importance of their association with marine organisms goes beyond their influence on the ecosystem. For example, microorganisms living in corals secrete antimicrobial molecules that allow their survival, thereby providing a competitive advantage among species (Giudice et al., 2007). They develop specific adaptive responses, such as the production of toxins, signaling molecules, and other secondary metabolites. They also act in defense, preventing colonization or growth of competitors (Egan et al., 2008). Thus, microorganisms associated with corals represent a reservoir for potential drugs, therapeutic agents, and bioactive molecules with applications in areas of economic interest (Egan et al., 2008).

The species *Siderastrea stellata* is a coral endemic to Brazilian waters and is widely distributed along the coast of Bahia state in northeastern Brazil. The coast of Bahia is known to have the highest concentration of coral reefs and the highest rate of coral species endemism in the South Atlantic Ocean (Leão et al., 2003).

The difficulties of novel antibacterial drug discovery and the increasing number of infections caused by multidrug-resistant bacteria have incentivized researches to find new antimicrobial compounds (Bax et al., 2000). Currently, new combinations of antimicrobials and the enhancement of known molecules is employed, since the last novel class of antibiotics was discovered in the 1980s (Boucher et al 2009; Durand et al., 2019; FDA, 2003; Slee et al., 1987). Thus, a systematic search for bioactive compounds in the environment is required (Donadio et al., 2010; Lam, 2007; Newman et al., 2003).

In the current study, the antimicrobial activity of microorganisms isolated from the coral *Siderastrea stellata* against pathogenic bacteria was screened. Among the tested

microorganisms, eight showed antimicrobial activity against pathogenic strains of *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus*, demonstrating distinct potential for use as sources of biocompounds. This is the first work reporting on the antimicrobial activity of *Bacillus* isolated from the coral *Siderastrea Stellata.*

2 MATERIALS AND METHODS

2.1 SAMPLE SOURCE

Sixty-eight bacterial strains isolated from the coral *Siderastrea stellata* (Sousa et al., 2016) were screened for antimicrobial activity. The strains were kept in marine broth (Difco) supplemented with 20% glycerol at -20° C.

For antimicrobial activity assays, microorganisms from the Instituto Nacional de Controle de Qualidade em Saúde (INCQS) of Fiocruz (Brazil) and the American Type Culture Collection (ATCC) were used. Antimicrobial activity was tested against gram-positive *Staphylococcus aureus* (INCQS 00186), and nine gram-negative bacteria: *Escherichia coli* (INCQS 0310); enterohemorrhagic *E. coli* - EHEC O157 H7 (INCQS 00171); enteropathogenic *E. coli* - EPEC O55 (INCQS 00181); enterotoxigenic *E. coli* - ETEC (INCQS 00218); enteroinvasive *E. coli* - EIEC (INCQS 00170); enteroaggregative *E. coli* - EAEC O111ab (INCQS 00180); *Salmonella enterica* subsp. Serovar Enteritidis phage type 4 (*S.* Enteritidis PT4); *S.* Enteritidis PT11; and *S.* Typhi (ATCC 5339).

2.2 CULTIVATION OF BACTERIA FROM THE *Siderastrea* CORAL AND PRODUCTION OF CRUDE EXTRACTS

Aliquots of 10 µL from the stock of isolated bacteria in marine broth (DIFCO) were transferred into 250 mL Erlenmeyer flasks containing 25 mL of seawater broth (AM) (seawater; Peptona 5 g/L and yeast extract 1 g/L). The flasks were kept under constant agitation in an incubator (Solab) at 120 rpm and 28 °C for 5 days. The growth medium was then centrifuged at 13,000 × *g* for 15 minutes. The supernatant, known as crude extract, was collected, and the pellets were discarded. Antimicrobial activity was tested using the agar-well diffusion method (Anand et al., 2016).

2.3 SCREENING FOR ANTIMICROBIAL ACTIVITY IN THE SUPERNATANT OF MARINE BACTERIA

Pathogenic strains were spread-plated on Muller-Hinton agar and incubated for 16 hours at 37 °C. They were then diluted in 0.9% sterile saline. Inoculum formation was

standardized by spectrophotometry (Thermo Fisher Scientific) to a concentration of 1 × 10^8 CFU/mL, equivalent to the 0.5 McFarland standard, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2012). Next, 100 µL of the pathogenic bacterial dilutions were swabbed across the surface of petri dishes. Using autoclaved tips, 6 mm holes were made in the agar and later inoculated with 100 µL of the crude extract obtained from the growth of coral bacterial isolates. The plates were incubated for 24 hours at 37 °C in a bacteriological incubator (Electrolab). As a control, sterile AM medium was used instead of the crude extract. The resulting halos were then measured with the aid of a millimeter ruler for determining the index of antimicrobial activity. The tests were performed in triplicate.

2.4 CULTIVATION OF CORAL-ISOLATED BACTERIA IN MUELLER-HINTON BROTH

In order to evaluate the production of antimicrobial compounds in different culture media, bacteria that presented antimicrobial activity were cultivated in Mueller-Hinton broth (MH). Bacteria were grown in this medium for 5 days under constant agitation in an incubator shaker (Solab) at 120 rpm and 28 °C. The growth medium was centrifuged at 13,000 × *g* for 15 minutes. The supernatant, called crude extract, was collected, and the pellets were discarded. Cell-free supernatants from each isolate were then evaluated by the well diffusion method, as previously described.

2.5 STABILITY AND STORAGE TIME ANALYSIS

The crude extract from AM medium was tested for shelf-life stability. For this purpose, aliquots of extracts were stored in a refrigerator (2 -8 °C) and in a -20 °C freezer for three months. Freshly obtained extracts were also autoclaved for 15 minutes at 121 °C. The inhibition test was performed by the well diffusion method with extracts from different treatments. The presence or absence of inhibition halos was observed. The assays were performed in triplicate.

2.6 MOLECULAR IDENTIFICATION OF ANTIMICROBIAL-PRODUCING BACTERIA

The bacterial isolates with antimicrobial activity were subjected to molecular characterization with the use of 16S ribosomal RNA sequencing. The total extraction of genomic DNA from bacterial isolates grown in AM medium was performed by the

phenol/chloroform extraction method (Santos et al., 2017). The 16S rRNA genes from the isolates were amplified using primers F27 (5'-AGAGTTTGATCGGCTCAG-3') and R1525 (5'-AAGGAGGTGTCCARCC-3') (Lane 1991). Polymerase chain reaction (PCR) was carried out in a thermocycler (Eppendorf, Hamburg, Germany) and contained 0.2 M of each primer, 0.2 mM of each dNTP, 0.03 U/µL Taq DNA polymerase (Invitrogen, São Paulo, Brazil), ~50 ng DNA, 3 mM $MgCl_2$, and 1× buffer. The thermocycler was programmed under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 3 min, followed by a final extension of 7 min. The amplicons were purified by cold salt precipitation and were then resuspended in sterile ultrapure water. All amplicons were sequenced at ACTGene Análises Moleculares Ltd. (Alvorada, RS, Brazil) using the ABI Prism 3500. The resulting isolate sequences were analyzed and compared to the GenBank nucleotide database using BLAST (Altschul et al., 1990).

3 RESULTS

3.1 SCREENING FOR ANTIMICROBIAL ACTIVITY

Among the sixty-eight microorganisms isolated from the *Siderastrea stellata* coral, eight (SS4, SS55, SS15, SS77, SS51, SS85, SS38, and SS69) produced active antimicrobial compounds that inhibited the growth of *S. aureus*, EHEC O157 H7, EPEC, EAEC, *S.* Enteritidis PT4, *S.* Enteritidis PT11, and *S.* Typhi. There was no growth inhibition observed in EIEC, ETEC, and *E. coli* (INCQS 0310) in experimental conditions. The antimicrobial activity index was determined by halo size (Table 1).

Table 1. Antimicrobial activity index by the agar-well diffusion method demonstrating the antimicrobial action of bacterial isolates from coral expressed as halo size in marine water (AM) and Mueller-Hinton (MH) medium. On the left, in purple, are the average values in AM. In orange, on the right, are the average values are in MH. The results represent the size of the average halos from the three replicates.

	Bacteria isolated from coral *Siderastrea stellata*							
Test bacteria	SS4	SS15	SS55	SS85	SS38	SS69	SS77	SS51
	Inhibition zone diameter (mm)							
EHEC	22|30	41|-	-|22	41|-	21|-	31|-	29|-	24|32
EPEC	39|-	39|-	35|-	28|33	44|35	35|37	-|36	35|-
EAEC	-|27	-|21	-|12	27|-	-|30	-|-	-|-	-|-
S. Enteritidis PT4	25|40	-|-	28|-	32|-	30|36	30|38	30|-	36|-
S. Enteritidis PT11	22|-	35|42	-|-	30|-	-|43	35|-	28|-	-|38
S. Typhi	-|-	-|-	-|23	-|29	15|-	-|-	-|-	-|-
S. aureus	-|22	-|-	-| 23	18|26	39|-	13|9	21|14	22|-

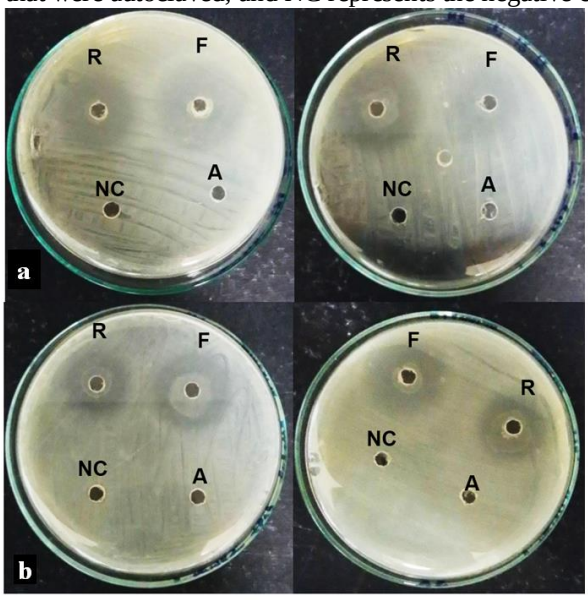
3.2 ANTIMICROBIAL COMPOUND PRODUCTION IN MUELLER-HINTON MEDIUM

The antimicrobial screening of Mueller-Hinton (MH) medium is shown in Table 1. When grown in AM medium, the SS55 isolate showed no antimicrobial activity against EHEC O157H7. However, it exhibited antimicrobial activity when cultivated in MH medium. The crude extract of the SS77 isolate showed antimicrobial activity against EPEC O55 only when cultivated in MH medium. An increase of the biocidal spectrum against the bacterium EAEC was observed since only one isolate produced an antimicrobial agent in AM medium while four isolates inhibited the growth of this pathogen when cultured in MH medium.

3.3 TABILITY ANALYSIS AND STORAGE TIME

Considering their performance in antimicrobial screening assays for *Salmonella*, the SS85 and SS69 isolates were selected for evaluation of their stability. Antimicrobial compounds showed stability throughout storage time but were sensitive to the humid heat of autoclaving. Antimicrobial assays against *S.* Enteritidis PT4 and *S.* Enteritidis PT11, performed using extracts that had been stored for three months in the refrigerator (2 to 8 °C) and freezer (-20 °C), presented inhibition halos (Figure 1). After autoclaving, no halos were observed (Figure 1).

Figure 1 - Stability test of compounds derived from SS69 and SS85 isolates. In (a) are the extracts from isolates SS85 (left) and SS69 (right) against *S.* Enteritidis PT4. In (b) are extracts from isolates SS85 (left) and SS69 (right) against *S.* Enteritidis PT11. R indicates the compounds that were stored in a refrigerator (2 to 8 °C), and F indicates the compounds that were stored for three months in the freezer (-20 °C). A indicates the compounds that were autoclaved, and NC represents the negative control.



3.4 SEQUENCING OF SELECTED ISOLATES IN SCREENING

The results obtained by 16S rRNA sequencing of positive isolates compared to sequences from the National Center for Biotechnology Information GenBank are shown in Table 2. Local sequence alignment performed with BLASTn demonstrated that all isolates matched *Bacillus* species with a similarity with sequences similarity with GenBank entries ranging from 97 to 100%.

Table 2. Taxonomic identification of antimicrobial-producing bacterial isolates from marine coral.

Bacterial isolate	Taxonomic Identification	Access	Similarity (%)
SS4	*Bacillus pumilus*	NR_112637	99
SS15	*Bacillus safensis*	KX809601	97
SS55	*Bacillus safensis*	NR_113945	99
SS85	*Bacillus stratosphericus*	NR_042336	98
SS38	*Bacillus altitudinis*	NR_042337	97
SS69	*Bacillus stratosphericus*	NR_042336	99
SS77	*Bacillus altitudinis*	NR_042337	99
SS51	*Bacillus safensis*	NR_113945	99

4 DISCUSSION

Eight isolated bacteria were identified to be capable of inhibiting at least two of the ten pathogenic bacteria used in the current work, that is indicative of the biotechnological potential of these strains isolated from coral *Siderastrea stellata*. Our previous study on a metagenomic library from tissues of this coral found that coral-associated microorganisms are rich sources of cellulolytic enzymes, but that no antimicrobial activity was detected (Sousa et al., 2016); however, antimicrobial activity was detected in clone with proteolytic activity in further assays (Costa et al., 2020).

A total of 11.76% of isolates obtained had antimicrobial activity, in contrast to the somes findings (ElAhwany et al., 2013) in which twenty strains associated with the soft coral *Sarcophyton glaucum* were isolated and 80% of obtained isolates possessed antimicrobial activity. Although we found a relatively smaller portion, more strains were isolated, and more bacterial tests were performed in the antimicrobial assay. Unlike other studies (ElAhwany et al., 2013), our isolates with antimicrobial activity were able to inhibit the growth of at least two of the tested bacteria.

The number of isolates producing bioactive molecules with antibiotic activity was higher than the 5.8% found in previous works from the *Oculina patagonica* coral (Nissimov et al., 2009). It has already been suggested that bioactivity assessment depends on the isolation method and assay used. Other factors that may cause changes in antimicrobial activity are the species and amount of test bacteria used for screening Shnit-Orland and Kushmaro, 2009).

In order to mimic the natural environment, a medium containing seawater was created. The media were called marine water broth (AM) and marine water agar (AAM), for the liquid and solid media, respectively. Microorganisms isolated from coral *Siderastrea stellata* were cultivated under these conditions. The AM and AAM media are practical and cheaper alternatives for the cultivation of marine bacteria.

Antimicrobial activity was different when isolates were cultured in MH, demonstrating that the culture medium directly affects the production of bioactive molecules capable of competitive inhibition of other microorganisms. We suggest that MH stimulated the production of bioactive molecules in some cases, such as in isolates 38 and 51 that only showed antimicrobial activity against *S.* Enteritidis PT11 when they were cultured in MH. Similarly, antimicrobial activity was demonstrated by isolates A55 and 85 against *S.* Typhi (ATCC 5339) and by AC4 and A55 against *S. aureus* INCQS 00186 exclusively in MH. These findings indicate that the use of more than one medium allows the identification of diverse antimicrobial activities of bacterial isolates, thereby corroborating the findings of other authors (Houang et al., 1983; Sawer et al., 1997).

A reduction of the biocidal spectrum when cultured in different media was observed. These results indicate that MH is useful for antimicrobial compound production for a few isolates, which do not produce antimicrobial compounds in AM. However, when compared to MH media, AM was overall more efficient for the production of antimicrobial compounds. These findings show that the use of seawater was effective, favored the growth of isolates, and also enhanced the production of more antimicrobial compounds compared to that of commercial media. Seawater is cheaper, easily accessible, and very efficient compared to the conventional means available for the isolation and cultivation of marine bacteria for biotechnological purposes.

Temperature and storage stability experiments were made with isolates capable of inhibiting *S.* Enteritidis. As isolates 69 and 85 presented the best halos for *S.* Enteritidis PT4 and *S.* Enteritidis PT11, they were chosen for the thermal stability and storage assays. The observed stability as demonstrated by preserved antimicrobial activity makes these

isolates good candidates for biotechnological use as they remain stable through temperature changes and time. Sensitivity to humid heat resulting in a loss of activity due to autoclaving may arise from a loss of metabolite structure, as is the case with protein antimicrobials. However, further studies are needed to elucidate the complete chemical structure of the antimicrobial compounds in question.

The high sequence similarity of the isolate sequences suggests that some *Bacillus* isolates are closely related to *B. pumilus* and are not easily distinguished from each other by the 16S rRNA sequence alone. The *B. pumilus* group contains five species, *B. pumilus*, *B. safensis*, *B. stratosphericus*, *B. altitudinis*, and *B. aerophilus* (Liu et al., 2013). These are almost identical in the 16S rRNA sequence, sharing a similarity of 99.5%. Such high sequence similarity would confirm that a species belongs to this complex. This finding is in agreement with the relevant literature that has described a broad spectrum of antimicrobial activity against pathogenic bacteria by *Bacillus* sp. (Sumi et al., 2015). *Bacillus* species can produce structurally diverse secondary metabolites that exhibit a diverse spectrum of antibiotic activity (Li et al., 2012; Motta et al., 2008; Paik et al., 1997; Sabaté and Audisio 2013). Furthermore, the bacterial isolates from *Siderastrea stellata* corals are likely to be new lineage symbionts of this coral.

Bacterial isolates of the coral *Siderastrea stellata* demonstrated antimicrobial activity against at least two of the ten pathogenic bacteria tested. The isolates have been identified as belonging to the genus *Bacillus* and are promising strains for the development of new antimicrobial drugs. Additionally, a new culture medium using seawater was produced, thereby presenting a cheaper and more efficient alternative for conventional bacterial isolates and bioprospection compared to conventional media. The results of this study highlight the importance of prospecting compounds from natural and unexplored environments and corroborate the fact that coral is a promising source of new antimicrobial compounds.

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DECLARATION OF COMPETING INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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