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Quorum sensing inhibition activity of marine Gammaproteobacteria

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ABSTRACT

Quorum sensing (QS) is a communication mechanism between bacteria, mediated by signals released at high cell densities, which regulates bioluminescence, virulence, and biofilm formation. By inhibiting QS, these processes can be controlled when they become undesirable, as in infectious diseases and during biodeterioration of materials. In this context, this study investigated the Quorum Sensing Inhibition (QSI) activity in 60 strains of marine Gammaproteobacteria isolated from samples of the South Atlantic Ocean. Initially, the bacterial strains were screened using plaque assays, with *Chromobacterium violaceum* LAMA 0447 and *Serratia marcescens* LAMA 1170 as indicator strains. Subsequently, seven selected bacteria were further evaluated via luminescence test with *Aliivibrio fischeri*, considering both cultivation time and mixed cultures with *A. fischeri*. Later, three strains were investigated regarding the chemical nature of their substances with QSI activity via ultrafiltration and proteinase K treatments. Finally, the genome of one of these strains was examined for the identification of genes related to QSI activity. In total, 16 strains exhibited QSI activity in plaque assays, and the activity of seven of these strains was further assessed in quantitative assays. Higher activity was observed in supernatants obtained after 48 hours of cultivation for six strains and from mixed cultures with *A. fischeri*. Treated supernatants of three strains allowed us to infer that, for *Halomonas olivaria* LAMA 0626, the substances responsible for QSI are probably enzymes. For the other two strains, belonging to the *Marinobacter* genus, more than one type of substance seems to be involved: (1) at least one enzyme and (2) non-protein low molecular weight molecules (< 10 kDa). Genes identified in *M. excellens* LAMA 0842 support this hypothesis. In summary, marine bacteria from the Gammaproteobacteria class can disrupt the communication of other bacteria, which could form the basis for the development of novel microbial control products.

Keywords: Oceanic bacteria; Marinobacter; Quorum-quenching

INTRODUCTION

Quorum sensing (QS) is a mechanism employed by bacteria to communicate with each other and to synchronize their behavior via production, secretion, and detection of signaling molecules (Eickhoff and Bassler, 2018). This mechanism was originally discovered in *Aliivibrio fischeri* as a system regulating bioluminescence. This bacterial species produces a QS signaling molecule, *N*-acyl homoserine lactone (AHL), synthesized by the LuxI protein (Yao et al., 2019). As the population density increases, the concentration of AHL accumulates. When it exceeds a certain concentration, it binds to a QS specific regulator

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protein, LuxR, which activates the transcription of bioluminescence-regulated *lux* genes (Whiteley et al., 2017). When the density of the bacterial population is low, the concentration of AHL is also low, and the transcription of *lux* genes is repressed (Liao et al., 2018).

After the discovery of QS in *A. fischeri*, further studies revealed its involvement in the regulation of virulence-related gene transcription of pathogenic bacteria, such as the production of proteases in *Pseudomonas aeruginosa* (O'loughlin, 2013) and of toxins in *Clostridioides difficile* and *Staphylococcus aureus* (Tripathi et al., 2023). The regulation of virulence factors by QS in *P. aeruginosa* involves, as in *A. fischerii*, the production of autoinducers by the AHL synthase LasI, which activate the transcription of several virulence genes and other genes via the regulator LasR (Tripathi et al., 2023). In *S. aureus*, which exemplifies QS mechanisms in Gram-positive bacteria, the production of virulence factors is regulated by the Agr system, which involves autoinducers composed of peptides and their perception by a phospho-relay or two-component system (Oliveira et al. al., 2023).

Other microbial behaviors, such as conjugative plasmid transference, motility, differentiation, aggregation, bioluminescence, siderophore production, antibiotic biosynthesis, symbiosis, biofilm maintenance, and pigmentation, have also been reported as regulated by quorum-sensing (Romero et al., 2011; Hmelo, 2017). Quorum sensing can also impact antimicrobial resistance by regulating the expression of antibiotic efflux systems and the formation of biofilms (Zhao et al., 2020). This has even been reported for pathogens in the ESKAPE group (Odularu et al., 2022). Biofilms can promote greater resistance of pathogens to antibiotics by limiting contact with these molecules, in addition to facilitating the acquisition of resistance genes among members of this microbial community (Zhao et al., 2020; Odularu et al., 2022).

Marine bacteria produce a wide variety of structurally diverse and biologically active secondary metabolites (Wietz et al., 2013). Among these, molecules inhibiting the QS can be achieved and may contribute to the development of several biological products, which present low toxicity, are effective in low concentrations, and show easy degradation in case of environmental contamination (Munn, 2019). These products may be applied for the control of undesired QS-dependent behaviors, such as biofilm formation and the production of virulence factors (Hmelo, 2017; Boursier et al, 2019; Sun et al., 2019).

In total, three mechanisms may be involved in the destabilization of QS: the inhibition of autoinducer synthesis, the inhibition of the autoinducer-receptor binding, and the enzymatic degradation of the autoinducer (Tay and Yem, 2013; Zhu et al., 2023). This set of processes responsible for inhibiting QS is named quorum quenching (QQ) (Grandclément et al., 2016; Rather et al., 2022; Zhu et al., 2023). Molecules exhibiting the latter two mechanisms have already been reported, the first group consisting of algae, marine invertebrates, terrestrial plants, and bacteria, and the latter of mammals, plants, and bacteria (Romero et al., 2011). Molecules that inhibit the synthesis of autoinducers or the action of their receptors include peptides, amides, fatty acid derivatives, phenol derivatives, and AHL analogs, among others (Borges and Simões, 2019; Zhao et al., 2019).

There are three groups of enzymes known to degrade AHLs: the acylases, the lactonases, and the oxidoreductases. Acylases hydrolyze the amide bond in AHL, releasing homoserine lactone and the fatty acid chain. Lactonases hydrolyze the ester bond in the lactone ring. Oxidoreductases, including deaminases and decarboxylases, do not hydrolyze the molecule but instead modify it into a biologically inactive form (Tay and Yem, 2013; Romero et al. 2011).

Thus, considering the potential of the QS inhibitors as biotechnological products, this work focused on the selection of marine bacteria from the Gammaproteobacteria class with QS-inhibiting activity against model processes such as pigment production and bioluminescence. This was motivated by the fact that this bacterial class is one of the most cultivated from marine samples, in addition to its recognized biotechnological potential (Bollinger

et al., 2018; Kizhakkekalam and Chakraborty, 2020; Tikhonova et al., 2023). However, studies related to its QS inhibitory potential are relatively scarce. These studies lack information on many of the important bacterial genera of this class, such as *Marinobacter* and *Halomonas*, among others. This represents an opportunity to search for new ways to combat microbial growth when undesirable, such as in biofilms, in which, as explained previously, antimicrobial resistance of pathogens hinders the control of infectious diseases (Zhao et al., 2020; Odularu et al., 2022). In this research, we observed that the capacity of inhibiting QS is common in marine bacteria. The genera *Halomonas* and *Marinobacter* stood out for their high inhibitory activity, making them of interest for further investigations aimed at their future application in the control of biofilms and pathogens.

METHODS

Strains used

In total, 60 strains of marine Gammaproteobacteria isolated from sediment and water samples of the South Atlantic Ocean were included in this study (Table 1). These strains were obtained from the collection of the Laboratory of Applied Microbiology located at the Vale do Itajaí University (Itajaí, SC, Brazil). The details of their origin and isolation procedures can be found elsewhere (Odisi et al., 2012; Da Silva et al., 2013). For the various assays (Figure 1), the following strains were also used: *Chromobacterium violaceum* LAMA 0447 and *Serratia marcescens* LAMA 1170, both obtained from the Laboratory of Applied Microbiology collection, and *Alivibrio fischeri* NRRL B-11177, acquired from Unwelt Biotecnologia Ambiental.

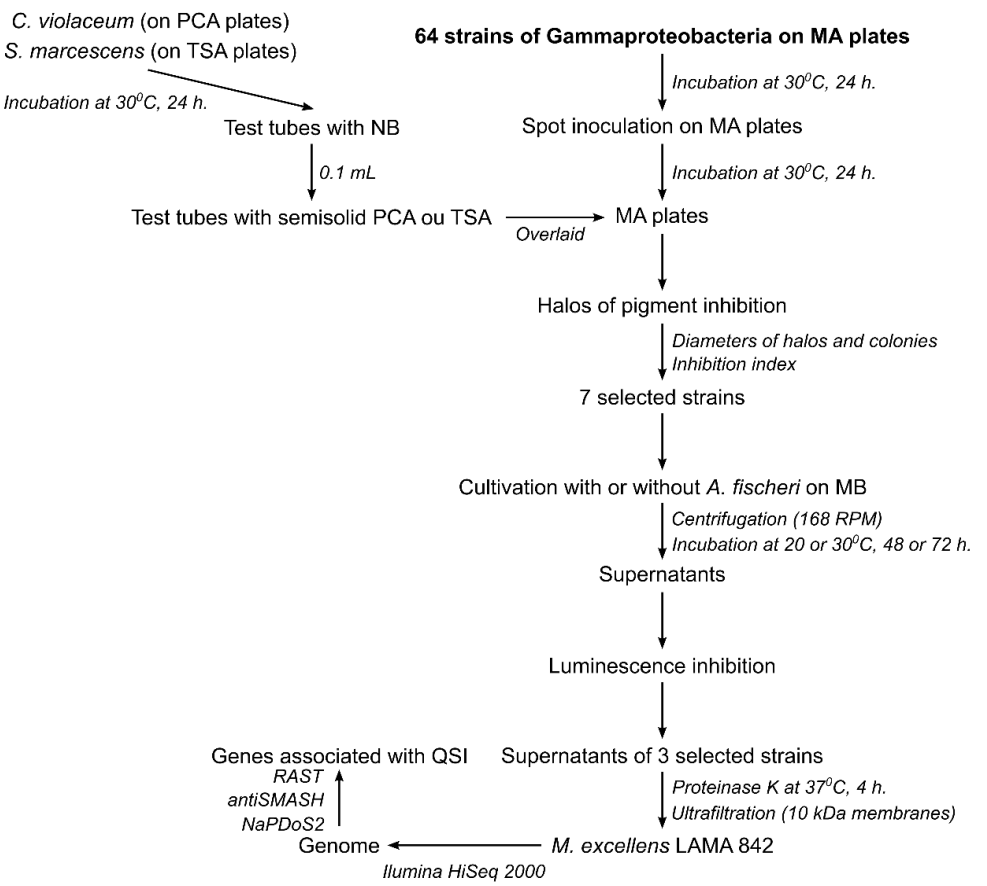

Figure 1. Scheme of the different phases of this research. The methods employed in these phases are described in the next subsections.

Table 1. Marine Gammaproteobacteria strains investigated in this study with the NCBI accession number of their partial 16S gene sequences (Da Silva et al., 2013).

Strain	Species	NCBI
LAMA 0622	*Cobetia marina*	JX860197.1
LAMA 0624	*Psychrobacter* sp.	JX860198.1
LAMA 0625	*Cobetia marina*	JX860199.1
LAMA 0626	*Halomonas olivaria*	OQ442357.1
LAMA 0627	*Halomonas boliviensis*	JX86020.11
LAMA 0631	*Pseudoalteromonas spiralis*	OQ442358.1
LAMA 0632	*Halomonas sulfidaeris*	JX860202.1
LAMA 0633	*Halomonas sulfidaeris*	JX860203.1
LAMA 0634	*Halomonas* sp.	JX860204.1
LAMA 0636	*Halomonas sulfidaeris*	JX860205.1
LAMA 0638	*Halomonas sp.*	JX860207.1
LAMA 0639	*Psychrobacter nivimaris*	JX860208.1
LAMA 0641	*Psychrobacter nivimaris*	JX860209.1
LAMA 0642	*Halomonas* sp.	JX860210.1
LAMA 0646	*Halomonas boliviensis*	JX860214.1
LAMA 0685	*Halomonas hydrothermalis*	OQ442359.1
LAMA 0686	*Halomonas hydrothermalis*	OQ442360.1
LAMA 0691	*Idiomarina loihiensis*	JX860218.1
LAMA 0719	*Stutzerimonas zhaodongensis*	OQ442361.1
LAMA 0723	*Psychrobacter* sp.	JX860228.1
LAMA 0734	*Halomonas boliviensis*	JX860230.1
LAMA 0761	*Halomonas* sp.	OQ442362.1
LAMA 0774	*Halomonas hydrothermalis*	OQ442363.1
LAMA 0784	*Pseudoalteromonas issachenkonii*	JX860241.1
LAMA 0786	*Halomonas sulfidaeris*	JX860242.1
LAMA 0794	*Halomonas boliviensis*	JX860244.1
LAMA 0796	*Halomonas* sp.	JX860245.1
LAMA 0797	*Halomonas boliviensis*	JX860246.1
LAMA 0799	*Psychrobacter piscatorii*	OQ442364.1
LAMA 0802	*Halomonas boliviensis*	JX860248.1
LAMA 0805	*Halomonas alkaliantarctica*	OQ442365.1
LAMA 0807	*Halomonas boliviensis*	JX860249.1
LAMA 0809	*Halomonas* sp.	JX860250.1
LAMA 0810	*Halomonas boliviensis*	JX860251.1
LAMA 0811	*Halomonas alkaliantarctica*	OQ442366.1
LAMA 0818	*Psychrobacter nivimaris*	JX860253.1
LAMA 0835	*Idiomarina loihiensis*	OQ442367.1
LAMA 0837	*Halomonas* sp.	JX860258.1
LAMA 0838	*Halomonas sulfidaeris*	JX860259.1

[continued]

Strain	Species	NCBI
LAMA 0842	*Marinobacter excellens*	JX860260.1
LAMA 0879	*Halomonas* sp.	JX860262.1
LAMA 0885	*Psychrobacter proteolyticus*	OQ442368.1
LAMA 0900	*Stenotrophomonas maltophilia*	KC583188.1
LAMA 0903	*Halomonas meridiana*	KC583189.1
LAMA 0905	*Halomonas meridiana*	KC583190.1
LAMA 0916	*Halomonas axialensis*	KC583201.1
LAMA 0917	*Halomonas meridiana*	KC583202.1
LAMA 0918	*Halomonas meridiana*	KC583203.1
LAMA 0919	*Salinicola salarius*	KC583204.1
LAMA 0931	*Halomonas axialensis*	KC583213.1
LAMA 0936	*Halomonas axialensis*	KC583217.1
LAMA 0939	*Salinicola salarius*	KC583220.1
LAMA 0940	*Pseudoalteromonas mariniglutinosa*	KC583221.1
LAMA 0946	*Halomonas axialensis*	KC583225.1
LAMA 0947	*Salinicola salarius*	KC583226.1
LAMA 0954	*Marinobacter flavimaris*	KC583232.1
LAMA 0956	*Idiomarina zobellii*	KC583234.1
LAMA 0964	*Halomonas axialensis*	KC583241.1
LAMA 0982	*Psychrobacter celer*	OQ442369.1
LAMA 1006	*Halomonas meridiana*	OQ442370.1

Strains used

To analyze quorum sensing inhibitory (QSI) activity, two indicator bacteria were used to screen in plate assays, *C. violaceum* LAMA 0447 and *S. marcescens* LAMA 1170 (Mclean et al., 2004). Initially, cultures of all marine bacterial strains were prepared in Petri dishes containing Marine Agar (peptone, 5.0 g; yeast extract, 1.0 g; ferric citrate, 0.1 g; bacteriological agar, 15.0 g; seawater, 750 mL; distilled water, 250 mL) (MA), then incubated at 30°C for 24hours. In parallel, cultures of indicator bacteria were also prepared, Plate Count Agar (enzymatic digest of casein, 5.0 g; yeast extract, 2.5 g; glucose, 1.0 g; bacteriological agar, 15.0 g; distilled water, 1,000 mL) (PCA; *C.violaceum*) and Tryptic Soy Agar (pancreatic digest of casein, 15.0 g; papaic digest of soya bean, 5.0 g; sodiumchloride, 5.0 g; bacteriological agar 15.0 g; distilled water, 1,000 mL) (TSA; *S. marcescens*).

After the incubation period, new Petri dishes containing Marine Agar (MA) were spot inoculated, in triplicate, with the aid of an inoculation loop, with the 60 marine bacteria and incubated at 30°C for 24 hours. Each bacterial strain occupied two plates. Indicator microorganisms were transferred from plates to test tubes containing 5 mL of Nutrient Broth (beef extract, 3.0 g; peptone, 5.0 g; distilled water, 1000 mL) (*C. violaceum*) and Tryptic Soy Broth (TSA without bacteriological agar) (*S. marcescens*), which were also incubated at 30°C for 24 hours.

After this new incubation period, test tubes containing liquefied semisolid Plate Count Agar (PCA with 5 g/L bacteriological agar) were inoculated with 100 µL of cultures of indicator microorganisms (60 for each indicator microorganism), then homogenized and overlaid on the plates of MA containing the marine strains inoculated punctually. Subsequently, these plates containing the overlays were incubated at 30°C for 24 hours. After incubation, the inhibitory activity of quorum sensing was observed by the formation of halos of pigmentation inhibition of the indicator

microorganisms present in the overlay (Mclean et al., 2004). The diameters of the generated halos and the colonies were then measured using a caliper. The measurements were used to estimate an inhibition index by dividing the diameter of the halo by the diameter of the colony that produced it.

Assessment of QSI activity by the bioluminescence inhibition assay

Of the 60 strains tested, seven were selected based on two criteria: their confirmed QSI activity on both indicator bacteria and the highest inhibition index values for at least one of the indicator microorganisms. These seven bacteria were evaluated in more detail with the luminescent bacteria test, following the procedures described in the International Organization for Standardization (ISO) (2018), using the "Freshly prepared bacteria" method. The microorganism *A. fischeri* NRRL B-11177 and the illuminometer LUMIStox 300 (DR Lange) were employed in these experiments. After these tests, luminescence inhibition percentage values were estimated, all according to the Draft International Standard guidelines.

A total of two rounds of experiments were conducted. In the first round, QSI activity of the selected bacterial strains was evaluated in two cultivation times (48 and 72h) and in the absence or presence of *A. fischeri* NRRL B-11177 as a potentially competitive microorganism that could stimulate quorum inhibition sensing. In the second round of experiments, three bacterial strains, selected from the previous round, were evaluated to investigate some of the properties of their quorum sensing inhibiting substances. All incubation times were established based on the growth of these bacteria in Petri dishes containing Marine Agar during routine laboratory cultivation. These bacteria produced visible colonies within 24 to 48 hours of cultivation under the indicated conditions.

In the first round of experiments, the selected bacterial strains were cultivated alone (at 30°C) or with *A. fischeri* NRRL B-11177 (at 20°C) for 48 and 72 hours in Marine Broth (MA without bacteriological agar), under agitation (168 rpm). In this case, a lower incubation temperature was adopted for the cultures with *A. fischeri* NRRL B-11177 because this species is a psychrophile, an organism with optimum growth at cold temperatures. After incubation, the cultures were centrifuged at 8,962.71 × g (Eppendorf 5810R) at 4°C for 15 minutes to precipitate the cells, as experimentally determined in the laboratory, and the supernatants were recovered and stored frozen (–20°C) for later testing for luminescence inhibition, as described above.

In the second round of experiments, the supernatants containing quorum sensing inhibiting substances of three bacterial strains, selected from the first round, were subjected to protease treatment and ultrafiltration (Heredia-Castro et al., 2015; Robles-Hernández et al., 2021). For the experiments with protease treatment, the selected strains were cultivated in Marine Broth in the presence of *A. fischeri* NRRL B-11177 at 20°C for 48 hours. After the incubation, the supernatants were prepared as described in the previous round of experiments.

For the protease treatment, 0.5 mL of proteinase K (USB, 45.5 units/mg; 4 mg/mL) was added to 3.5 mL of the supernatant and the mixture was incubated at 37°C for 4 hours. Control consisted of two treatments: one in which the supernatant was replaced with Bovine Serum Albumin solution (INLAB, 0.25 mg/mL, diluted in Marine Broth) and another in which Marine Broth was used instead of the supernatant. Both controls were treated and incubated under the same conditions. Finally, the treated supernatants and controls were stored frozen and later evaluated for QSI activity by the luminescent bacteria test, as described previously. To verify the enzymatic activity of proteinase K, 0.25 mL of the enzyme was added to 1.75 mL of Bovine Albumin Serum - BSA (INLAB; 0.25 mg/mL diluted in Marine Broth). This procedure was performed twice, with the first incubated at 100°C for 10 minutes for enzyme inactivation and the second incubated at 37°C for 4 hours.

For the ultrafiltration procedure, 15 mL of supernatant were filtered in a stirred ultrafiltration cells kit (Micon 8200), with a membrane (Millipore) of 10 kDa, submitted to 1 kgf. This procedure generated the filtered supernatants that were stored frozen and later evaluated for QSI activity

by the luminescent bacteria test, as described previously. The culture diluted with sterile distilled water (1:1) and the unfiltered supernatant were also tested as controls. In all experiments, the estimated percentages of inhibition were normalized by the optical density (600 nm) of the cultures of selected strains. It was possible to do that because all bacterial species studied produce rod-shaped cells with similar sizes. This was performed to facilitate the comparisons between different organisms.

Identification of genes involved in the inhibition of quorum sensing in Marinobacter excellens LAMA 0842

The strain *M. excellens* LAMA 0842, whose genome has been sequenced, annotated, and publicly deposited in the GenBank of the National Center for Biotechnology Information (GenBank Accession Number: LOCO00000000.1), was investigated to identify genes associated with quorum sensing or QSI activity. The genome sequencing data were obtained using an Illumina HiSeq2000 system, employing 101-nucleotide paired-end read sequencing from a genomic library with insert sizes ranging from 350 to 550 bp. Raw data comprised 16,515,704 reads, totaling 1.66 Gb, with 92.72% of reads achieving a Phred score ≥ Q30. Sequence data quality trimming, performed using CLC Genomics Workbench (v. 6.5.1) with a threshold of 0.05, resulted in 16,515,964 reads, which were subsequently employed for *de novo* assembly in the same software. The assembly produced 59 contigs. The Rapid Annotation using Subsystem Technology server (RAST) (Aziz et al., 2008) was employed as a primary tool for annotation, and the proteins identified as "hypothetical" were further annotated with the aid of the CLC Genomics Workbench software (v. 6.5.1) (Lima et al., 2013). This strain was chosen because it stood out among the most active on all indicator bacteria used. The genome of this strain is being published by the research group in parallel to the present study. In this analysis, we searched for genes encoding enzymes, metabolic pathways, and regulatory mechanisms that could suggest the possible mechanisms of QSI present in *M. excellens* LAMA 0842.

Further analysis was performed on the genome of *M. excellens* LAMA 0842 employing bioinformatics approaches. Secondary metabolite and natural product domains analyses were conducted using the antibiotics & Secondary Metabolite Analysis Shell (antiSMASH) v 6.0 (Blin et al., 2021) and the Natural Product Domain Seeker 2 (NaPDoS2) (Klau et al., 2022). Based on the results generated by the analysis platforms, biosynthetic pathways and enzymes that could be related to the inhibitory activity of the LAMA 0842 on quorum sensing were sought. The analysis was performed following the guidelines provided on their respective websites, and the results were retrieved from the final records obtained.

Data analysis

For data analysis, data normality was initially assessed using the Shapiro-Wilk test. Data sets whose *p*-values were greater than 0.05 were considered normal. The percentages of luminescence inhibition, obtained in assays involving the competitor microorganism, were analyzed using two-way ANOVA (culture time and presence/absence of *A. fischeri*). On the other hand, the percentages of luminescence inhibition, obtained in tests involving ultrafiltration and treatment with proteinase K, were compared with the untreated supernatant for each of the three selected bacterial strains separately. For this analysis, the non-parametric test of Kruskal-Wallis and pairwise comparisons of Mann-Whitney were used. In all tests, *p*-values below 0.05 were considered significant. All statistical analyses were performed using the free software Past, version 4.11 (Hammer et al., 2001).

RESULTS

Screening experiments

Out of the sixty bacterial strains tested, 16 (26.67%) showed QSI activity against at least one of the tested indicator strains (*S. marcescens* and *C. violaceum*) (Table 2). The breakdown of QSI activity is as follows: eight strains (13.33%) inhibited only the pigmentation of *S. marcescens*; two strains (3.33%) inhibited only the pigmentation of *C. violaceum*; and six strains (10%) inhibited the

pigmentation of both indicator microorganisms. These strains with QSI activity belonged to seven genera: *Halomonas* (n = 8), *Marinobacter* (n = 2), *Salinicola* (n = 2), *Idiomarina* (n = 1), *Pseudoalteromonas* (n = 1), *Stenotrophomonas* (n = 1), and *Stutzerimonas* (n = 1).

Among the *Halomonas* strains, QSI activity was found to vary. The strains *H. alkaliantarctica* LAMA 0626, *H. meridiana* LAMA 0918, and *Halomonas* sp. LAMA 0837 exhibited QSI activity against both *S. marcescens* and *C. violaceum*. *H. boliviensis* LAMA 0646 was active exclusively against *C. violaceum*, whereas the remaining four *Halomonas* strains only inhibited *S. marcescens*.

The highest QSI index against *S. marcescens* was observed in the strain *H. hydrothermalis* LAMA 0685, and it inhibited only this indicator microorganism. On the other hand, the highest QSI index against *C. violaceum* was observed in the strain *Halomonas* sp. LAMA 0837, but it also inhibited *S. marcescens*. All six strains, plus *H. hydrothermalis* LAMA 0685, were selected for further experiments.

Table 2. Bacterial strains that showed QSI activity for at least one of the indicator microorganisms used, with their mean index of activity and standard deviations (SD).

Strain	*S. marcescens* LAMA 1170		*C. violaceum* LAMA 447	
	Mean	SD	Mean	SD
***H. olivaria* LAMA 0626**	5.49	0.42	0.58	0.76
H. boliviensis LAMA 0646	-	-	2.84	0.33
***H. hydrothermalis* LAMA 0685**	6.45	0.72	-	-
***H. meridiana* LAMA 0918**	2.89	0.38	1.82	0.16
H. sulfidaeris LAMA 0633	1.59	0.17	-	-
Halomonas sp. LAMA 0917	3.31	0.31	-	-
Halomonas sp. LAMA 0811	1.53	0.21	-	-
***Halomonas* sp. LAMA 0837**	2.08	0.15	3.63	0.41
I. loihiensis LAMA 0835	-	-	1.69	0.21
***M. excellens* LAMA 0842**	4.27	0.64	2.84	0.33
***M. flavimaris* LAMA 0954**	1.67	0.17	3.36	0.23
P. siralis LAMA 0631	1.46	0.04	-	-
S. zhaodongensis LAMA 0719	1.72	0.10	-	-
S. maltophilia LAMA 0900	1.11	0.05	-	-
***S. salarius* LAMA 0939**	2.74	0.42	1.95	0.50
S. salarius LAMA 0947	1.90	0.11	-	-

Competitor and different cultivation times experiments

The seven selected strains were further evaluated using the bioluminescence inhibition assay. However, the highest activities were found when the competitor strain was present and peaked at different cultivation times, within a two-factor experimental design (Figure 2). For six out of the seven strains, QSI activity was higher in 48 hours of cultivation, regardless of the presence of the competitor microorganism. In the case of *H. hydrothermalis* LAMA 0685, the highest QSI activity was observed after 72 hours of cultivation in the absence of the competitor microorganism. However, in the presence of the competitor, the highest activity was observed after 48 hours of cultivation. In all cases, the highest activity was observed in the presence of the competitor microorganism for all seven strains, regardless of the cultivation time (Figure 2).

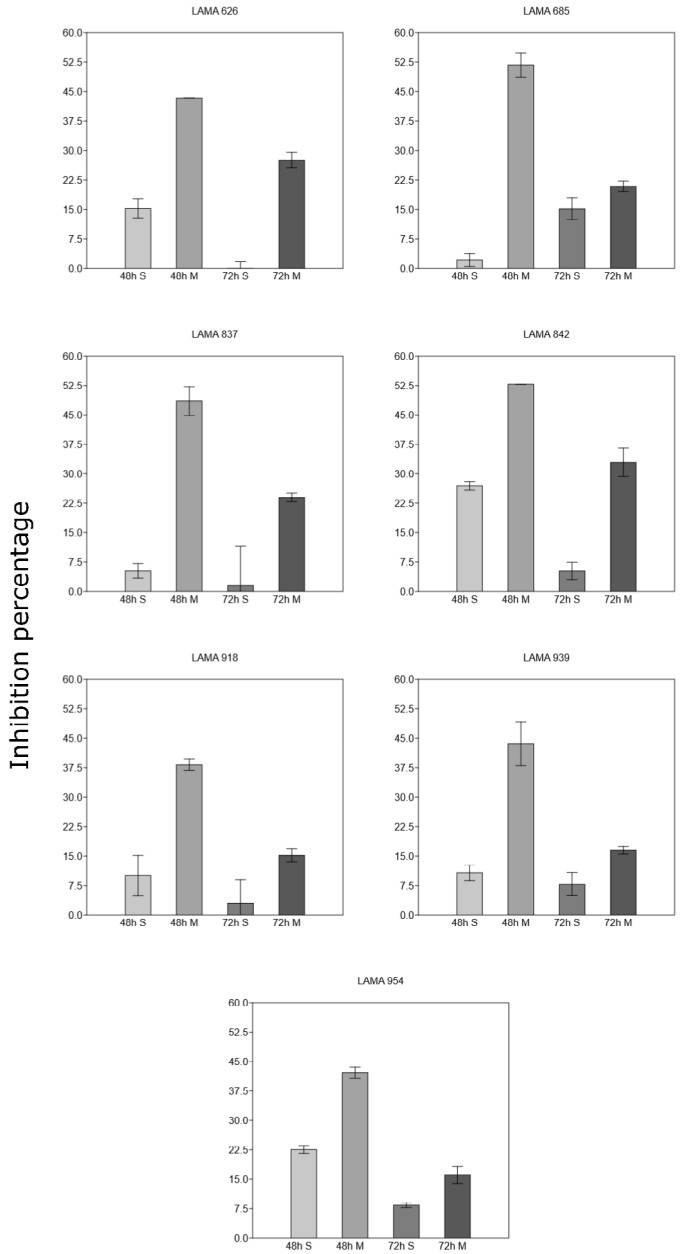

Figure 2. Mean bioluminescence inhibition percentage of selected bacterial strains cultivated singly (S) or in mixed cultures with *A. fischerii* (M), as a function of cultivation time. The vertical bars represent the standard deviations.

These observations were supported by statistical analysis. First, the data was tested for normality, and it was found that all data sets generated from these experiments were normally distributed (Shapiro-Wilk $W > 0.918$; $p > 0.1566$). Significant differences were then found for both factors—the presence of competitor and cultivation time—for all bacterial strains evaluated, confirmed by the two-way ANOVA ($F > 13.38$; $p < 0.0216302$). Additionally, a significant interaction between the two factors was only detected for the strains *H. hydrothermalis* LAMA 0685 ($F = 183.25$; $p < 0.001$), *S. salarius* LAMA 0939 ($F = 26.28$; $p = 0.0068541$), and *M. flavimaris* LAMA 0954 ($F = 34.11$; $p = 0.0042850$).

Experiments with proteinase K and ultrafiltration treatments

In the second round of experiments, three strains, including *H. olivaria* LAMA 0626, *M. excellens* LAMA 0842, and *M. flavimaris* LAMA 0954, were examined for the impact of proteinase K and ultrafiltration on the QSI activity of their supernatants, using the luminescent bacteria test (Figure 3). Supernatants of *H. olivaria* LAMA 0626 lost all QSI activity when treated with proteinase K or ultrafiltration. On the other hand, a reduction, but not the complete loss, of QSI activity was observed in the supernatants subjected to proteinase K or ultrafiltration treatments for the other two bacterial strains examined.

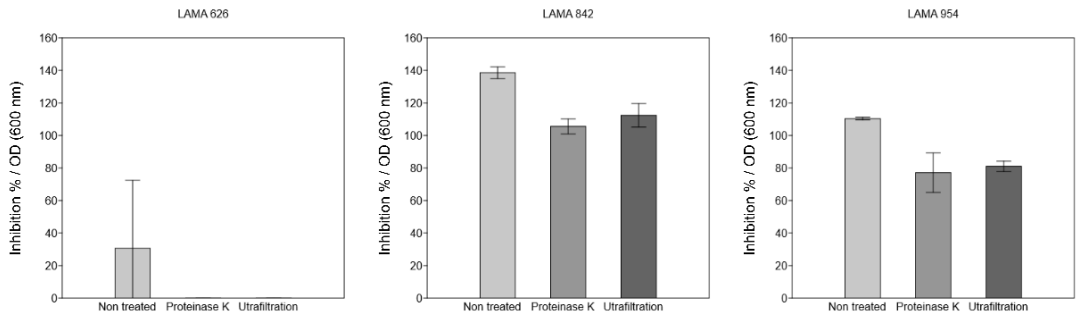

Figure 3. Mean inhibition percentage of bioluminescence, normalized by the optical density (600 nm), of supernatants non-treated, treated with proteinase K, and ultra-filtered, from the three selected strains. The vertical bars represent the standard deviations.

When the strains *M. excellens* LAMA 0842 and *M. flavimaris* LAMA 0954 were compared using two-way ANOVA, a significant effect was observed for both factors, treatment ($F = 62.1$; $p < 0.001$) and strains ($F = 127.1$; $p < 0.001$), but no interaction was found between these factors ($F = 0.1558$; $p = 0.8568$). Pairwise comparisons further determined that proteinase K and ultrafiltration treatments differed significantly from non-treated supernatants for both strains ($Q > 12.3$; $p < 0.001$). A significant difference between these two *Marinobacter* strains was also observed ($Q = 15.94$; $p < 0.001$).

The QSI activity of non-treated supernatants of the three strains was compared by one-way ANOVA. Since these data did not present homogeneity of variances ($p = 0.01363$ in the Levene's test), a corrected (Welch) F test was used instead of the usual F value. Regardless, a significant effect was observed in this test ($F = 21.2$; $p < 0.001$). Pairwise comparisons revealed significant differences between the non-treated supernatants of *H. olivaria* LAMA 0626 and the other two strains ($Q > 6.573$; $p = 0.003098$). On the other hand, the supernatants of *M. excellens* LAMA 0842 and *M. flavimaris* LAMA 0954 did not differ significantly in this test ($Q = 2.325$; $p = 0.2777$).

Genomic analysis of *M. excellens* LAMA 0842

In the genome of *M. excellens* LAMA 0842, 80 open reading frames (ORFs) potentially related to quorum sensing or QSI activity were identified (Figure 4). Among these, five ORFs belonged to the LuxR family of regulatory proteins. The 75 remaining ORFs were related to enzymes belonging to six different categories. Oxidoreductases stood out among these enzymes in the genome of *M. excellens* LAMA 0842. This is a broad category of enzymes associated with various functions; however, since certain oxidoreductases can also inhibit QS, they were specifically searched in the genome of *M. excellens* LAMA 0842.

Using antiSMASH, seven biosynthetic gene clusters were identified in the genome of *M. excellens* LAMA 0842 (Figure 5). A cluster was associated with the synthesis of RiPP-like peptides. Moreover, two beta-lactone biosynthetic gene clusters were also identified, which produce protease inhibitors with potential as antimicrobial agents. The production of ectoine was associated with two ectoine biosynthetic gene clusters. Finally, a NI-siderophore biosynthetic gene cluster

was identified producing NRPS-independent, IucA/IucC-like siderophores.

Phylogenetic analyses and the presence of ketosynthase (KS) and condensation (C) domains, which are highly informative of gene architecture and function, were evaluated using NaPDoS2. The presence of two KS domains from the Class Type II FAS (FASII) was identified, which are discrete, monofunctional proteins commonly observed in bacteria and archaea. However, no hits were found for the C domains following BLAST searches in NaPDoS2.

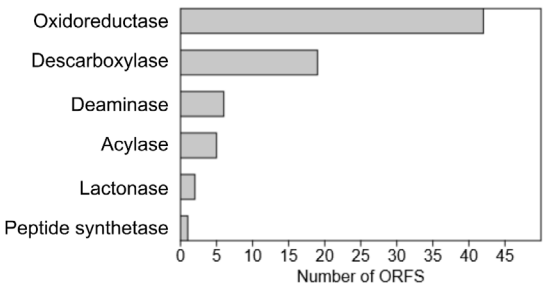

Figure 4. Number of ORFs codifying enzymes that may be involved in QSI identified in the genome of *M. excellens* LAMA 0842.

Identified secondary metabolite regions using strictness "relaxed"

LOCO01000001.1

Region	Type	From	To	Most similar cluster	Similarity
Region 1.1	redox-cofactor	115,660	137,829	lankacidin C NRP+Poliketide	13%
Region 1.2	NI-siderophore	266,101	297,758		

LOCO01000002.1

Region	Type	From	To	Most similar cluster	Similarity
Region 2.1	betalactone	126,127	150,375		

LOCO01000003.1

Region	Type	From	To	Most similar cluster	Similarity
Region 3.1	ectoine	199,996	210,391		

LOCO01000005.1

Region	Type	From	To	Most similar cluster	Similarity
Region 5.1	ectoine	200,311	210,706		

LOCO01000006.1

Region	Type	From	To	Most similar cluster	Similarity
Region 6.1	betalactone	1	15,518	plipastatin NRP	15%

LOCO01000026.1

Region	Type	From	To	Most similar cluster	Similarity
Region 26.1	RiPP-like	31,179	43,365	cittilin A/cittilin B RiPP	7%

Figure 5. Biosynthetic cluster for secondary metabolites identified in the genome of *M. excellens* LAMA 0842.

DISCUSSION

Detection of QSI activity on plate assays

During the screening phase, 16 (26.67%) marine strains of Gammaproteobacteria were identified as active against QS of *C. violaceum* and/or *S marcescens* strains. On the one hand, the frequency of marine bacteria with QSI activity in this study was slightly lower when compared to collections of isolates obtained from seawater of the Mediterranean Sea (38.24%; Muras et al., 2018) and diverse marine samples from Egypt (35%; El-Kurdi et al., 2021). On the other hand, it was higher than collections of strains isolated from marine animals (19.1%; Reina et al., 2019), coastal habitats (18%; Romero et al., 2011), and epibionts from brown algae (12%; Kanagasabhapathy et al., 2009).

The differences observed between the results of this study and those of previous reports can be explained by taxonomic and source differences between the collections of strains analyzed. This study focused on Gammaproteobacteria isolated from marine water and sediments, whereas the other studies analyzed bacteria from various classes, not restricted to Gammaproteobacteria, from various sources. Moreover, different assays of QSI activity were employed, although usually with the same bacterial indicators.

Therefore, considering that seven of the nine genera of bacteria analyzed showed QSI on at least one of the indicator strains, this study confirmed that this type of activity is a common attribute of easily cultivated bacteria within the Gammaproteobacteria class. It can also be concluded that bacteria belonging to the Gammaproteobacteria class may be a source of bioactive molecules that may be employed in the control of undesirable bacterial growth. In agreement with this, another study has reported the presence of QSI-related genes in other bacteria from the Gammaproteobacteria class. Wang et al. (2022) reported the presence of multiple genes encoding fatty acyl-CoA ligases and AHL acylases, responsible for QSI in *Pseudomonas nitroreducens* HS-18. Ye et al. (2020) reported the fadY gene, encoding fatty acyl-CoA synthetase responsible for QSI in *Acinetobacter lactucae* QL-1. Genes encoding AHL acylases have also been reported in the genome of *Pseudomonas aeruginosa* Strain MW3a (Chan et al., 2014). Since bacteria belonging to Gammaproteobacteria are among the most common cultivable bacteria from marine sources (Romero et al., 2011; El-Kurdi et al., 2021), the present work also contributed to indicate this group of microorganisms as an easily obtainable source of bioactive molecules.

From the 60 analyzed strains, 37 (61.67%) belonged to the genus *Halomonas*. Out of these, eight (21.62%) showed QSI activity of different specificities. Other authors have also reported QSI activity in bacteria from *Halomonas*. Abed et al. (2013) have detected QSI activity in three strains of *Halomonas* from hypersaline environments. Woods et al. (2022) have reported QSI activity of *Halomonas hibernica*, isolated from brines, over *S. marcescens* but not *C. violaceum*. Romero et al. (2011) have isolated one strain of *Halomonas taeanensis* from fish tank sediments that exhibited QSI activity over *C. violaceum*. Although the *Halomonas* strains examined in the study belonged to different species, it can be concluded that QSI activity is not uncommon in the genus *Halomonas* and may be mediated by different mechanisms. These potentially include enzymatic destruction of the quorum sensing signals or production of other signals that interfere with the quorum sensing signals of other organisms (Tay and Yem, 2013; Zhu et al., 2023).

In this study, both analyzed strains of *Marinobacter* showed QSI activity over both indicator microorganisms employed. QSI was previously reported for *Marinobacter hydrocarbonoclasticus* isolated from marine sediments (Mithya et al., 2010). The bacterium inhibited the pigmentation in *C. violaceum* and biofilm formation in *Pseudomonas aeruginosa*. Inhibition of QS regulated processes of *S. marcescens* (swarming and biofilm formation) was also reported by Alagely et al. (2011) for all their *Marinobacter* strains. Lastly, *Marinobacter* strains from hypersaline environments also exhibited QSI activity (Abed et al., 2013). In this work,

QSI activity was evaluated with *C. violaceum* CV017, identifying two active strains of the genus *Marinobacter*, both phylogenetically related to the species *Marinobacter zhanjiangensis*. In one of the strains, SK-3, four diketopiperazines were isolated and identified, three of which showed QSI activity (Abed et al. 2013).

Both strains of *S. salarius* examined in this study also showed QSI activity. However, one of them (*S. salarius* LAMA 0947) was only active over *S. marcescens*. QSI activity of *S. salarius* had only been reported previously by Romero et al. (2012). These authors observed that two strains identified as *S. salaries* were able to enzymatically degrade QS signals bearing four, six, ten, or twelve carbon atoms. *S. marcescens* produces acylated homoserine lactones (AHLs) containing four carbon atoms, whereas *C. violaceum* produces AHLs containing six or eight carbon atoms (Gutiérrez-Barranquero et al., 2017). Thus, the results of the present study agree with the results of Romero et al. (2012), except for strain *S. salarius* LAMA 0947. This suggests that QSI potential may vary between strains of the same species, as observed for other adaptative attributes (Rossum et al., 2020).

The remaining four strains with QSI activity belonged to four different genera. On the one hand, the QSI activity of *Pseudoalteromonas*, *Stenotrophomonas*, and *Stutzerimonas* (formerly *Pseudomonas*) has been documented by other authors (Kanagasabhapathy et al., 2009; Pan et al., 2019; Reina et al., 2019). On the other hand, as far as we know, QSI activity has not been previously reported for *Idiomarina*, although it has been reported as capable of QS (Charlesworth et al., 2019). Therefore, this study contributes to the expansion of activities associated with the genus *Idiomarina*, indicating its potential in controlling processes that involve the intracellular communication of bacteria, such as the formation of biofilms and infections in humans.

Influence of cultivation time and presence of a competitor on the QSI activity

In total, seven bacterial strains were selected to be further investigated regarding the impact of cultivation time and presence of competitor microorganism on the QSI activity. We found a significant impact of cultivation time on the QSI activity of all strains. Generally, QSI activity was higher within 48 h of cultivation and lower within 72 h. A similar result was reported for *Bacillus* strains from soils that exhibited higher QSI activity within 24 h compared to 48 h (Wahman et al., 2015). Lower QSI activity within longer periods of cultivation can be explained by the cell mortality and/or degradation of the molecules responsible for the QSI activity while the culture proceeds to the death phase. However, the differences between the results of this study and those found in the study by Wahman et al. (2015) are probably related to the growth rate of the strains examined. *Bacillus* strains are typically fast-growing microorganisms: the species *Bacillus cereus*, for instance, showed a maximum growth rate of 3.46 doublings per hour (Benedict et al., 1993), whereas *M. excellens* LAMA 0842, one of the strains examined in this work, grows slower at 0.42 doublings per hour (Delabary et al., 2020).

In the experiments conducted, QSI activity was always higher when the active strain was cocultured in the presence of *A. fischerii*. A similar effect was reported for antibacterial activity in bacterial strains isolated from a marine sponge (Kanagasabhapathy and Nagata, 2008) and for the enhancement or induction of QSI activity, antimicrobial activity, and surfactant production of epibiont marine bacteria in cocultures (Dusane et al., 2011).

Kanagasabhapathy and Nagata (2008) suggested that the enhancement or induction of activity of one bacterium by another is due to a response to a chemical stimulus generated by the competing organism. In this context, Roy et al. (2010) reported that *Escherichia coli* produces autoinducer-2 (AI-2), a quorum sensing signal synthesized by more than 80 bacterial species that may mediate cross-species communication, as also reported by Liaqat et al. (2014) and Majumdar and Pal (2017). Similarly, *A. fischerii*, the competing species used in the present study, can produce AI-2 as well as two other quorum sensing signals (Verma and Miyashiro, 2013). Besides, strains of *Halomonas* and *Marinobacter*

were already reported as AI-2 producers (Liaqat et al., 2014), although the gene coding for the synthesis of AI-2 was not detected in another strain of *Marinobacter* (Pinto et al., 2021) nor annotated in the genome of *M. excellens* LAMA 0842, one of the strains used in the present study. However, *M. excellens* LAMA 0842 has in its genome one ORF annotated as "Two-component system response regulator QseB" (contig 3, start: 56277; stop: 55609; antisense strand). This protein is part of a system of *E. coli* that regulates several functions in response to AI-2 (Sperandio et al., 2002). Thus, it is possible that the product of this ORF is responsible for perceiving AI-2 produced by *A. fischeri*. Considering all this information, we can conclude that AI-2 may be one of the mechanisms responsible for the enhancement of QSI activity in some Gammaproteobacteria, but not the sole one. Other mechanisms of cross-species communication must exist at least in some of these bacteria, involving, for instance, electric signaling (Majumdar and Pal, 2017) and small RNAs or extracellular vesicles (Cai et al., 2019).

Chemical Nature of Substances Responsible for QSI Activity

Three of the seven selected strains were further investigated regarding the chemical nature of their substances with QSI activity. The supernatant of *H. olivaria* LAMA 0626 completely lost its QSI activity when treated with proteinase K or subjected to ultrafiltration. This suggested that the substance with QSI activity in this strain is an extracellular protein with a molecular mass > 10 kDa, which agrees with the reports by Maisuria and Neruskas (2015) and Rehman and Leikness (2018).

Both strains belonging to the genus *Marinobacter* exhibited a significant reduction in QSI activity with proteinase K or ultrafiltration treatments, although they maintained most of their QSI activity. This indicates that both strains show more than one QSI mechanism. The reduction of QSI by both treatments suggests that a potential extracellular protein may be one of these mechanisms in these strains, as previously reported in two *Marinobacter* strains from the Mediterranean Sea (Kem et al., 2015). Genes homologous to other enzymes involved in QSI activity were also identified in the genome of *M. excellens* LAMA 0842, which supports the results of the experiments conducted. For instance, an ORF, identified in the genome of LAMA 0842 as coding for a beta-lactamase (contig 21; start: 78648; stop: 78007; antisense strand), could be involved in breaking the lactone ring present in autoinducers produced by indicator bacteria, interrupting QS. Another ORF (contig 12; start: 297998; stop: 297285; sense strand), which also encodes a lactonase, could act in a similar way.

Although a potential protein may be contributing to QSI in *Marinobacter* strains, some studies have pointed that small non-protein molecules are the main responsible for their activity. For instance, Abed et al. (2013) identified four diketopiperazines in a strain of *Marinobacter* isolated from a hypersaline cyanobacterial mat that showed QSI activity on *C. violaceum*. Diketopiperazines are secondary metabolites widespread in microorganisms that show a wide range of biological activities (Jia et al., 2019). Their synthesis is accomplished by nonribosomal peptide synthetases (NRPSs) or cyclodipeptide synthases (CDPSs) (Harken and Li, 2021). As stated before, one BGC was associated with the synthesis of RiPP-like peptides, which generate antimicrobial action and may impact quorum sensing in some bacteria (Whiteley et al., 2017). Besides this, other BGCs were also identified, highlighting *M. excellens* LAMA 0842's potential to produce a variety of secondary metabolites and natural products with diverse biological activities. Further research is necessary to explore the potential of these compounds as antimicrobial agents or as inhibitors of quorum sensing in bacterial populations.

CONCLUSION

Marine bacteria obtained from water and sediment samples of the Atlantic Ocean can inhibit the cellular communication mechanism of various bacteria such as *C. violaceum* LAMA 0447, *S. marcescens* LAMA 1170 and *A. fischeri* NRRL B-11177. Higher QSI activity was observed after 48 hours of cultivation and in mixed cultures

with *A. fischeri* NRRL B-11177. The molecules responsible for QSI activity of *H. olivaria* LAMA 0626 were probably enzymes. In the case of *Marinobacter* strains, both enzymes and non-protein low molecular weight (< 10 kDa) molecules were involved. Genes that may encode these molecules or their synthesis were identified in the genome of *M. excellens* LAMA 0842. Finally, this work contributes to the search for innovative techniques for controlling microbial growth when it is unwanted by selecting QS inhibitor organisms via screening assays with different bacterial indicators. This work can also stimulate research into QS and QSI activities in other microorganisms and samples from the South Atlantic Ocean, including prospecting for genes using cultivation-independent techniques, as already used for other molecules of biotechnological interest.

Data availability statement

Experimental data can be accessed via the following link: https://univali-my.sharepoint.com/:x:/g/personal/marcus_silva_univali_br/EXcb5GEiZRZBh_z404VqYYcBxeZ0eH1kRi8rN5tzeVV-og?e=0Dx0M0.

Supplementary material

There is no supplementary material for this article.

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Author contributions

V. R.: Conceptualization; Investigation; Writing – original draft; Writing – review and editing.

C. A. S., T. M. de M.: Investigation.

A. O. de S. L.: Investigation; Writing – review and editing.

M. A. C. da S.: Supervision; Conceptualization; Investigation; Writing – original draft; Writing – review and editing.

Conflicts of interest

The authors declare no conflicts of interest.

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