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Bioprospecting the Cultivable Bacteria Associated with *Trididemnum maragogi* from the Saint Peter and Saint Paul's Archipelago (Brazil) for Bioactive Metabolites

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Abstract

Ascidians and their associated microbiota represent one of the most prolific sources of novel bioactive compounds with application in marine biotechnology. This study investigates the pharmacological potential and metabolic diversity of the bacterial microbiota associated with the ascidian *Trididemnum maragogi*, collected at Saint Peter and Saint Paul's Archipelago (SPSPA), Brazil. A total of 45 bacterial strains were taxonomically identified via 16S rRNA gene sequencing into 11 distinct genera, with a predominance of *Bacillus* and actinomycetes such as *Micromonospora*, *Salinispora*, and *Streptomyces*. Crude organic extracts from bacterial cultures were evaluated for antiproliferative activity against human cancer cell lines (501-Mel and HCT-116). Extracts from *Micromonospora* spp. (BRB-810, BRB-814, BRB-832) and *Salinispora* spp. (BRB-803, BRB-808) exhibited potent cytotoxic activity. Metabolomic profiling via HPLC-MS/MS and Feature-Based Molecular Networking (GNPS) enabled the annotation of staurosporines, rifamycins, diketopiperazines, and other bioactive classes, while revealing that approximately 95% of detected features remain unannotated. Furthermore, whole-genome sequencing of *Micromonospora aurantiaca* BRB-832 unveiled 20 biosynthetic gene clusters (BGCs) related to macrolides, alkylresorcinols, anthracyclines, and novel uncharacterized pathways. These findings highlight *T. maragogi* and its microbial symbionts as a valuable, underexplored source of novel bioactive natural products with biotechnological potential.

Keywords: Tunicate-associated bacteria; Marine Natural Products; Antiproliferative activity; Metabolomics; Genome mining.

Introduction

Ascidians (Tunicata: Ascidiacea) are sessile marine invertebrates that typically colonize hard substrates in shallow marine waters. These filter-feeding organisms are characterized by a cellulosic tunic that involves either a solitary individual or a colony of zooids. Furthermore, ascidians are a staple host to bioactive natural products, sourcing a myriad of compounds with pharmacological potential (Dou and Dong, 2019). In fact, trabectedin (Yondelis™), lurbinectedin (Zepzelca™) and plitidepsin (Aplidin™) represent natural molecules, or derived thereof, isolated from ascidians that are clinically approved drugs for anticancer treatments (Jimenez et al. 2020; Papon et al. 2022; Haque et al. 2022; Jimenez et al. 2023).

Mass spectrometry-based metabolomics has become a powerful approach for the chemical investigation of natural products, enabling rapid profiling of complex biological extracts and facilitating the discovery of bioactive metabolites (Brunetti et al. 2019; Nunes et al. 2014; Gauglitz et al. 2022). In particular, tandem mass spectrometry (MS/MS) combined with feature-based molecular networking have been improving dereplication strategies, allowing the early identification of known compounds and prioritization of potentially novel metabolites for isolation and characterization (Aksenov et al. 2017). Molecular networking organizes MS/MS spectra according to their fragmentation similarity, clustering related metabolites into molecular families that can be visually explored and annotated. One important example of a platform used is the Global Natural Products Social Molecular Networking (GNPS), a collaborative and community-driven environment that enables the comparison of experimental spectra with public libraries and shared datasets (Wang et al. 2016). Importantly, molecular networking also facilitates the exploration of the large proportion of unannotated MS/MS signals often referred to as "metabolomic dark matter", allowing researchers to infer structural relationships between known and unknown metabolites and guide targeted discovery efforts (Watrous et al. 2012; Quinn et al. 2017). These advances have significantly accelerated the study of chemically rich systems such as marine invertebrates and their associated microbiota, which represent an important source of structurally diverse natural products with biotechnological application.

In this context, *Tridemnum* is a notable ascidian genus in the pharmacological realm. Indeed, *Tridemnum solidum*, a species from the Caribbean, is the source of didemnins, a series of cyclic depsipeptides with anticancer, antiviral and immunosuppressive properties described in

the early 1980s (Rinehart et al. 1981; Ali et al. 2025). Among these, didemnin B displayed the highest potency and was forwarded to clinical trials for the treatment of various types of cancer (Chun et al. 1986), only to be withdrawn after concluding phase II due to poor efficacy and intolerable toxicity (Ali et al. 2025). Still, didemnin B was the first natural marine compound to enter clinical trials, and the lessons learned during decades of studies early in the field have been resonating throughout the marine pharmacology timeline (Chun et al. 1986; Vera and Joullié, 2002; Sahm et al. 2022).

Subsequent evidence has linked the production of didemnins to bacteria associated with the ascidian, following reports on free-living bacteria of the genus *Tistrella* spp. producing these compounds (Xu et al. 2012). Indeed, the biosynthesis of didemnins is carried out through a hybrid microbial pathway, encoded by nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) gene clusters (Xu et al. 2012; Zou et al. 2024). Findings like these reveal both ecological and pharmacological relevance of the microbiota associated with ascidians.

With a coastline of over 8,000 km and four oceanic islands, Brazil harbors a particularly diverse ascidian fauna, where endemism is highly acknowledged. In the past 10 years, 13 new species were described for different parts of the Brazilian coastline and islands (Rocha et al. 2024). In this scenario, *Tridemnum maragogi* (Figure 1A and 1B) is an endemic ascidian from the Northeastern Brazilian coast and equatorial oceanic islands, first described in 2002 from the State of Alagoas (Rocha, 2002). In 2015, the occurrence of *T. maragogi* was reported at Rocas Atoll, an atoll of volcanic origin and coralline formation established as a Marine Protected Area, located approximately 78 nmi off the northeast coast of Brazil (Paiva et al. 2015). Herein, we report the occurrence of this species at the Saint Peter and Saint Paul's Archipelago (SPSPA; Figure 1C), a collection of islets located on the Equatorial Mid-Atlantic ridge, nearly 590 nmi off the Brazilian coast.

T. maragogi is a conspicuous element of the benthic coverage at the SPSPA, forming large patches over the rocky bottom. Although there is no published inventory on the diversity of ascidians found at ASPSP, many studies have associated this region with a rich and peculiar biodiversity, with promising biotechnological features to be explored (Wilke et al. 2021; Ferreira et al. 2016; Trindade-Silva et al. 2017). In the present study, *T. maragogi* was assessed for the anticancer potential of its culturable associated bacteria, which were described for their taxonomic,

cytotoxicity potential and metabolomics features. Furthermore, strain BRB-832 was selected for whole-genome sequencing and mining of biosynthetic gene clusters related to production of potential active compounds.

Materials and Methods

Collection and taxonomic identification of the ascidian

The collection of *T. maragogi* specimens at Saint Peter and San Paul Archipelago took place in 4 different expeditions by the team of the long-term ecological project in the Brazilian oceanic islands (PELD-ILOC), occurred in May and October of 2022 and 2023, under national authorization for scientific activity (SISBIO-ICMBio No. 41327-46). Ascidians were visually identified by snorkeling divers during low tide at the "Enseada" site (0° 55' 1.5" N, 29° 20' 43.9" W), followed by collection with the help of surgical forceps. Samples were placed into sterile whirl-pak bags and kept frozen at -20°C for storage and transportation, up until laboratory processing. Divers wore gloves during the collection and storage of samples.

Isolation, culture and preservation of bacteria strains

Once at the laboratory, samples remained stored in an ultrafreezer (-80ºC) for a maximum of 90 days until processing for bacteria isolation. Ascidians were macerated within a sterilized crucible and pestle and 1 mL of 75% sterile-filtered seawater was used to dilute samples before transfer to a labeled tube. The crucible was washed again with another 500 µL of seawater to recover all the macerated material. Tubes followed incubation in a dry bath at 55ºC for 15 minutes. For plating, the homogenate of each sample was added in triplicate utilizing three different volumes (10 µL, 50 µL and 100 µL) to four culture media: SWA (seawater 75% and agar 18 g/L), TMA (seawater 75%, glucose 0.1 g/L, yeast extract 0.1 g/L, K_2HPO_4 0.5 g/L, Na_2HPO_4 0.7 g/L, KNO_3 0.1 g/L, NaCl 0.3 g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L, $CaCl_2 \cdot 2H_2O$ 0.02 g/L, trace metal solution 1% containing $FeSO_4 \cdot 7H_2O$ 0.2 g/L, $ZnSO_4 \cdot 7H_2O$ 0.18 g/L, $MnSO_4 \cdot 4H_2O$ 0.02 g/L, $CuSO_4 \cdot 5H_2O$ 0.09 g/L, $CoSO_4 \cdot 7H_2O$ 0.1 g/L, H_3BO_3 0.2 g/L and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.05 g/L, and agar 18g/L), A1 (seawater 75%, starch 10g/L, yeast extract 4g/L, peptone 2g/L and agar 18g/L) and SCA (seawater 75%, starch 10g/L, casein 1g/L and agar 15g/L). Samples were spread across the

plates with the aid of a sterile microbiological loop. Growth colonies were observed between 7 and 90 days.

Bacteria isolation and cultivation was carried in A1 plates using simple streak seeding technique, with the aid of a sterile toothpick, and then through the depletion technique utilizing a sterile bacteriological loop. Pure cultures grown on solid media were transferred to 250 mL Erlenmeyer flasks containing 100 mL of A1 broth and incubated at 28°C under agitation at 140 rpm for 7–32 days. Strain BRB-803 was additionally cultivated in SWB (75% seawater broth) under the same conditions. Aliquots of well-grown cultures were cryopreserved in the in-house marine bacteria biobank (MicroMarin - USP) in 25% glycerol and stored at -80ºC.

16S rRNA taxonomic identification and phylogenetic analysis of recovered bacteria

Taxonomic and phylogenetic assessments of the recovered bacterial strains were carried out based on the partial sequence of the 16S rRNA gene. Therefore, a 3 mL aliquot of well-grown cultures of each strain in A1 broth was subjected to genomic DNA extraction. Given the diversity of the isolates, a sequential extraction strategy was applied to ensure adequate DNA yield and quality across all samples. Initially, the "salting out" technique (Kieser et al. 2000) was employed as the standard method. For isolates that proved recalcitrant, yielding insufficient DNA, the DNeasy Blood and Tissue kit (QIAGEN) was utilized. Finally, for any remaining challenging isolates, a standard phenol-chloroform extraction (Green and Sambrook, 2017) was adopted. Quantification of the extracted DNA was performed using a BioTek Synergy HTX Multimode Reader. Partial amplification of the 16S rRNA gene was performed via PCR (GoTaq Master Mix, Promega) using universal primers 27F and 1492R. The PCR products were purified with ExoSAP-IT (Affymetrix) and sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems).

The sequences were analyzed using Geneious Prime 2025.0.3 software and compared with sequences previously deposited in public databases using the Basic Local Alignment Search Tool (BLAST), from NCBI, and EzBioCloud (Yoon et al. 2017). Representative type strain sequences showing the highest similarity to each isolate were retrieved and aligned together with the sequences obtained in this study using the MAFFT algorithm implemented in UGENE v51.0. Phylogenetic relationships were inferred by the Maximum Likelihood method using RAxML-HPC BlackBox available through the CIPRES Science Gateway, employing the GTRCAT evolutionary

model and 1,000 rapid bootstrap replicates. The resulting phylogenetic tree was visualized and edited using the Interactive Tree of Life (iTOL) v6 platform (Letunic and Bork 2024).

Production of organic extracts from bacterial isolates

Organic extracts were obtained from well-grown cultures of each isolate in A1 liquid media. After the respective growth period, which varied between 7 and 32 days, 100 mL of ethyl acetate (1:1) were added to the broth and returned for agitation for 1 h. Next, the solution was partitioned in a separation funnel, where the aqueous phase and biomass were discarded and the organic phase was recovered. Solvent removal was carried out at reduced pressure using a rotary evaporator, then completely dried in a sample concentrator to obtain the crude extracts. These extracts were then subjected to cytotoxicity and metabolomics assessments.

Analysis of cytotoxicity of extracts obtained from bacterial isolates

Cytotoxicity assessments were carried out by the MTT assay (Mosmann 1983) . In an initial qualitative analysis, bacterial extracts were evaluated for their antiproliferative effect against human melanoma (501-Mel, RRID:CVCL_4633, ATCC), and colorectal carcinoma (HCT-116, RRID: CVCL_0291, BCRJ) tumor cell lines. The cells (80% density) were seeded in 96-well plates and treated with the extracts at 5 and 50 µg/mL for 72 h. The extracts that demonstrated excellent inhibition of tumor cell growth (> 70% at a concentration of 50 µg/mL) were furthered to a quantitative cytotoxicity assessment against same tumor cell line and in a non-tumoral murine fibroblast cell line (NHI-T3T, RRID: CVCL_0594, BCRJ). In such cases, cells were exposed to seven concentrations of each extract, between 0.003 and 50 µg/mL for 72 h, to enable calculations of the IC_{50} values of the extracts.

In any case, cell proliferation was quantified by the ability of viable cells to reduce MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to formazan. Once the incubation period was over, the wasted culture medium was replaced with fresh medium containing 0.05 mg/mL of MTT in every well. After 2 h of reincubation, to allow for MTT conversion to formazan, the medium was removed and the formazan product was diluted with DMSO. Absorbance was measured using a BioTek Synergy HTX Multimode Reader multiplate

reader, at 570 nm. The percentage of viable cells in treated wells, considering the qualitative assessment, was determined proportionally to the absorbance of control wells, obtained by exposing cells to 0.5% DMSO (the dilution vehicle for the extracts). For the quantitative evaluation, the IC_{50} and respective confidence intervals (CI 95%) for the selected extracts were obtained by nonlinear regression using the software GraphPad Prism 10.0.0 for Windows. Relative potency was assessed by calculating fold differences based on IC_{50} ratios between extracts. The selectivity index (SI) was calculated as the ratio between the IC_{50} value obtained for non-tumoral cells and the IC_{50} value obtained for tumoral cells as follows:

$$SI = \frac{IC50\ non-tumoral\ cells}{IC50\ tumoral\ cells}$$

Metabolomics analysis

HPLC-MS/MS analyses

The dried extracts of BRB samples, and samples of culture media A1 and SWA were solubilized in methanol:water (1:1) at a final concentration of 1 mg/mL, and homogenized under vortex mixing. Afterwards, all solutions were filtered with 0.22 µm PTFE syringe filters into 1 mL vials. The diluent methanol: water (1:1) was used as blank. The volume of 3 µL of each sample was injected in a HPLC-MS/MS timsTOF fleX MALDI-2 system, operating with the electrospray ionization (ESI). Chromatographic separation was performed in a C18 column (Kinetex XB^{TM}, Phenomenex, 100 × 2.1 mm; particle size 2.6 µm) connected to a guard cartridge with the same material. The mobile phase was water (A): acetonitrile (B), both with formic acid 0.1% (v/v), at flow rate of 0.35 mL min^{-1}, using the following gradient: 0–23 min, 5–100% B; 23–27 min, 100% B; 27–28 min, 100–5% B; 28–33 min, 5% B. The oven temperature was set at 40 °C. MS and MS/MS spectra were acquired between *m/z* 100 and 1000 with ESI in positive mode. The ESI-MS parameters were capillary voltage, 3.5 kV; End Plate Offset, 500 V; dry temperature, 250 °C; gas flow 9.0 L/min; pressure, 4.0 bar. N_2 was used as drying and nebulizer gas. MS/MS was acquired in Auto MS/MS mode.

Spectral similarity network

Raw data from HPLC-MS/MS analyses were convert to .mzML format using the TIMSCONVERT workflow (Luu et al. 2022). The .mzML files were processed by the software MZmine v. 4.4.3 (BMC Bioinformatics, United Kingdom) with the following parameters: (I) Mass detection (MS level 1): Mass detector – centroid, Noise level: 1.0×10^3; (II) Mass detection (MS level 2): Mass detector – centroid, Noise level: 2.0×10^2; (III) Chromatogram builder: Minimum consecutive scans – 3, Minimum intensity for consecutive scans - 1.0×10^3, Minimum absolute height - 1.5×10^3, *m/z* tolerance (scan-to-scan) – 0.02 *m/z*; (IV) Local minimum feature resolver: Chromatographic threshold – 85%, Minimum search range RT/Mobility (absolute) - 0.05, Minimum relative height – 1.0×10^3, Min ratio of peak/top edge – 1.70, Peak duration range (min/mobility) – 0.00–1.50, Minimum scans (data points) – 3; (V) 13C isotope filter (formerly: isotope grouper): *m/z* tolerance (intra-sample) – 0.002 *m/z*, Retention time tolerance – 0.2 minutes; maximum charge – 2; (VI) Join aligner: *m/z* tolerance (sample-to-sample) – 0.02 *m/z*, Weight for *m/z* – 75, Retention time tolerance – 0.3 minutes, Weight for RT – 25; (VII) Feature list row filter: Keep rows that match all criteria – Feature with MS2 scan. After this processing, the feature list was exported as .mgf (spectral data) and .CSV (data table with RT, precursor ions and Peak area of features). Then, the .mgf and .CSV files were analyzed by FBMN in GNPS2 (https://gnps2.org/workflowinput?workflowname=feature_based_molecular_networking_workflow). The Precursor Ion Tolerance and Fragment Ion tolerance were set to 0.02 Da. Connection between consensus spectra were set to minimum of 6 matched fragment ions and cosine score above than 0.7. Matches between acquired MS/MS spectra and GNPS 2 spectral libraries were set to cosine score above 0.7 and the minimum of 5 matched fragment ions. The molecular networks were visualized into Cytoscape software v. 3.10.1 (Shannon et al. 2003). Structural classification of annotated compounds was performed by the NPClassifier tool (Kim et al. 2021).

MicrobeMASST analysis

The occurrence of MS/MS spectra of annotated compounds in microorganism datasets were visualized with the microbeMASST tool (https://masst.gnps2.org/microbemasst/). Searches were performed by manually entering the Universal Spectrum Identifier (USI) of selected nodes from the spectral similarity network.

Genome sequencing and analysis

Full genomic DNA sequencing was performed for the strain BRB-832. Genomic DNA extraction was carried out following a 7-day cultivation period in A1 media, utilizing Genomic DNA Purification Kit (Promega). DNA quality was assessed in 1% agarose gel electrophoresis and Nanodrop (DeNovix). Genome sequencing and assembly were performed by Plasmidsaurus (South San Francisco, CA, US). Sequencing was accomplished using Oxford Nanopore Technology (ONT), with R10.4.1 flow cells. Basecalling was realized by Dorado v4.3 Super-Accurate Basecalling and raw data filtering was performed by Filtlong v0.2., removing reads <300 bp and keeping the best 95% quality reads. Assemblies were created using three different assemblers - Flye v2.9.6+ (Kolmogorov et al. 2019), Hifiasm (Cheng et al. 2021) and Plassembler v1.8.0+ (Bouras et al. 2023) - and a consensus assembly was generated by Autocycler (Wick et al. 2025). Genome annotation was obtained with Bakta v1.11 (Schwengers et al. 2021) and genome completeness and contamination were determined by Busco (Seppey et al. 2019) and CheckM (Parks et al. 2015). Additional technical information about the sequencing and assembly service can be found in Plasmidsaurus web-page (https://plasmidsaurus.com/technical-documentation/genome).

Initial digital DNA-DNA hybridization (dDDH) (Auch et al. 2010) values were calculated with the Genome-to-Genome Distance Calculator (GGDC) server (Meier-Kolthoff et al. 2013; 2022), including all 1150 *Micromonospora* genomes deposited into NCBI in the comparisons. Strains that showed dDDH values ≥70% had their Average Nucleotide Identity (ANI) value (Goris et al. 2007) calculated as well, using the FastANI tool (Jain et al. 2018), hosted in the Galaxy Australia server. The 5 strains with the highest dDDH and ANI values were included in a Type Strain Genome Server (TYGS) (Meier-Kolthoff et al. 2022) phylogeny, constructed using the Genome Blast Distance Phylogeny (GBDP) approach (Meier-Kolthoff et al. 2013). Finally, biosynthetic gene cluster (BGC) identification was executed using antiSMASH 8.0.4 (Blin et al. 2025) with the "relaxed" parameter setting. Novelty of the BGCs and comparison with characterized BGCs was performed with the KnownClusterBLAST algorithm, using the MiBIG (Zdouc et al. 2025) database as a source of reference BGCs.

3. Results and Discussion

3.1. Taxonomic identification of T. maragogi *from SPSPA.*

Specimens of *T. maragogi* were collected from SPSPA on four expeditions and examined following the typical dissection procedures for the group. The species typically forms aggregations of small colonies as large patches over 20cm in diameter (Figure 1). The color varies from light grey to dark brown, and colonies are usually 1-4 cm in length and 2-3mm in thickness. Their tunic is packed with small, rounded spicules with many short conical rays. All morphological details match the description for Rocas Atoll specimens presented by Paiva et al. (2015). The overall aspect of the colony and the clear presence of *Prochloron* and other symbionts make it very similar to *T. solidum*. However, there are important differences in the size and shape of the spicules, as well as in the number of sperm duct coils (Rocha, 2002). Additionally, a phylogeny including some of the Atlantic species of *Trididemnum*, recovered *T. solidum* as a close sister species of *T. maragogi* (Oliveira et al. 2017). This is the third record of the species, which appears to be endemic to the northeast of Brazil and the oceanic islands of the Rocas-Noronha-SPSPA chain.

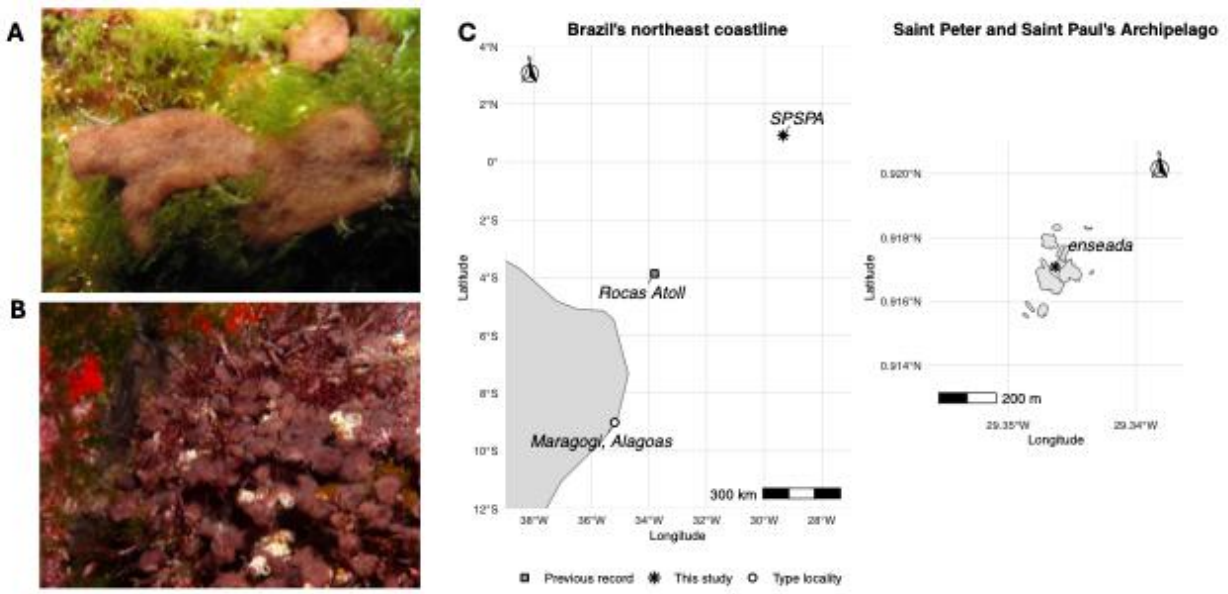


Figure 1. *In situ* photographs and geographic distribution of *Trididemnum maragogi*. **(A)** Detail of a colony and **(B)** aggregation of colonies as typically observed in the Saint Peter and Saint Paul Archipelago (SPSPA). **(C)** Geographic distribution of *T. maragogi* along the northeastern Brazilian coastline and oceanic islands. The inset shows the sampling site at "Enseada", SPSPA. Symbols indicate previous records

(square), the record reported in the present study (asterisk), and the type locality (open circle). The map was created in R using the sf, ggplot2, and rnaturalearth packages.

2.2. Cultivation and isolation of culturable bacteria

For bacteria recovery, specimens of *T. maragogi* obtained through 4 sampling expeditions to SPSPA, were processed for the isolation and cultivation of their associated microbiota. Samples from May (n=3) and October (n=3) 2022 were stored in an ultra-freezer at -80ºC until they were processed in early 2023. The first bacterium strain isolated from this sample took about 60 days to appear on culture plates, and the efforts on this material yielded a total of 21 strains. In May 2023 a new collection returned with only one specimen of *T. maragogi* (n=1) for bacterial isolation, which was immediately processed, yielding 4 recovered strains. In October of the same year, a new collection returned with fresh colonies of the ascidian (n=5) from which 25 isolated strains were obtained. In total, 50 bacterial isolates associated with the ascidian *T. maragogi* collected at SPSPA were recovered herein. Details on the original isolation media for each strain are provided in Table S1.

2.3 Taxonomic and phylogenetic assessments of bacterial isolates assessment

Out of the 50 strains recovered, 45 had a successful outcome in their taxonomy quality, and the obtained contig sequences were used for taxonomic assignment based on 16S rRNA gene analysis (Figure 2; Table S1). Based on the topology of the clustering, the 45 analyzed bacteria were organized into eleven distinct genera: *Micromonospora* (12)*, Serratia* (3)*, Bacillus* (13)*, Paenibacillus* (2), *Sporosarcina* (1)*, Rhodococcus* (1)*, Tsukamurella* (1)*, Streptomyces* (2)*, Microbacterium* (3)*, Brucella* (5)*,* and *Salinispora* (2). These genera belong to bacterial classes known for their symbiotic associations with ascidians, particularly *Alphaproteobacteria, Gammaproteobacteria,* and *Actinomycetia* (Bauermeister et al. 2024). The predominant presence of actinomycetes, such as *Streptomyces, Salinispora* and *Micromonospora*, indicates the effectiveness of the bacterial isolation methods applied herein, which were intended to favor the recovery of members of this taxon, as actinomycetes are highly significant sources of secondary metabolites with biotechnological potential, including anticancer, antimicrobial, and immunosuppressive properties (Ruwandeepika et al. 2022). The observed taxonomic diversity also

suggests that symbiotic bacteria play complementary roles in the ecological adaptation of ascidians, contributing both to their chemical defense and broader microbial interactions (Tianero et al. 2015).

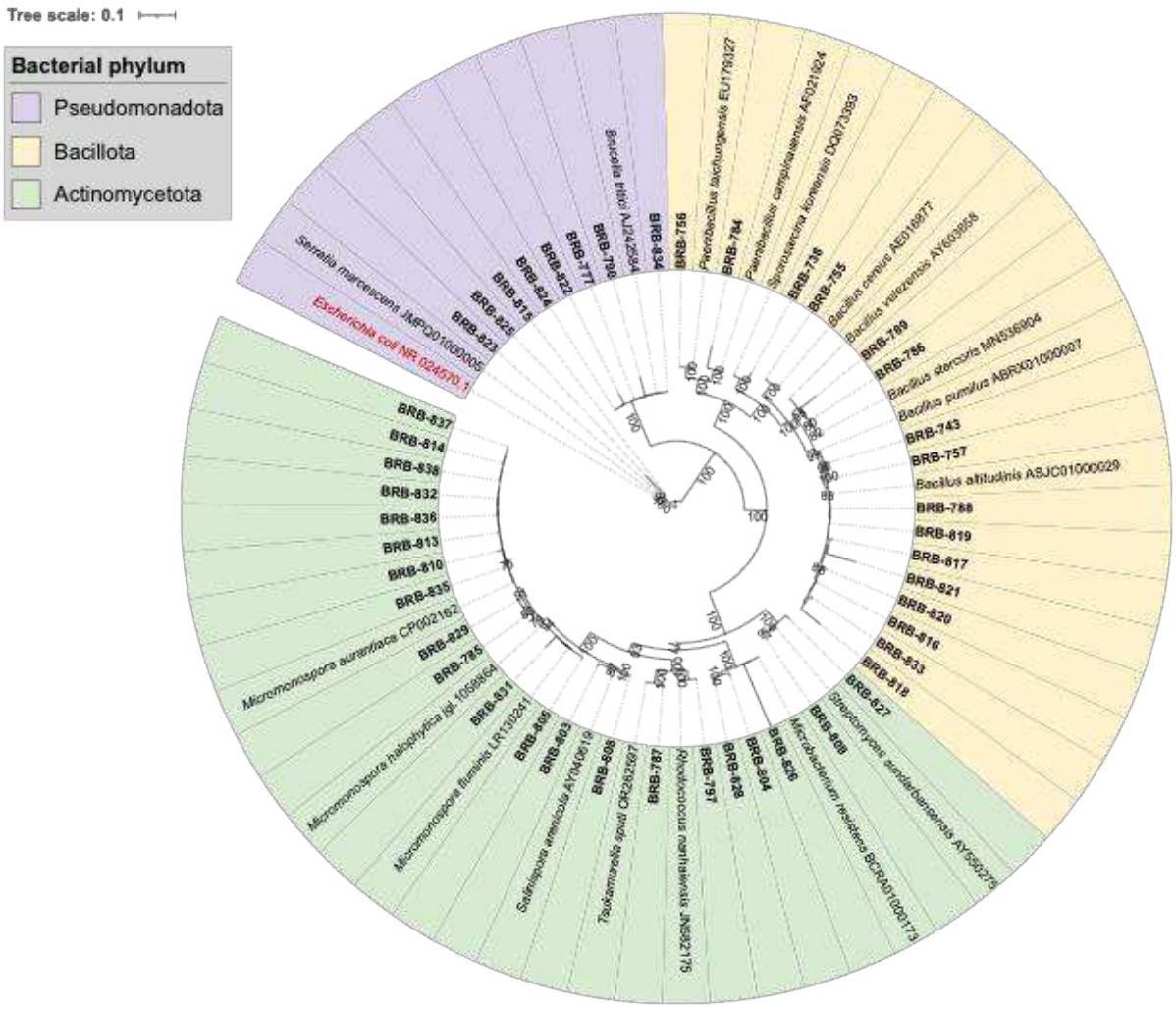


Figure 2. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of 45 bacterial strains isolated from the ascidian *Trididemnum maragogi* and their closest type strains. Sequences were aligned using the MAFFT algorithm and phylogenetic inference was performed using RAxML-HPC BlackBox under the GTRCAT evolutionary model. Bootstrap support values (>70%) are indicated at the nodes. *Escherichia coli* NR_024570.1 was used as the outgroup. Branches are colored according to bacterial phylum affiliation: Actinomycetota (green), Bacillota (yellow), and Pseudomonadota (purple). Strains

isolated in this study are shown in bold. The phylogenetic tree was visualized and edited using the Interactive Tree of Life (iTOL) platform.

The culturable microbiota associated with ascidians has been investigated by different research groups around the world, revealing a diverse assemblage of bacteria, fungi, and cyanobacteria isolated from these invertebrates (Chen et al. 2017; 2018). A review on the topic reported that 19 ascidian genera have served as sources for the isolation of these microorganisms, including representatives of the family Didemnidae, such as *Didemnum* and *Tridemnum* (Chen et al. 2018). Bacterial strains isolated from *Tridemnum* specimens include *Tristella mobilis*, associated with *T. solidum* from Japan and the Red Sea (Tsukimoto et al. 2011), as well as two actinomycete strains, *Nocardia* sp. and *Solwaraspora* sp., isolated from *T. orbiculatum* collected in the Florida Keys, USA (Wyche et al. 2012; Ellis et al. 2014). A greater diversity of bacterial genera has been cultivated from *Didemnum* sp. specimens collected on the southeastern coast of Brazil, including *Bacillus*, *Paenibacillus*, *Ruegeria*, and *Staphylococcus*, as well as actinomycetes such as *Arthrobacter*, *Gordonia*, *Micrococcus*, and *Nocardia* (Menezes et al. 2010). Herein, isolates from *T. maragogi* were predominantly represented by *Bacillus* sp. (28.9%) and *Micromonospora* sp. (26.7%), followed by *Brucella* sp. (11.1%), *Serratia* sp. and *Microbacterium* sp. (6.7% each), and *Streptomyces* sp. and *Salinispora* sp. (4.4% each). This taxonomic composition differs from those previously reported for other members of the family Didemnidae, providing new insights into the cultivable microbiota associated with *T. maragogi* and contributing to the understanding of microbial diversity in the SPSPA.

Regarding its biotechnological potential, cultures of the genus *Serratia* have been isolated from the gut of the ascidian *Halocynthia roretzi* collected in Shandong Province, China, where they appear to contribute to the host's environmental adaptation by providing antibiotic protection (Yang et al. 2022). In addition, *Micromonospora* spp. strains isolated from the ascidian *Eudistoma vannamei*, from the northeastern coast of Brazil, have demonstrated antiproliferative activity against tumor cells (Sousa et al. 2012; Jimenez et al. 2013), highlighting the well-established capacity of this genus to produce biologically active secondary metabolites (Yan et al. 2022). Along with *Streptomyces* and *Bacillus*, which are also widely recognized for their remarkable biosynthetic potential for bioactive compounds, these findings broaden the understanding of

symbiotic interactions between bacteria and ascidians and reinforce the biotechnological potential of this association, particularly in the search for novel bioactive compounds from ascidian-associated microbiota.

2.4 Evaluation of cytotoxic activity of organic extracts obtained from bacterial isolates

All extracts produced by the 50 bacterial isolates were tested for their antiproliferative effect against 501-Mel and HCT-116 tumor cell lines. Extracts obtained from BRB-789, BRB-808, BRB-810, BRB-814, BRB-832, and BRB-837 induced over 75% inhibition of 501-Mel cell growth at a concentration of 50 µg/mL (Table S2; Figure 3A). Meanwhile, extracts from BRB-738, BRB-803, BRB-808, BRB-810, BRB-814, BRB-832, BRB-834, BRB-836, and BRB-837 were active against HCT-116 (Table S2; Figure 3B). It is worth mentioning that a second extract of strain BRB-803 was produced from a culture grown in SWB medium, the medium originally used for strain isolation, to assess whether returning to the original isolation conditions could improve bacterial growth. However, no apparent improvement in growth was observed under this condition. Nevertheless, the resulting extract also displayed noteworthy activity against the HCT-116 cell line.

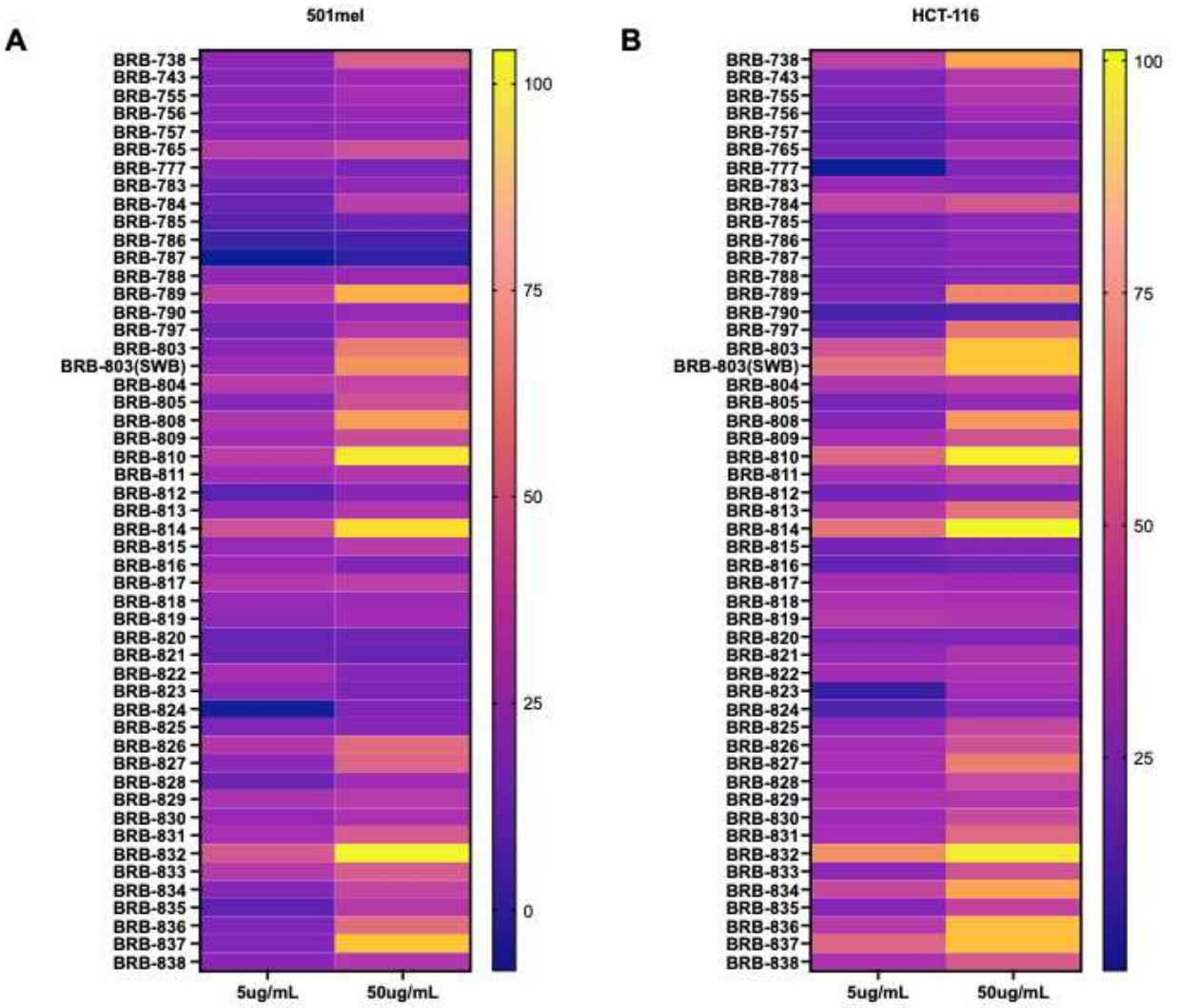


Figure 3. Inhibition of cancer cell proliferation (%) induced by bacteria extracts at 5 and 50 µg/mL against **(A)** 501mel and **(B)** HCT-116 cell lines. Cytotoxicity of extracts was evaluated by the MTT assay after 72 hours of incubation. Extracts that reduced at least 75% of cell proliferation were considered cytotoxic. Heatmap represents the mean of three independent experiments (n=3) analysed at GraphPad Prism v.10.0.

Extracts with recognized biological activity were subjected to cytotoxicity testing at different concentrations to determine the IC_{50} of the extract in both tumoral cell lines and in the non-tumoral NIH-T3T cell line. However, not all extracts retained the potency initially observed in the screening assays, possibly due to instability of molecular structures of their bioactive compounds. Therefore, only the extracts that retained biological activity at the highest

concentration tested were selected for further analyses, namely BRB-803, BRB-803-SWB, BRB-808, BRB-810, BRB-814, and BRB-832 (Table 1; Figure S1).

In light of these results, it is worth highlighting that the extracts produced by *Micromonospora* spp. strains BRB-810, BRB-814, and BRB-832 were the most promising from a pharmacological perspective, as they exhibited the lowest IC_{50} values, markedly in HCT-116. Additionally, extracts of BRB-803 produced in different culture media exhibited very similar activity profiles, although the extract obtained in SWB medium showed slightly higher potency (~ 1.3-fold) against HCT-116 cells. Furthermore, both BRB-803 extracts, as well as the BRB-808 extract, exhibited a cytostatic effect below 50% cell growth inhibition on the non-tumoral NIH-3T3 cell line, as observed in Figure S1-C.

Table 1. Half-maximal inhibitory concentration (IC_{50}), 95% confidence interval (CI95%) and coefficient of determination (R^2) of bacteria extracts ($\mu g.mL^{-1}$) against tested cell lines (501Mel, HCT-116, and NHI-T3T), determined by the MTT assay after 72hs of incubation. Values were calculated by non-linear regression using GraphPad Prism software v.10.0, and reflected the mean of three independent experiments (n=3).

	501mel		HCT-116		NHI-T3T	
Extracts	IC_{50} (CI95%)	R^2	IC_{50} (CI95%)	R^2	IC_{50} (CI95%)	R^2
BRB-803	NT	-	35.78 (22.6 - 74.4)	0.8427	>50 (ND)	-
BRB-803 SWB	NT	-	28.26 (15.2 - 69.7)	0.8393	>50 (ND)	-
BRB-808	>50 (46.0 - ND)	0.7695	32.84 (22.3 - 59.0)	0.8630	>50 (ND)	-
BRB-810	35.13 (26.9 - 44.1)	0.8918	17.22 (ND - 21.3)	0.9655	26.42 (21.9 - 31.6)	0.9561
BRB-814	40.98 (34.5 - 48.0)	0.9360	18.95 (15.7 - 22.8)	0.9662	33.30 (27.0 - 41.1)	0.9317
BRB-832	33.30 (26.2 - ND)	0.9147	16.60 (ND - 20.6)	0.9586	24.76 (18.6 - 32.7)	0.8853

NT = Not Tested; ND = Not Determined by Non-linear Regression.

The selectivity index (SI) was determined for the BRB-810, BRB-814, and BRB-832 extracts by comparing the most sensitive tumor cell line, HCT-116, with the non-tumoral NIH-3T3 cell line, resulting in SI values of 1.53, 1.75, and 1.49, respectively (Table 2). Although SI values greater than 1 suggest some degree of selectivity toward tumor cells, the values obtained were relatively low, preventing the confirmation of pronounced selective activity in any of the evaluated extracts.

Table 2. Selectivity index (SI) values of bacterial extracts against HCT-116 cells after 72 h of exposure. SI was calculated as the ratio between the IC_{50} values obtained for the non-tumoral NIH-3T3 cell line and the tumoral HCT-116 cell line.

SI	BRB-810	BRB-814	BRB-832
NHI-T3T/HCT-116	1.53	1.75	1.49

2.5 Metabolomic analysis of the active organic extracts obtained from bacterial isolates

HPLC-MS/MS data from the active extracts were organized into a spectral similarity network, available at https://gnps2.org/status?task=d41f9b41b13c45e7881b575a855cda90. Molecular networking facilitates the analysis of large sets of MS/MS spectra by grouping metabolites that share similar fragmentation profiles (Watrous et al. 2012; Yang et al. 2013). The similarity (cosine score) between MS/MS spectra is represented by the thickness of the edges connecting two nodes, with thicker lines indicating greater similarity (Figure 4). Compound annotations potentially related to cytotoxicity were based on matches against GNPS2 spectral libraries (Table S3). Furthermore, the molecular network allowed visualization of the distribution of annotated compounds across the samples (Figure 4).

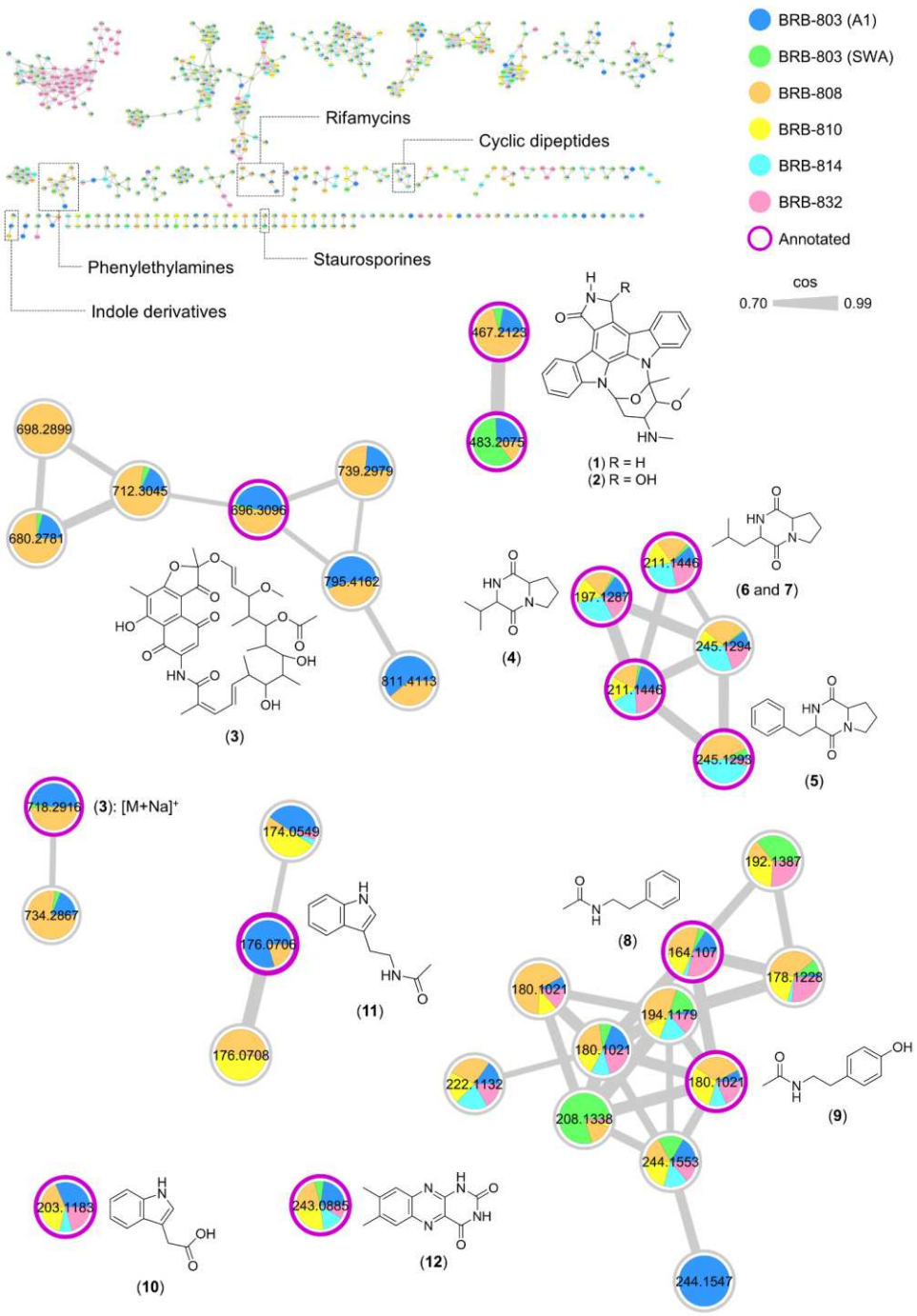
In this analysis, we observed that only approximately 5% of the detected nodes could be annotated through spectral library matches, highlighting the large fraction of chemically uncharacterized signals present in the dataset. Such low annotation rates are commonly observed in untargeted metabolomics and natural product studies and reflect the existence of the so-called *metabolomic dark matter*, which comprises detected but structurally uncharacterized molecules (da Silva et al. 2015). Large-scale metabolomics studies have demonstrated that, even in curated datasets, more than 80% of molecular entities remain unannotated, emphasizing the current limitations of spectral libraries and structural databases (El Abiead et al. 2025). Similarly, isotope-labeling experiments in model organisms have shown that 52–87% of detected metabolites cannot be assigned to known compounds, even after extensive data processing and ion deconvolution (Mahieu and Patti 2017; Gao et al. 2025). In addition, annotation success strongly depends on metabolite prevalence and database coverage, with rare metabolites often displaying annotation

rates below 5%, further reinforcing the vast unexplored chemical diversity present in biological systems (El Abiead et al. 2025).

Considering these limitations, the approach employed here allowed the annotation of different chemical classes of compounds, including staurosporines (**1** and **2**), rifamycins (**3**), diketopiperazines (**4–7**), phenylethylamines (**8** and **9**), tryptophan alkaloid derivatives (**10** and **11**), and a degradation product of riboflavin, lumichrome (**12**) (Figure 4).

For instance, staurosporines (**1** and **2**), found only in extracts of *Salinispora* spp. strains (BRB-803, grown in both A1 and SWB media, and BRB-808), are a family of indolocarbazole alkaloids with broad-spectrum protein kinase inhibitory activity (Gani and Engh 2010) and are well known for inducing apoptosis in cancer cells (Malsy et al. 2019). Rifamycin S (**3**) was annotated in clusters containing nodes corresponding to the precursor ion m/z [M+H]+ and the respective adduct m/z [M+Na]+ of this compound (Figure 4), along with other potentially unannotated rifamycins and their structural analogues. These compounds were found mainly in *Salinispora* spp. strains grown in A1 medium (BRB-803 and BRB-808). Rifamycins are a group of antibiotic metabolites produced by several actinomycetes, including *Salinispora* species (Hewavitharana et al. 2012; Bose et al. 2015; Duncan et al. 2015; Bauermeister et al. 2018; Velasco-Alzate et al. 2019; Van Anh et al. 2023). In addition to their established antibacterial activity, the *in vitro* cytotoxicity of rifamycins against cancer cells has also been reported (Matsuda et al. 2009; Van Anh et al. 2023b).

Compounds (**4–12**) were broadly distributed across the analyzed extracts. Previous studies have also reported members of these chemical classes as cytotoxic compounds. Cyclic dipeptides containing a 2,5-diketopiperazine ring, as observed for compounds **4–7**, are known to exhibit a broad range of biological activities, including cytotoxicity against several cancer cell lines (Bojarska et al. 2021). Antitumor activities have also been reported for the phenylethylamines **8** and **9** (Zhao et al. 2010; Lee et al. 2021), as well as for lumichrome (**12**) (Chantarawong et al. 2019). Nonetheless, the presence of these compounds may contribute to the observed cytotoxicity but does not preclude the presence of other bioactive compounds that could not be annotated.



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495 **Figure 4.** Molecular networking of BRB extracts, (HPLC-ESI-MS/MS, positive mode), highlighting the
496 clusters and singletones with annotations of staurosporines (**1** and **2**), rifamycin S (**3**), cyclic dipeptides (**4**
497 to **7**), phenylethylamines (**8** and **9**), indole derivatives (**10** and **11**), and lumichrome (**12**).

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In addition, searches performed using the microbeMASST platform revealed that the MS/MS spectra of staurosporine (**1**) and rifamycin S (**3**) had previously been detected in strains of marine actinobacteria belonging to the genus *Salinispora*, including *S. pacifica*, *S. arenicola*, and *S. oceanensis*, as well as in members of the genus *Streptomyces* (Figure S2). Complementarily, previous studies conducted by our group recovered several *Salinispora* spp. strains from sediment samples collected at SPSPA, which were also shown to produce staurosporines and rifamycins (Ferreira et al. 2016; Bauermeister et al. 2018). These findings reinforce *Salinispora* species as sources of compounds with pharmacological potential. Moreover, our group has isolated other *Salinispora* strains associated with the ascidian *T. maragogi* collected at Rocas Atoll, as reported by Velasco-Alzate et al. (2019).

Finally, the predominance of unannotated features observed in the molecular network also highlights the remarkable chemical diversity associated with marine invertebrates and their microbiota. Symbiotic microorganisms are recognized as important producers of structurally diverse secondary metabolites, many of which remain absent from current metabolite databases (Atanasov et al. 2021). In marine systems, microbial symbionts frequently contribute to the biosynthesis of specialized metabolites originally attributed to the host organism, expanding the chemical repertoire detected in metabolomics analyses (Dou and Dong, 2019). In this context, molecular networking has emerged as a powerful strategy to explore this hidden chemical diversity, enabling the visualization of molecular families and facilitating the prioritization of potentially novel metabolites for further investigation (Watrous et al., 2012; Wang et al., 2016). Therefore, the large proportion of unannotated nodes detected in the present study suggests that *T. maragogi* and its associated bacteria may represent a promising source of previously undescribed metabolites with potential pharmacological relevance.

2.6 Genome sequencing and analysis of BRB-832

Given the potent cytotoxic activity of BRB 832 and the apparent novelty of its metabolites, evidenced by the lack of annotation and the unique clustering pattern of most BRB832-derived compounds (Figure 4), the strain was selected for whole-genome sequencing to explore its biosynthetic potential. After raw data filtering, a 95x coverage of the full genome sequence was achieved. The genome was assembled in a single scaffold, with 6803145 base pairs in length and

72,97% GC content. Its completeness was assessed as 98,3% via Busco (using "actinobacteria class" as a reference) and 100% via CheckM ("Actinomycetales" reference), whereas CheckM pointed to 0% contamination.

dDDH values were calculated comparing BRB-832 to all the genomes deposited as *Micromonospora* genus in NCBI, composed of 1150 accessions at the date of elaboration of this manuscript. All the strains that showed dDDH values ≥70% either belonged to the *Micromonospora aurantiaca* species or did not have species delineation concluded (Table S3). In a phylogeny based on the TYGS database (Figure 5), BRB-832 was placed on a clade with only *Micromonospora aurantiaca* strains and one strain without an identified species. Considering these results and adopting the >70% and >95% dDDH and ANI thresholds, respectively, for species delineation (Wayne 1988; Richter and Rosselló-Móra 2009; Meier-Kolthoff et al. 2013), this isolate can be placed within the *M. aurantiaca* species.

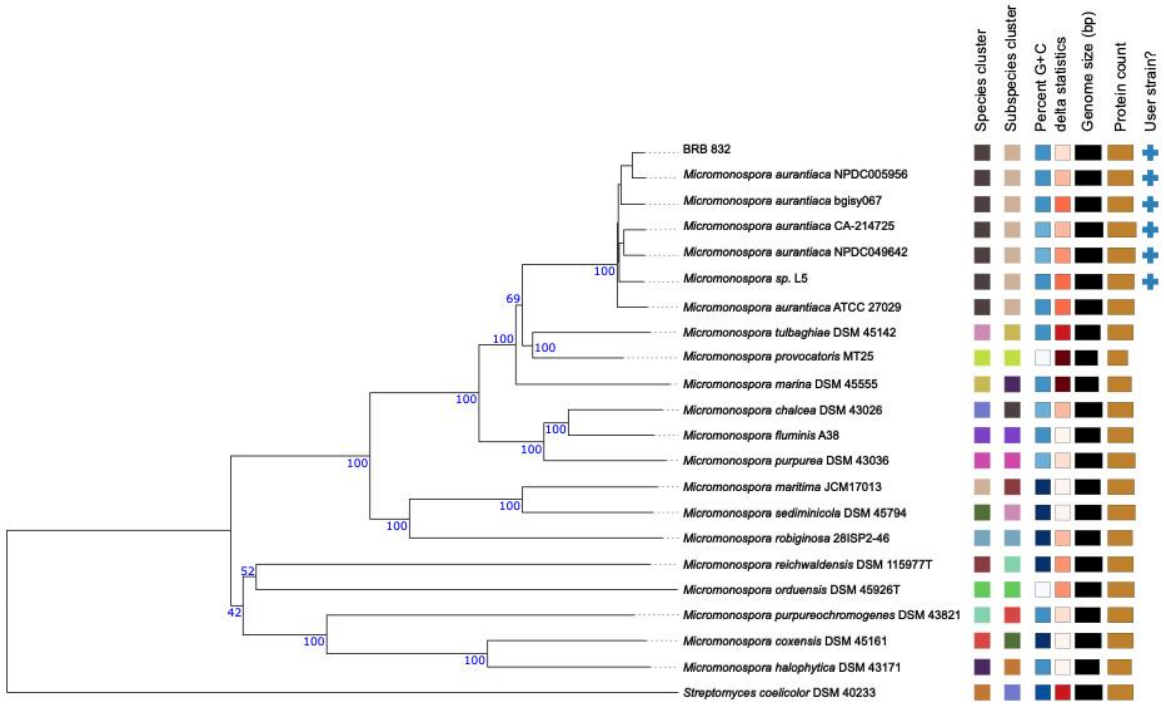

Figure 5. Phylogenomic tree of BRB-832 constructed on TYGS, with the addition of the 5 strains with highest dDDH values obtained in the GGDC calculations for the *Micromonospora* genera, rooted with the *S. coelicolor* DSM 40233 strain. Branch lengths were scaled based on the GBDP pseudo-bootstrap support values.

M. aurantiaca (Sveshnikova et al. 1970; Kasai et al. 2000) research initially had an emphasis in its biodegradation capabilities and applications (Berekaa 2000; Linos et al. 2000; Rose and Steinbüchel 2002; Otto et al. 2013; Forsberg et al. 2018; Guo et al. 2024), however, more recent studies have identified the production of cytotoxic compounds through biotransformation of culture media components into cytotoxic molecules (Wang et al. 2019), the detection of antibiotic gene clusters and the production of kanamycin (Hu et al. 2020), and the production of amicoumacin A e B - protease inhibitors with possible cytotoxic, antibiotic, antifungal and anti-inflammatory activities (Handel et al. 2022).

While a previous study has initiated the integration of metabolomic and genomic data for *M. aurantiaca* (Hu et al. 2020), the genome assembly used in that analysis showed a high fragmentation level. This could have led to a possible over-estimation of the number of biosynthetic gene clusters (BGCs) present in the strain, as 77 BGCs have been reported in *M. aurantiaca* sp.01, placing it far above the normally observed number of BGCs for the phylum, that tends to vary between 20 and 30 BGCs (Doroghazi and Metcalf 2013; Bhattacharjee et al. 2023; Kitikhun et al. 2023; Jørgensen et al. 2024). When submitting BRB-832's assembly to a BGC search in the antiSMASH platform, 20 regions containing proto-clusters were located, where the predominant BGC class was terpenes. Out of these regions, only 9 had identifiable matches in MiBIG's database: one region with a high level of similarity confidence, 3 regions classified as medium level of confidence and the remaining 5 were marked as low level of confidence (Table S4).

Upon more detailed verification of the obtained matches, nearly identical cluster structure was seen for regions 1.4, 1.6 and 1.20 and a high similarity was observed for regions 1.14 and 1.15 (Figure 6), despite only 1.20 being classified as showing a high confidence in match similarity. Region 1.6 contained a siderophore producing BGC and its low similarity score is probably related to antiSMASH's conservative BGC delineation approach, which often leads to the inclusion of more genes than necessary for the biosynthesis of the molecule.

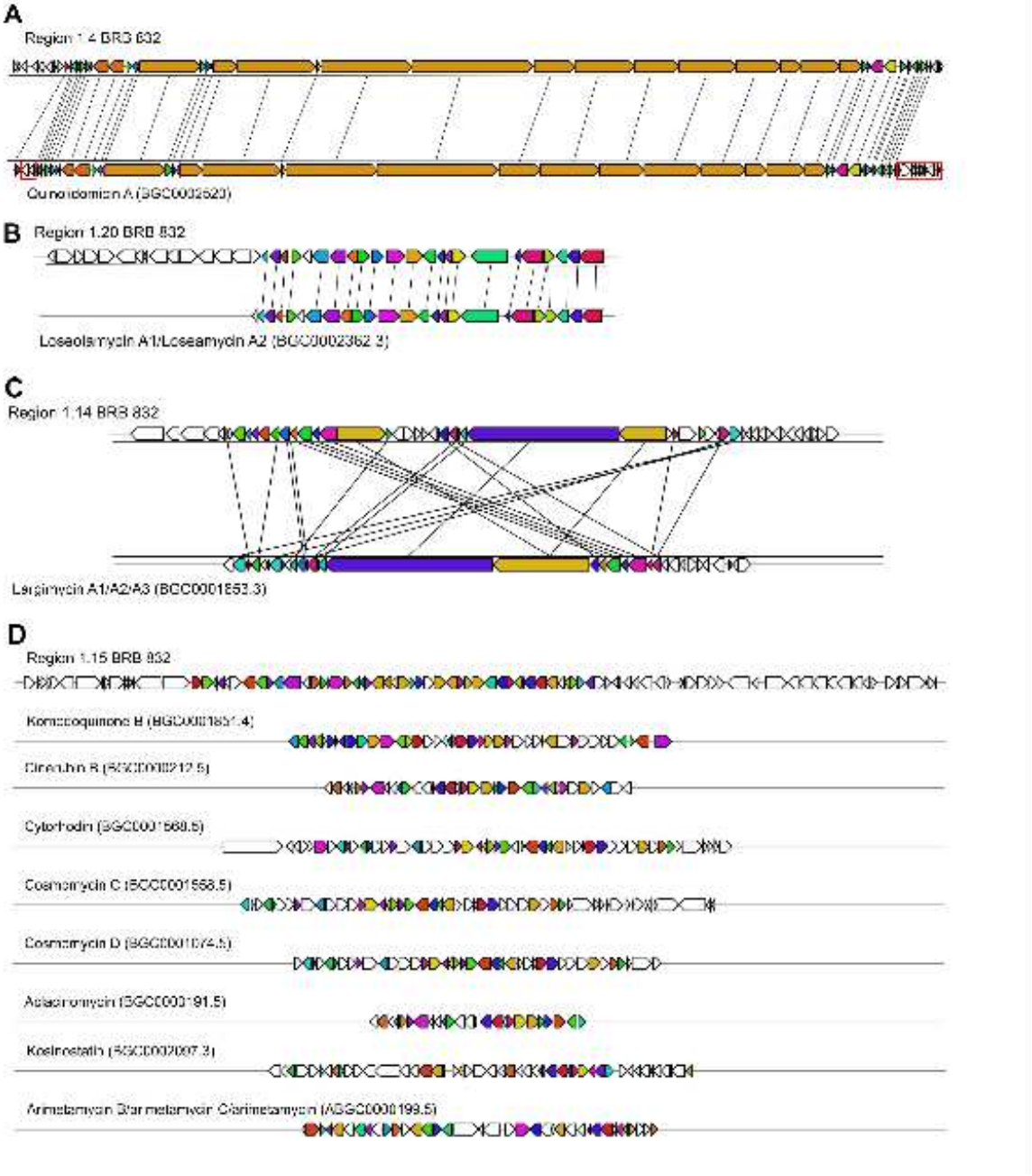

Figure 6. Comparison between BRB-832's genomic regions containing BGCs with identified matches in MiBIG's database. Matching colors identify genes with detected similarity that share the same biosynthetic functions within the BGCs, whereas ORFs with no color identify genes with no matches found. **(A)** Comparison between region 1.4 and the quinolidomicin A BGC. The red squares indicate the peripheral genes with no matching entries in region 1.4. Out of these 9 ORFs, 3 did not have annotation beyond "hypothetical protein". **(B)** Comparison between region 1.20 and the loselamycin BGC. The 2 ORFs from the reference BGC that were not present in BRB-832 were marked as hypothetical proteins. **(C)** Comparison

between region 1.14 and the largimycin BGC. More variation is observed in these BGCs, with additional tailoring genes in the center of BRB 832's BGC. **(D)** Resulting matches for region 1.15 after KnownClusterBLAST search. All the matches show both conserved features and variations between each other and all belong to the anthracycline class.

Region 1.4 contained 55 annotated genes, totaling >200Kb in size. Its closest match was the quinolidomicin A (Hayakawa et al. 1993) BGC (MiBIG accession number: BGC0002520). The quinolidomicin A BGC, characterized in *Micromonospora chalcea* AK-AN57, was also encoded in a >200Kb region, producing a macrolide with a large ring composed of over 60 carbons, with cytotoxic activity (Hashimoto et al. 2018). All the core genes from BRB-832 showed >90% identity and >90% coverage with their respective match, while the genes that did not show significant similarity were peripheral genes mostly assigned as hypothetical proteins (Figure 6A). Additionally, the BGCs contained the same Non-Ribosomal Peptide Synthase (NRPS) and Polyketide Synthase (PKS) modules, indicating a high likelihood that BRB-832 is also a producer of quinolidomicin A.

Region 1.20 contained 37 genes, in a 41Kb region that matched the Type 3 PKS BGC to produce loseolamycin A1/A2 (BGC0002362). This molecule has displayed antibacterial activity against *B. subtilis* and herbicidal activity against *Agrostis stolonifera* (Lasch et al. 2020). The reference BGC was characterized in *Micromonospora endolithica* through heterologous expression of a randomly delimitated 24Kb region and, since no further studies have been published on this molecule's biosynthesis, the exact role of each on the production of the final product is still undetermined. Despite that, only 2 genes from BRB-832 did not match the reference BGC, both marked as hypothetical proteins (Figure 6B). The additional genes included in region 1.20 are likely a result from antiSMASH's BGC delimitation strategy. Therefore, it is also likely that BRB-832 produces the same molecule as *M. endolithica*.

Regions 1.14 and 1.15 showed similarity to known BGCs but displayed more significant composition differences in relation to their references. Region 1.14 displayed its highest similarity with the largimycin BGC (BGC0001853). Largimycin belongs to the family of the antitumoral leinamycin, although no bioactivity reports have been made for largimycin yet. While the core structure of largimycin's BGC is similar to the core of region 1.14, differences in tailoring genes

and detected PKS modules can be found (Figure 6C), pointing to the production of molecules that share similar cores with different additional features.

Region 1.15 had 10 matches from MiBIG, all of which produced anthracyclines or anthracyclinones (Figure 6D). This class of molecules is usually synthesized by smaller BGCs containing a small biosynthetic core with Type 2 Polyketide Synthesis enzymes. These BGCs reportedly show little variation amongst each other, with the structural differences between molecules being attributed to variations in a small subset of tailoring genes (Grocholski et al. 2019). Therefore, it is possible that a new anthracycline is produced by BRB-832, which would match the occurrence of antitumor activity without no known compound annotation. Additionally, the presence of 11 BGCs with no identifiable matches with significant similarity detected in MiBIG's database along with the metabolomic data strongly suggest the production of novel compounds with unique structures from this strain.

In light of these findings, it is key to consider the complementarity, rather than the clash between genome mining and metabolomics as strategies for bioprospecting bioactive natural products. In fact, these approaches examine different levels of the same biosynthetic system: the genotypic methodology is a prediction-driven approach and describes the biosynthetic capacity of a given organism, whereas the phenotypic approach, such as untargeted metabolomics, accesses the metabolites expressed under certain conditions (Wang et al. 2025; Sahana et al. 2024). Therefore, a genotype-phenotype agreement is not merely coincidental; such correlation materializes a genomic prediction evidenced by a chemical observation.

Still, the presence of a metabolite does not necessarily reveal a connected BGC, while analytical cutoffs can be blinded to compounds in very low yields, which will be interpreted as the absence thereof. Moreover, genome mining alone cannot establish that a BGC is expressed. This is particularly important when considering marine microorganisms, which carry a substantial amount of cryptic BGC, which remain mostly silent when cultivated under conventional laboratory conditions (Chu et al. 2020; Costatini 2020; Han et al. 2024). Herein, metabolomic analysis of the extract from *Micromonospora* sp. BRB-832 indicates a prevalent, mostly exclusive, and unannotated cluster of nodes, while genomic analysis reveals a diversified capacity to biosynthesize bioactive compounds along with many unmatched BGCs. While the data obtained does not consent to particular genotypic-phenotypic correlations, it does allow to regard BRB-832

as a biosynthetically prolific and biotechnologically relevant bacterial strain through both genomic and metabolomic appraisals, while bioactivity assessments provide a third feature of high interest.

Beyond the unprecedented data arisen from this study, these findings underscore the importance of supporting scientific investigation and sustainable access to the SPSPA and other Brazilian oceanic islands. Continued research efforts can contribute not only to the discovery and characterization of new bioactive natural products, but also to a broader understanding of the biological richness and the value of conservation of these regions. In this context, collaboration and field-based research are essential for documenting biodiversity at all levels, strengthening scientific knowledge, and supporting conservation-oriented approaches that recognize the ecological and biotechnological importance of such unique environments.

Conclusion

The results obtained in this study reinforce the potential of bacteria associated with the ascidian *T. maragogi* from the SPSPA as a valuable and largely unexplored source of natural products with pharmacological potential. The diversity of bacterial isolates and the detection of biologically active and chemically interesting metabolites highlight the relevance of host-associated microorganisms as promising targets for natural product bioprospecting in Brazil's oceanic islands.

The bioprospective strategy employed herein proved effective in integrating bacterial isolation, taxonomic characterization, biological activity screening, chemical annotation, genome mining, and prioritization of the most promising isolates. By combining these complementary approaches, it was possible to narrow down the microbial diversity to candidates of particular interest for further in-depth chemical, genomic and pharmacological investigations, demonstrating the value of a multidisciplinary workflow for the discovery of bioactive microbial metabolites.

Beyond the identification of promising bacteria and metabolites, this study contributes to expanding the knowledge of the microbiota associated with *T. maragogi* and, more broadly, to documenting the microbial and biotechnological diversity of the SPSPA. Such information is particularly relevant for environments that remain comparatively underexplored and may harbor microbial, chemical and biosynthetic diversity with biotechnological applications.

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Data Availability: The 16S rRNA gene sequences generated in this study have been deposited in NCBI GenBank database under the accession numbers provided in the Supplementary Information (Table S1). The complete genome sequence of strain BRB-832 has been submitted to the NCBI Genome database and is associated with BioSample accession SAMN63535796 (submission SUB16508548). The official genome accession number is expected to be assigned during the review process of the submitted manuscript and will be added to its final version upon acceptance. LC-MS/MS data are available at Zenodo and can be accessed through the following link: https://doi.org/10.5281/zenodo.20629427. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests: The authors declare no competing interests.

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