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Taxonomic Diversity and Metabolic and Pharmacological Profiles of Marine-Derived Actinomycetes from the Lisbon and Setúbal Coast, Portugal

Miguel P. Coelho [1,2], Pablo Suárez-Moo [3], Mariana Rocha [4], Artur O. G. Matos [4], Vanda Marques [5], Sara Margarida [1,2], Mário Mil-Homens [6], Alejandra Prieto-Davó [1,2,3], Cecília M. P. Rodrigues [5], Anelize Bauermeister [4], Rita G. Sobral [1,2] and Susana P. Gaudêncio [1,2,*]

1 Associate Laboratory i4HB, Institute for Health and Bioeconomy, NOVA School of Science and Technology, NOVA University of Lisbon, Campus Caparica, 2819-516 Caparica, Portugal; mp.coelho@campus.fct.unl.pt (M.P.C.); sara-margarida@live.com.pt (S.M.); apdavo@unam.mx (A.P.-D.); rgs@fct.unl.pt (R.G.S.)
2 UCIBIO—Applied Molecular Biosciences Unit, Chemistry and Life Sciences Departments, NOVA School of Science and Technology, NOVA University of Lisbon, Campus Caparica, 2819-516 Caparica, Portugal
3 Unidad Sisal, Facultad de Química, Universidad Nacional Autónoma de México, Sisal 97356, Yucatán, Mexico; pablolosuarez@gmail.com
4 Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes, São Paulo 05508-900, Brazil; mari_rocha.12@usp.br (M.R.); artur.matos@usp.br (A.O.G.M.); anelize@iq.usp.br (A.B.)
5 Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Professor Gama Pinto, 1649-003 Lisboa, Portugal; vismsmarques@ff.ulisboa.pt (V.M.); cmprodrigues@ff.ulisboa.pt (C.M.P.R.)
6 IPMA—Instituto Português do Mar e da At-mosfera, I.P., Divisão de Oceanografia e Ambiente Marinho, Avenida Doutor Alfredo Magalhães Ramal-ho, 6, 1495-165 Algés, Portugal; mario.milhomens@ipma.pt
* Correspondence: s.gaudencio@fct.unl.pt

Abstract

This study explores the taxonomic diversity, metabolic profile, and bioactivity of marine-derived actinomycetes isolated from sediments collected off the coast of Lisbon and Setúbal Peninsula, Portugal. The combined use of two sediment pre-treatments (heat shock and dry overnight) and four growth media with varying nutrient concentrations revealed that formulations 10% A1 and SWA were most effective for recovering diverse actinomycetes, including rare *Actinomadura*, resulting in a total of 142 cultivable strains closely related to 47 phylogenetic distinct species dominated by *Streptomyces* and *Micromonospora*. Antimicrobial screening against methicillin-resistant *Staphylococcus aureus* (MRSA, COL) and *Escherichia coli* (K12) identified 22 bioactive strains, with strain PTS-083 exhibiting the strongest activity against MRSA (MIC = 1.95 µg/mL) and a 98.30% 16S rRNA gene identity to *S. chumphonensis*, highlighting it as a strong candidate for further metabolite and genomic studies. Cytotoxicity assays against HCT-116 human colorectal adenocarcinoma cells revealed eight bioactive strains with potent anticancer activity for extracts from strains related to *S. sundarbansensis*, *S. violaceorubidus*, and *S. aculeolatus* (IC_{50} < 0.005–5.08 µg/mL). Untargeted LC-MS/MS metabolomic analysis uncovered a wide array of secondary metabolites, including macrolides, siderophores, fatty acids, and cyclic peptides. Comparative analyses with other Portuguese coastal studies revealed both shared and distinctive metabolomic profiles, emphasizing the importance of exhaustive sampling, even at nearby locations, since localized environmental conditions can influence metabolic diversity and are crucial for uncovering unique metabolites with potential biotechnological value. These findings highlight Portugal's coastal sediments as a rich and underexplored source of novel actinomycetes and bioactive compounds with promising pharmaceutical applications.

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1. Introduction

Actinomycetes have earned a central place in biotechnology due to their extraordinary ability to synthesize a vast array of bioactive natural compounds. These filamentous bacteria are prolific producers of secondary metabolites with applications across medicine, agriculture, and other industrial processes [1–3]. Their most celebrated contributions lie in the medical realm, where they serve as the primary source of antibiotics.

On land, terrestrial actinomycetes are responsible for producing nearly two-thirds of all known antibiotics. Within this group, the genus *Streptomyces* stands out, accounting for approximately 80% of these antibiotic discoveries. Historic breakthroughs include the isolation of actinomycin from *Streptomyces antibioticus*, streptothricin from *Streptomyces lavendulae*, and streptomycin from *Streptomyces griseus*, of which marked a milestone in the fight against infectious diseases [4–7].

Beyond terrestrial ecosystems, actinomycetes have also adapted to the complex and competitive environments of marine habitats. These ecosystems exert unique evolutionary pressures, driving marine organisms to develop specialized biochemical strategies for survival. Marine-derived actinomycetes have emerged as a rich source of novel compounds, with ongoing exploration revealing new genera and bioactivities.

A prime example is *Salinispora tropica*, a marine obligate actinomycete belonging to the *Salinispora* genus. This actinomycete produces salinosporamide A (Marizomib™), a potent γ-lactam-β-lactone proteasome inhibitor. This compound has shown great promise and is currently in phase III clinical trials for the treatment of various cancers, including lymphoma, pancreatic and lung cancers, melanoma and multiple myeloma [8–10].

The coastal and estuarine systems of Portugal, particularly around Lisbon and Setúbal, are shaped by a dynamic interplay of ocean currents, sedimentary processes, and estuarine hydrodynamics. Circulation within the Tagus and Sado estuaries promotes the transport of organic matter, nutrients, oxygen, and sediments. At the same time the mixing of freshwater and seawater leads to significant variations in physicochemical and biological conditions. The Tagus river estuary connects to the Atlantic Ocean through a 12 km long, 2 km wide, and 30 m deep channel, leading to a large bay, bordered by Raso Cape to the North and by Espichel Cape to the South [11]. Moreover, in the Sado river estuary, South of Espichel Cape, water movement follows a tidal regime through two main navigation channels, North and South, resulting in localized sediment transport and deposition [12,13].

The Portuguese continental margin near the mouths of the Tagus and Sado rivers features a narrow, canyon-incised shelf with extensive mud deposits. The Tagus estuary contributes significantly more suspended particulate matter than the Sado, with concentrations up to four times higher [12]. Seasonal variations intensify this contrast, with a surface nepheloid layer extending up to 30 km offshore in summer, while bottom sediments are often diverted toward the Cascais and the Lisbon submarine canyons [14]. Broader oceanographic forces further influence these estuarine and shelf systems.

The Azores current flows eastward from the mid-Atlantic and brings warm, saline waters that contribute to the Portugal Current. This affects stratification and nutrient transport near the Iberian coast [15]. Meanwhile the Canary Current, a cool southward flow along northwest Africa that drives seasonal upwelling. This enhances biological productivity and sediment redistribution along the Portuguese shelf [16–18]. The interaction of these

large-scale currents with local estuarine outflows modulates cross-shelf transport, sediment dispersal, and ecological connectivity.

These forces create a dynamic coastal regime in which tidal flows, river discharge, wave–current interactions, and regional ocean currents converge, shaping the sedimentary architecture and ecological character of the Tagus and Sado estuaries. This complexity supports diverse microbial communities and influences processes ranging from aquifer formation to the potential discovery of novel bioactive compounds in marine sediments.

Building on the ecological richness and microbial diversity shaped by Portugal's dynamic coastal and estuarine systems, this study aimed to explore the biological and chemical diversity of cultivable actinomycetes within this unique geographical region. Using culture-dependent isolation techniques alongside LC-MS/MS metabolomic profiling, the research aimed to identify and characterize actinomycete strains that produce bioactive metabolites with antimicrobial and anticancer properties.

2. Results and Discussion

2.1. Actinomycete Taxonomic Characterization and Phylogenetic Analysis

A total of 142 cultivable actinomycetes were successfully isolated from 22 marine sediment samples collected off the coast of Lisbon and Setúbal Peninsula, including the mouths of the Tagus and Sado rivers (Figure 1). These isolates revealed a microbial landscape dominated by three distinct genera: *Streptomyces*, *Micromonospora*, and *Actinomadura*. *Streptomyces* and *Micromonospora* were the most prevalent, reflecting their ecological adaptability in marine environments. Specifically, 73 isolates were identified as *Streptomyces*, followed closely by 67 isolates of *Micromonospora*, while *Actinomadura* was represented by two strains (Table 1).

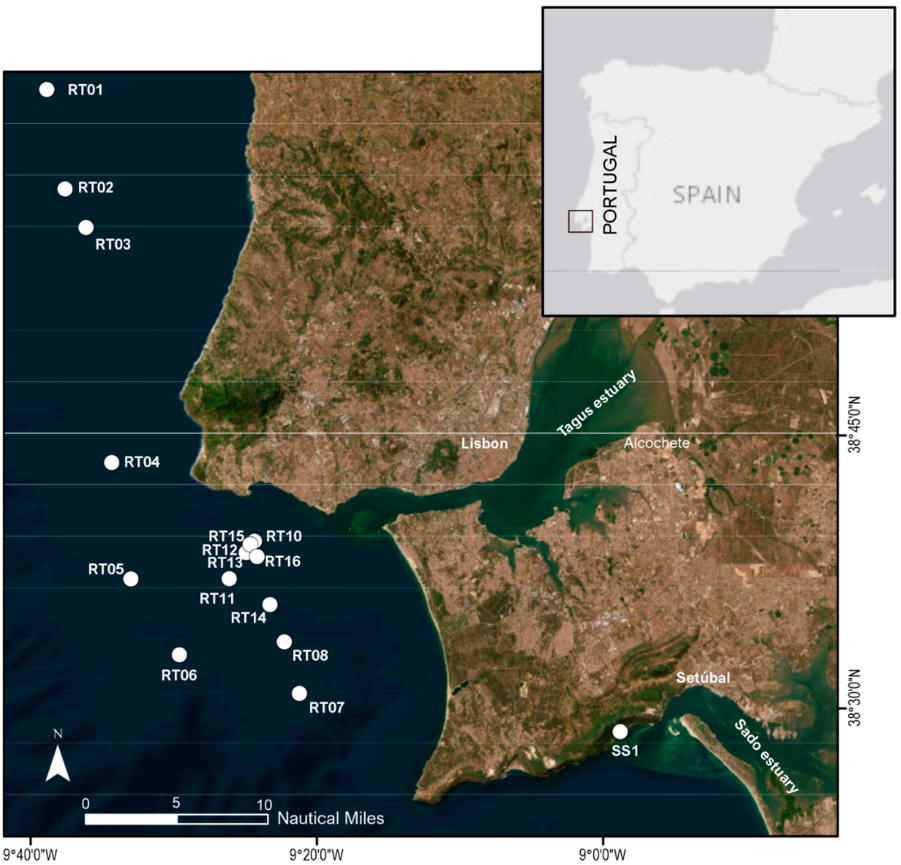

Figure 1. Marine sediments collection geographical locations off the Lisbon and Setúbal Peninsula coast. Twenty-two sediment samples were collected from 16 stations.

Table 1. Distribution of actinomycete isolates, genera, and number of species from Lisbon and Setúbal coastal sediments based on the pre-treatment techniques and growth media.

Genus	Number of Isolates	Number of Species	Isolates by Pre-Treatment Method		Isolates by Isolation Growth Media			
			P1	P2	A1	$^1/_2$ A1	10% A1	SWA
Streptomyces	73	27	25	48	21	12	27	13
Micromonospora	67	19	55	12	0	2	20	45
Actinomadura	2	1	0	2	0	0	1	1
Total	142	47	80	62	21	14	48	59

To maximize the recovery of cultivable actinomycetes from marine sediments, two distinct pre-treatment methods, P1 (heat shock) and P2 (dry overnight), were employed, each in four different growth media. P1 method yielded 80 strains, while P2 yielded 62 strains. Both approaches successfully recovered representatives of *Streptomyces* and *Micromonospora*, but the two *Actinomadura* strains were uniquely isolated using the P2 method, highlighting its potential to uncover less abundant taxa (Table 1).

The nutrient concentration in the growth media played a pivotal role in shaping the diversity and abundance of the recovered actinomycetes. Four growth media formulations, A1, $^1/_2$ A1, 10% A1, and SWA, were tested, each revealing distinct selectivity profiles. The A1 medium supported the growth of 21 strains, all belonging to *Streptomyces*, indicating its specificity for this genus. The $^1/_2$ A1 medium yielded 14 strains, including 12 *Streptomyces* and 2 *Micromonospora*. In contrast, the 10% A1 medium facilitated the growth of 48 strains spanning all three genera: *Streptomyces* (27), *Micromonospora* (20), and *Actinomadura* (1). The SWA medium proved most effective, yielding 59 isolates: *Streptomyces* (13), *Micromonospora* (45), and *Actinomadura* (1), also demonstrating broad-spectrum cultivation capacity.

Notably, the 10% A1 and SWA growth media, characterized by their lower nutrient concentrations, particularly promoted the growth of actinomycetes. These minimal growth media mimic the oligotrophic conditions of marine environments, suppressing the rapid growth of competing microorganisms and allowing slower-growing actinomycetes to thrive [19–21].

Spatial patterns in culturable actinomycete diversity from marine sediments collected off Lisbon-Setúbal are illustrated in Figure S1. The number of isolated strains varies with distance from the coastline. Still, no consistent trend is evident, suggesting that local environmental factors may play a stronger role than proximity to the shore.

Seawater requirement for growth was evaluated across all isolates, and 11 strains (7.75%) exhibited marine obligate behaviour [22,23], including 4 strains of *M. zingiberis* (PTS-031, PTS-040, PTS-042, and PTS-053), PTS-052 (*M. spongicola*), PTS-059 (*S. qinglanensis*), 2 strains of *S. chumphonensis* (PTS-070 and PTS-083), PTS-093 (*M. fluminis*), PTS-102 (*M. phytophile*), and PTS-143 (*S. karpasiensis*), marked with black stars in Figure 2 and Figures S2 and S3. Strains that successfully grew in the absence of seawater were classified as marine-derived, indicating they can tolerate non-marine conditions. In contrast, strains that failed to grow under these conditions were designated as marine-obligate, suggesting a strict requirement for seawater to support their development. This classification provides insight into the ecological adaptation of the isolates and may reflect differences in their metabolic profiles and potential for producing marine-specific bioactive compounds.

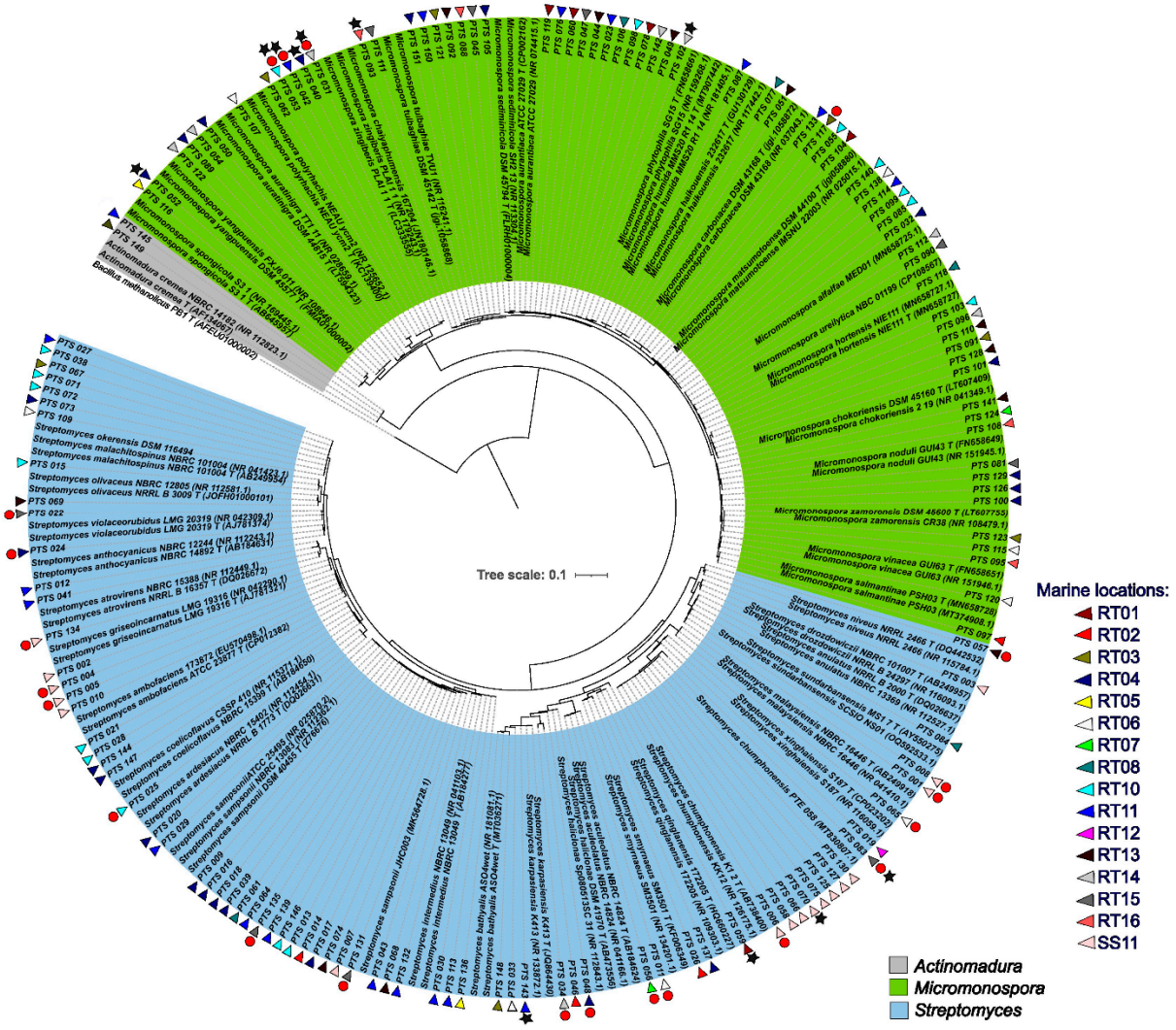

Figure 2. Phylogeny of the 142 isolated actinomycetes from the Lisbon and Setúbal coast, the BLASTn top-hit sequences from NCBI (GenBank) rRNA database and their type-strain sequences retrieved from EzBioCloud. All PTS numbers refer to internal reference collection codes. The colors in the triangle symbols represent the origin of the isolated PTS actinomycete from Lisbon and Setúbal coast. Red circle symbols represent the bioactive PTS strains. Black star symbols represent the strains that require seawater for growth. *Bacillus methanolicus* PB1 (AFEU010000002) was used as an outgroup.

The phylogenetic analysis revealed that none of the isolates in this study belonged to any previously reported marine obligate genera, such as *Salinispora* (Figure 2 and Table S1). However, the isolation of marine-obligate strains suggests adaptations from these groups to the marine environment.

The taxonomic analysis revealed high diversity within the two most prevalent genera *Streptomyces* and *Micromonospora* (Figure 2 and Figures S2 and S3, and Table S1).

The 73 isolated *Streptomyces* strains were closely related to 27 previously reported species, including: *S. aculeolatus*, *S. albogriseolus*, *S. ambofaciens*, *S. anthocyanicus*, *S. anulatus*, *S. ardesiacus*, *S. atrovirens*, *S. bathyalis*, *S. chumphonensis*, *S. coelicoflavus*, *S. drozdowiczii*, *S. griseoincarnatus*, *S. haliclonae*, *S. iconiensis*, *S. intermedius*, *S. karpasiensis*, *S. malachitospinus*, *S. malaysiensis*, *S. marinus*, *S. niveus*, *S. olivaceus*, *S. qinglanensis*, *S. sampsonii*, *S. smyrnaeus*, *S. sundarbansensis*, *S. violaceorubidus*, and *S. xinghaiensis* (Figures 2 and S2, and Table S1).

In the case of *Micromonospora*, the 67 isolated strains were closely related to 19 species, including: *M. aurantiaca*, *M. auratinigra*, *M. carbonacea*, *M. chokoriensis*, *M. fluminis*, *M. haikouensis*, *M. humida*, *M. matsumotoense*, *M. noduli*, *M. phytophila*, *M. polyrhachis*, *M. saelicesensis*,

M. sediminicola, *M. spongicola*, *M. tulbaghiae*, *M. vinacea*, *M. yangpuensis*, *M. zamorensis*, and *M. zingiberis* (Figures 2 and S3, and Table S1).

Only 2 isolated strains were associated with representatives from *Actinomadura*, both related to the same species, *Actinomadura cremea* (Figure 2 and Table S1).

The similarity between 16S rRNA sequences was expressed as percent identity, which reflects the genetic distance between the isolated strains consensus sequence and the nearest neighbor strain reported in the NCBI (GenBank) rRNA database [24,25]. Identities above 99% are generally considered to represent same species, while those with more than 95% identity are generally considered as members of same genus [26]. Although the percent identity cutoff value to determine species from 16S rRNA sequences was originally set at 97% (based on DNA:DNA hybridization assays), a more conservative value of >99% is now used for species-level designations [26–28]. Strains PTS-041 (*S. atrovirens*), PTS-059 (*S. qinglanensis*), and PTS-083 (*S. chumphonensis*) shared less than 99% sequence identity with their nearest BLASTn neighbors, suggesting that they may represent novel species [27]. In addition, PTS-059 and PTS-083 are marine obligate strains, underscoring their potential as a source of novel metabolites resulting from their adaptations to oceanic environments. Nevertheless, comprehensive genomic and chemical analyses are required to fully assess the biosynthetic potential of these strains.

In 2022, our research group conducted a comprehensive investigation of actinomycete diversity from marine sediments collected from the Estremadura Spur pockmarks, located approximately 145 km North of the present sampling site, using the same actinomycete isolation methodology as applied in the present study. It led to the isolation of actinomycete strains spanning nine distinct genera, with *Streptomyces* and *Micromonospora* emerging as the most dominant taxa [20].

Echoing these findings, the present study also revealed a strong prevalence of *Streptomyces* and *Micromonospora* in the marine sediments collected off the Lisbon and Setúbal Peninsula coast. However, taxonomic classification at the species level revealed a greater diversity within these genera. Among the 27 *Streptomyces* and 19 *Micromonospora* identified in this study, 22 and 10, respectively, were closely related to species not observed in the Estremadura Spur dataset. These include *S. ambofaciens*, *S. anthocyanicus*, *S. anulatus*, *S. ardesiacus*, *S. atrovirens*, *S. bathyalis*, *S. chumphonensis*, *S. coelicoflavus*, *S. drozdowiczii*, *S. griseoincarnatus*, *S. haliclonae*, *S. iconiensis*, *S. karpasiensis*, *S. malaysiensis*, *S. marinus*, *S. niveus*, *S. olivaceus*, *S. qinglanensis*, *S. smyrnaeus*, *S. surdarbnasensis*, *S. violaceorubidus*, *S. xinghaiensis*, *M. carbonacea*, *M. fluminis*, *M. haikouensis*, *M. humida*, *M. phytophila*, *M. polyrhachis*, *M. sediminicola*, *M. spongicola*, *M. yangpuensis*, and *M. zingiberis.* Additionally, the *Actinomadura* species, *A. cremea*, was also not observed in the Estremadura Spur findings.

Furthermore, in a study published by dos Santos et al. in 2024 [29], the submerged upper delta of the Tagus river estuary off the Alcochete region, located approximately 24 km East of the river mouth (Figure 1), was explored for actinomycete diversity. It similarly reported a predominance of the genera *Streptomyces* and *Micromonospora*, along with a single representative of *Saccharomonospora* [29]. Despite these similarities, the current study reveals a distinct microbial signature, identifying strains related with species not previously documented by dos Santos et al. (Figure S4). Specifically, among the 27 *Streptomyces* and 19 *Micromonospora*, 18 and 14, respectively, were closely related to species not previously reported from the Alcochete dataset. These include *S. aculeolatus*, *S. ambofaciens*, *S. anthocyanicus*, *S. anulatus*, *S. ardesiacus*, *S. atrovirens*, *S. bathyalis*, *S. chumphonensis*, *S. coelicoflavus*, *S. drozdowiczii*, *S. haliclonae*, *S. iconiensis*, *S. malachitospinus*, *S. marinus*, *S. niveus*, *S. olivaceus*, *S. smyrnaeus*, *S. surdarbansensis*, *M. auratinigra*, *M. carbonacea*, *M. chokoriensis*, *M. fluminis*, *M. haikouensis*, *M. humida*, *M. matsumotoense*, *M. phytophile*, *M. polyrhachis*, *M. saelicesensis*, *M. sediminicola*, *M. spongicola*, *M. zamorensis*, and *M. zingiberis.*

In addition to the overall diversity of actinomycete strains recovered in this study, a distinct clade comprising five isolates (PTS-031, PTS-040, PTS-042, PTS-053, and PTS-062) was identified within the genus *Micromonospora* (Figures 2 and S3). This clade includes four obligate marine strains, three of which exhibited varying levels of bioactivity in the assays performed, including the primary inhibition-halo screening. Members of the genus *Micromonospora* are increasingly recognized as a prolific yet still underexplored source of structurally diverse secondary metabolites, particularly in marine environments, where they have yielded antibiotics, cytotoxic agents, and other bioactive compounds spanning polyketide, non-ribosomal peptide, and hybrid biosynthetic classes [30–32]. Phylogenetically, the closest related type strain to this clade is *M. zingiberis*, an actinomycete originally isolated from the roots of *Zingiber montanum*, a plant traditionally used for medicinal purposes [33]. The observed bioactivity (Figure 2 and Figure S3), together with the established biosynthetic richness of marine *Micromonospora* and the growing success of genome-guided and metabolomics-assisted discovery approaches in this genus [30–32], highlights these strains as promising candidates for further chemical characterization and genomic analyses aimed at assessing their potential for natural product discovery and biotechnological applications.

These findings reinforce the ecological prominence of *Streptomyces* and *Micromonospora*, suggesting that, either the cultivation techniques employed selectively favor these genera, or that their natural abundance is inherently higher in these environments. The limited recovery of *Actinomadura* may reflect its lower adaptability to the applied culture conditions or its genuinely reduced prevalence in marine influenced regions. The data highlights the profound influence of localized ecological factors in shaping actinomycete diversity, while highlighting the unique microbial signatures of the Lisbon–Setúbal coastal sediments. These findings not only reveal the untapped potential of Portugal's coastal microbiomes for bioprospecting novel bioactive compounds but also emphasize the critical importance of regional sampling and the use of diverse cultivation methods to uncover previously unreported actinomycete species with promising biotechnological relevance.

2.2. Antimicrobial Activity Evaluation

2.2.1. Evaluation of Growth Inhibition Halo

To gain an initial insight into the antimicrobial potential of the isolated marine-derived actinomycetes, all 142 strains were screened using a growth inhibition halo assay. This assay served as a measure of the antagonistic behaviour of active isolates against two clinically relevant human pathogens: methicillin-resistant *Staphylococcus aureus* (MRSA, strain COL) and *Escherichia coli* (strain K12). The strains that produced visible growth inhibition for at least one of the pathogens, highlighted with red circles in Figures 2, S2 and S3, were selected for further evaluation, including determination of the minimum inhibitory concentration (MIC) of their crude extracts and metabolomic profiling.

From this preliminary screening, 22 strains demonstrated antibacterial activity. All 22 inhibited MRSA, while six also showed activity against *E. coli* (Table S2). Notably, four of these bioactive strains (PTS-031, PTS-043, PTS-053 and PTS-083) exhibited a strict requirement for seawater to support their growth (Figure 2), suggesting a strong adaptation to marine conditions.

2.2.2. MIC Determination

Crude extracts were produced for the 22 bioactive strains resulting from the growth inhibition halo assay. The minimum inhibitory concentration (MIC) values for MRSA ranged from 1.95 to 250 µg/mL (Table 2). The most potent antibacterial activity was observed for the extract from PTS-083 (*S. chumphonensis*), which exhibited a MIC of 1.95 µg/mL. This

was followed by PTS-011 (*S. aculeolatus*) and PTS-022 (*S. violaceorubidus*), both with MICs of 3.91 µg/mL. Extracts from PTS-056 (*S. aculeolatus*) and PTS-065 (*S. malaysiensis*) exhibited MICs of 7.81 µg/mL. An MIC $\leq$ 10 µg/mL is generally considered indicative of suitability for potential clinical development [34].

Table 2. Antimicrobial and anticancer activity of the marine-derived Lisbon and Setúbal coast actinomycetes crude extracts against methicillin-resistant *Staphylococcus aureus* (MRSA, strain COL) and *Escherichia coli* (strain K12), and against HCT-116 cells, respectively. NA—Not active. (–) symbol—Not determined.

Strain/Extract Code	Species Name (Best Match % Identity in the NCBI Database)	MIC MRSA (µg/mL)	MIC *E. coli* (µg/mL)	IC_{50} HCT-116 (µg/mL)
PTS-001	*Streptomyces sundarbansensis* (99.8%)	NA	NA	<0.005
PTS-004	*Streptomyces ambofaciens* (99.55%)	NA	NA	>100
PTS-005	*Streptomyces ambofaciens* (99.55%)	NA	NA	–
PTS-006	*Streptomyces chumphonensis* (99.85%)	NA	NA	–
PTS-008	*Streptomyces sundarbansensis* (99.7%)	NA	–	0.58
PTS-011	*Streptomyces aculeolatus* (99.77%)	3.91	–	5.08
PTS-022	*Streptomyces violaceorubidus* (99.85%)	3.91	–	1.76
PTS-024	*Streptomyces anthocyanicus* (100%)	125	–	–
PTS-025	*Streptomyces ardesiacus* (100%)	125	–	–
PTS-031	*Micromonospora zingiberis* (99.70%)	NA	–	–
PTS-034	*Streptomyces haliclonae* (98.05%)	NA	–	–
PTS-042	*Micromonospora zingiberis* (99.77%)	NA	–	>100
PTS-048	*Streptomyces haliclonae* (99.85%)	125	–	–
PTS-053	*Micromonospora zingiberis* (99.77%)	NA	–	–
PTS-056	*Streptomyces aculeolatus* (100%)	7.81	–	16.46
PTS-057	*Streptomyces niveus* (99.62%)	250	NA	–
PTS-065	*Streptomyces malaysiensis* (99.89%)	7.81	–	30.58
PTS-083	*Streptomyces chumphonensis* (98.3%)	1.95	–	30.28
PTS-117	*Micromonospora carbonacea* (99.48%)	31.25	–	16.11
PTS-131	*Streptomyces sampsonii* (99.70%)	125	–	–
PTS-134	*Streptomyces griseoincarnatus* (100%)	NA	NA	–
PTS-135	*Streptomyces sampsonii* (100%)	250	–	–
Vancomycin	Positive control	1.95	–	–
Tetracycline	Positive control	–	3.91	–
5-Fluorouracil	Positive control	–	–	10 µM

Moderate activity was observed for PTS-117 (*M. carbonacea*) with an MIC of 31.25 µg/mL, as well as for PTS-024 (*S. anthocyanicus*). In contrast, strains PTS-025 (*S. ardesiacus*), PTS-048 (*S. haliclonae*), and PTS-131 (*S. sampsonii*), each with MICs of 125 µg/mL, and strains PTS-057 (*S. niveus*) and PTS-135 (*S. sampsonii*), both with MICs of 250 µg/mL showed no bioactivity.

Marine obligate PTS-083 stands out for its strong antimicrobial activity, which combined with its 16S rRNA gene divergence from *S. chumphonensis* (98.30% identity), highlights it as a promising source of novel bioactive metabolites and a strong candidate for further genomic and metabolic investigation.

Ten crude extracts from strains that initially exhibited activity against MRSA, and six that were active against both MRSA and *E. coli* in the growth inhibition halo assay, did not demonstrate inhibitory activity in the subsequent MIC assays. This discrepancy may reflect limitations in the extraction process, particularly the use of ethyl acetate, a moderately polar solvent. Bioactive metabolites with very low or very high polarity could have remained in the aqueous phase and thus were absent from the final extract.

Alternatively, the MIC of these extracts may have exceeded the highest concentration tested, preventing detectable inhibition under the assay conditions.

The majority of the active crude extracts were derived from *Streptomyces* strains. This genus is globally recognized for its exceptional biosynthetic capacity and as a prolific source of clinically important antibiotics [35,36]. Several of the identified species in this study are associated with the production of diverse secondary metabolites with potent bioactivities: *S. chumphonensis* (PTS-083), is known to produce piericidins, aromatic acids, and leucine derivatives, which exhibit antimicrobial, cytotoxic, and antihyperlipidemic properties [37,38]. *S. aculeolatus* (PTS-011 and PTS-056), produces meroterpenoids, such as napyradiomycins and marinone derivatives, hybrid isoprenoids with broad-spectrum activities including antibacterial, antifungal, antitumor, antibiofilm, and antifouling effects [39–46]. *S. malaysiensis* (PTS-065), is a known source of thioholgamides A and B, ribosomally synthesized peptides with notable cytotoxic and antimicrobial activities [47]. *S. anthocyanicus* (PTS-024), also known as *S. violaceoruber*, produces kendomycin, a macrolide with antibacterial and antitumor properties, and granaticin, a benzoisochromanequinone antibiotic with broad antimicrobial activity [48,49]. *S. ardesiacus* (PTS-025) synthesizes phenazine derivatives with moderate antimicrobial effects, as well as complex polyketides like diastaphenazine and angucycline glycosides, which possess antibacterial and cytotoxic properties [50,51]. *S. sampsonii* (PTS-131 and PTS-135), also known as *S. albidoflavus*, is known to produce antimycin A, an antifungal and antibacterial compound, and albaflavenone, a sesquiterpene ketone with antibacterial activity [52,53]. *S. niveus* (PTS-057), is the producer of novobiocin, an aminocoumarin antibiotic also known as albamycin, which has been used clinically for decades [54,55].

In contrast, the bioactivity of *S. violaceorubidus* (PTS-022) and *S. haliclonae* (PTS-048) has not been previously reported in the literature, suggesting that these strains may harbor novel biosynthetic pathways and warrant further chemical investigation.

Beyond *Streptomyces*, one additional active extract derived from other actinomycetes of the *Micromonospora* genus, *M. carbonacea* (PTS-117), is known to produce several potent antibiotics, including tetrocarcin, arisostatin, everninomicin, and orthosomycin, all of which exhibit antibacterial activity [56–60].

Collectively, these findings underscore the remarkable potential of marine-derived actinomycetes to produce structurally diverse and pharmacologically valuable secondary metabolites. While many of the identified compounds have known therapeutic applications, further exploration of lesser-studied strains, particularly *S. haliclonae*, may lead to the discovery of new, previously undescribed molecules with significant pharmaceutical promise.

2.3. Anticancer Evaluation

Among the 22 microbial extracts evaluated for anti-tumor activity against HCT-116 human colorectal adenocarcinoma cells, eight demonstrated positive cytotoxic effects (Table 2).

The most potent extract was derived from PTS-001 (*S. chumphonensis*), with an IC_{50} of less than 0.005 μg/mL, reflecting exceptionally strong anti-tumor potential. Other highly active strains included PTS-008 (*S. sundarbansensis*), with an IC_{50} of 0.58 μg/mL; PTS-022 (*S. violaceorubidus*), with an IC_{50} of 1.76 μg/mL; and PTS-011 (*S. aculeolatus*), with an IC_{50} of 5.08 μg/mL. These strains exhibited low IC_{50} values ($\leq$10 μg/mL a range considered suitable for clinical development [34]) and therefore represent promising candidates for further investigation as sources of novel anticancer compounds.

Several other strains showed moderate cytotoxicity, including PTS-056 (*S. aculeolatus*), PTS-117 (*M. carbonacea*), PTS-065 (*S. malaysiensis*), and PTS-083 (*S. chumphonensis*), with IC_{50}

values ranging from approximately 16 to 30 μg/mL. Strains PTS-004 (*S. drozdowiczii*) and PTS-042 (*M. zingiberis*) IC_{50} values exceeded 100 μg/mL, indicating no cytotoxicity.

The data highlights several strains with compelling antitumor properties, particularly PTS-001, PTS-008, PTS-022, which merit further chemical and pharmacological exploration for potential development into therapeutic agents targeting colorectal cancer.

Extracts from four actinomycete strains demonstrated cytotoxic effects against HCT-116 human colorectal adenocarcinoma cells but showed no antimicrobial activity against MRSA or *E. coli*. These include the potent PTS-001 (IC_{50} >0.005 μg/mL) and PTS-008 (IC_{50} = 0.58 μg/mL) strains and the strains PTS-004 and PTS-042 that displayed no activity (IC_{50} >100 μg/mL). These findings highlight the importance of thoroughly studying coastal and ocean locations for actinomycete cultivation, and to perform a broad range of biological testing to fully understand the potential of marine-derived actinomycete strains.

As mentioned above, *S. aculeolatus* (PTS-011 and PTS-056), *S. violaceorubidus* (PTS-022), *S. malaysiensis* (PTS-065), *S. chumphonensis* (PTS-083), and *M. carbonacea* (PTS-117) are known to produce a diverse array of bioactive secondary metabolites, several with cytotoxic activity. *S. sundarbansensis* (PTS-001 and PTS-008), is known for producing antibacterial poliketides [61]. *S. ambofaciens* (PTS-004), has been reported to synthesize telomycin-like cyclic depsipeptides, specifically ambobactin, which exhibited antibacterial activity [62].

2.4. Metabolomic Profiling

Using LC-MS/MS data, a molecular network was constructed on the GNPS platform for the 22 extracts, which revealed bioactivity in the halo growth inhibition assay, and that were previously evaluated for antimicrobial and anticancer activity (Figure 3). The network revealed 4629 nodes (ions), of which 281 (approximately 6%) were annotated through spectral matching with known compounds from GNPS libraries [63], facilitating dereplication [64,65].

The dataset revealed a diverse array of chemical classes, each represented by several compounds that were annotated using GNPS libraries, considering annotation levels 2 and 3 of Metabolomics Standards Initiative (MSI) [66], including macrolides (e.g., bafilomycin C1, rosaramicin, cirramycin A), piericidins (e.g., 7-demethyl-piericidin A1, glucopiericidin), antimycins (e.g., antimycin A1), prodigiosins (e.g., prodiginines, undecilprodigiosin, streptorubin), peptides and cyclic peptides (e.g., surugamide, surfactin, cyclo(phenylalanyl-4-hydroxyproline)), siderophores and chelating agents (e.g., desferrioxamine, dehydroxynocardamine), porphyrins (e.g., coproporphyrin I), fatty acids and derivatives (e.g., linoleic acid, 9(10)-EpOME), indole derivatives (e.g., indole-3-acetamide), quinoline derivatives (e.g., 4-quinolinecarboxylic acid), phenylethylamides (e.g., N-phenethylacetamide), and cytokinins (e.g., zeatin riboside). Several of these compounds have been reported to exhibit antimicrobial and cytotoxic activity (Table S3).

The molecular network also revealed several molecular families that are strain-specific among the 22 evaluated extracts, each uniquely associated with a single taxonomic group (Figure 4). Bafilomycins were exclusively linked to *S. surdarbansensis*, antimycins to *S. sampsonii*, surfactins to *S. ambofaciens*, and rosaramicins and cirramycins to *M. carbonacea*.

In addition to these identified molecular families, the network presents other strain-specific clusters of yet-unidentified metabolites, highlighted in red boxes in Figure 3. Moreover, spectrorubins, surugamides, and glucopiericidin were found only in two *Streptomyces* species (Figure 5).

As mentioned above, *S. sampsonii* is known to produce antimycin A, which is consistent with the molecular network [52,53].

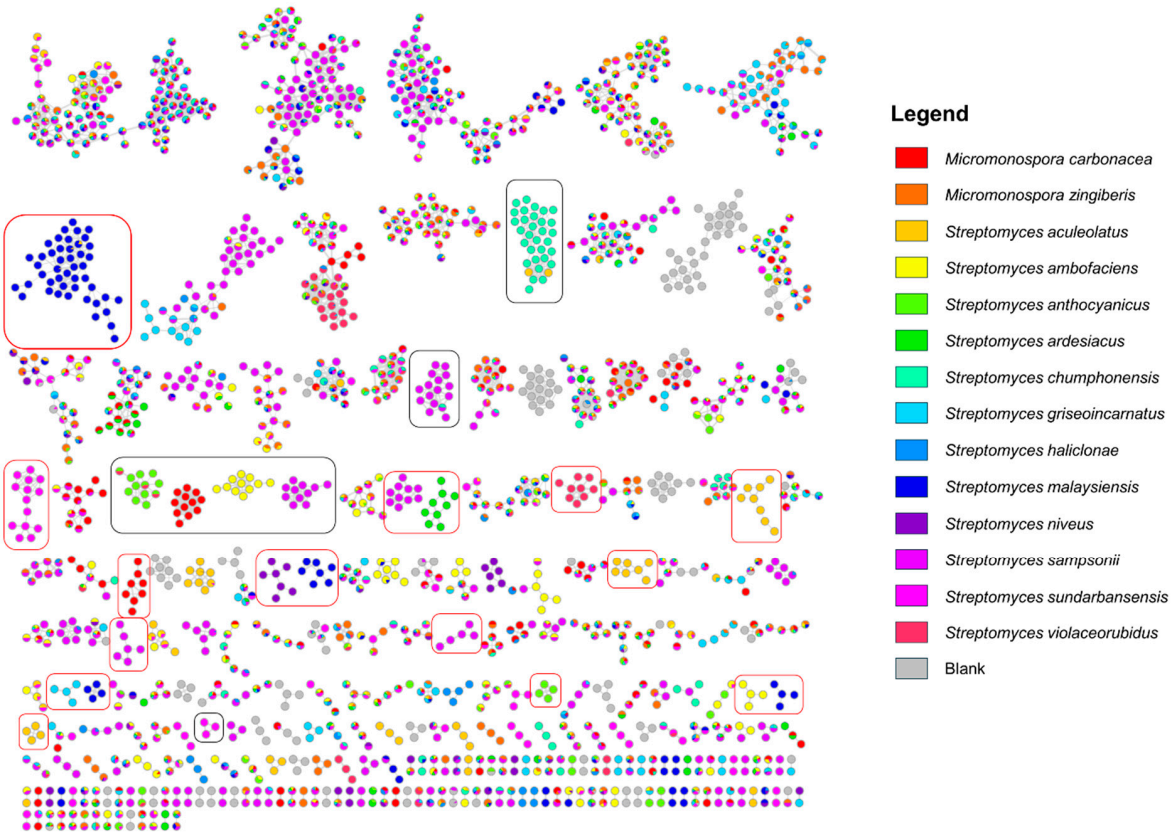

Figure 3. Molecular networking for the Lisbon and Setúbal coastal bioactive species using MS/MS data (ESI+). Nodes represent parent ions, and edge indicates the chemical similarity between the MS/MS spectra. Node colors represent the species according to the legend. Only networks containing at least two nodes are shown. Nodes highlighted in black boxes will be presented in Figures 4 and 5. Nodes highlighted in red represent unidentified strain-specific molecular families.

Importantly, none of the other molecular families annotated in our extracts correspond to previously reported metabolites described in Sections 2.2 and 2.3 for strains of the same associated species.

This study reveals a rich and diverse chemical profile, including both shared and distinctive compounds when compared to the Estremadura Spur study [20]. Unique to this dataset were compounds such as streptorubin and prodigiosins, glucopiericidin A, rosaramicin, dehydroxynocardamine, desmethylenylnocardamine, promicroferrioxamine, coproporphyrin I, surfactin, zeatin riboside, 4-quinolinecarboxylic acid, N-phenethylacetamide, indole-3-acetamide, and the cyclic peptides cyclo(phe-4-hyp) and cyclo(phe-pro).

Shared molecular families with the Estremadura Spur dataset include surugamides, antimycins, desferrioxamines, piericidines, and various fatty acids and lipid derivatives such as linoleic acid.

Surugamides were also reported from the Rocas Atoll (Brazil) and the Madeira Archipelago (Portugal) Atlantic actinomycete strains [21,67–69].

In contrast, the Estremadura Spur study reported additional molecular families not reported in this dataset, including distinct classes of peptides and alkaloids, such as etamycins, physostigmines, ikarugamycins, rakicidins and was reported as particularly rich in fatty acids [20,70].

Molecular differences are further corroborated by comparing our results with dos Santos et al. 2024 study, from the Alcochete location [29]. Exclusive to our study were macrolides such as rosaramicin, cirramycin, piericidins including glucopiericidin A and 7-demethyl-piericidine A1, and siderophores like promicroferrioxamine, and desmethylenyl-

nocardamine. Biosurfactants such as surfactin, plant hormone analogs like zeatin riboside, and cyclic peptides including cyclo(phe-4-hyp) were also exclusive to this dataset.

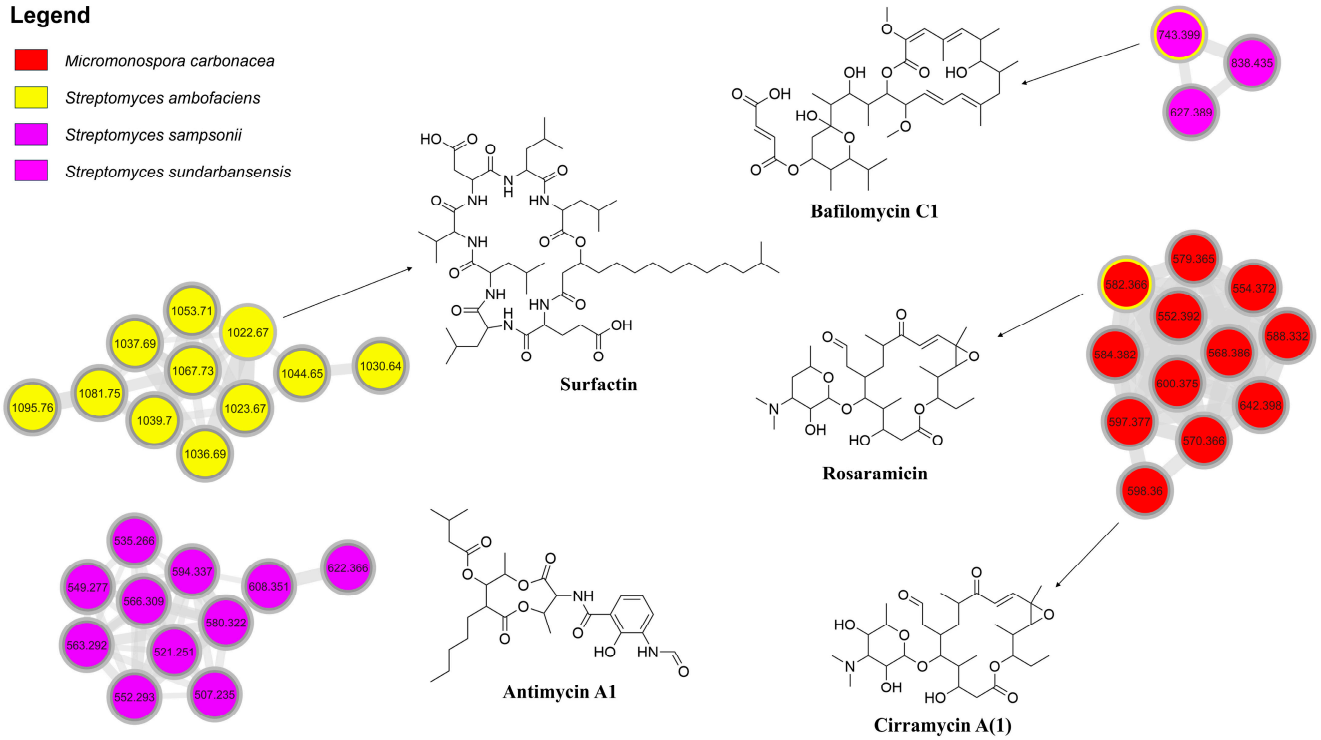

Figure 4. Cluster from antimycins, baflomycins, rosaramicins, and surfactins produced by Lisbon-Setúbal coastal actinomycete strains. Node colors represent the extracts according to the legend. Nodes represent parent ions, and edge strength indicates the chemical similarity between the MS/MS spectra. Nodes highlighted in yellow circles presented a spectral match with the library.

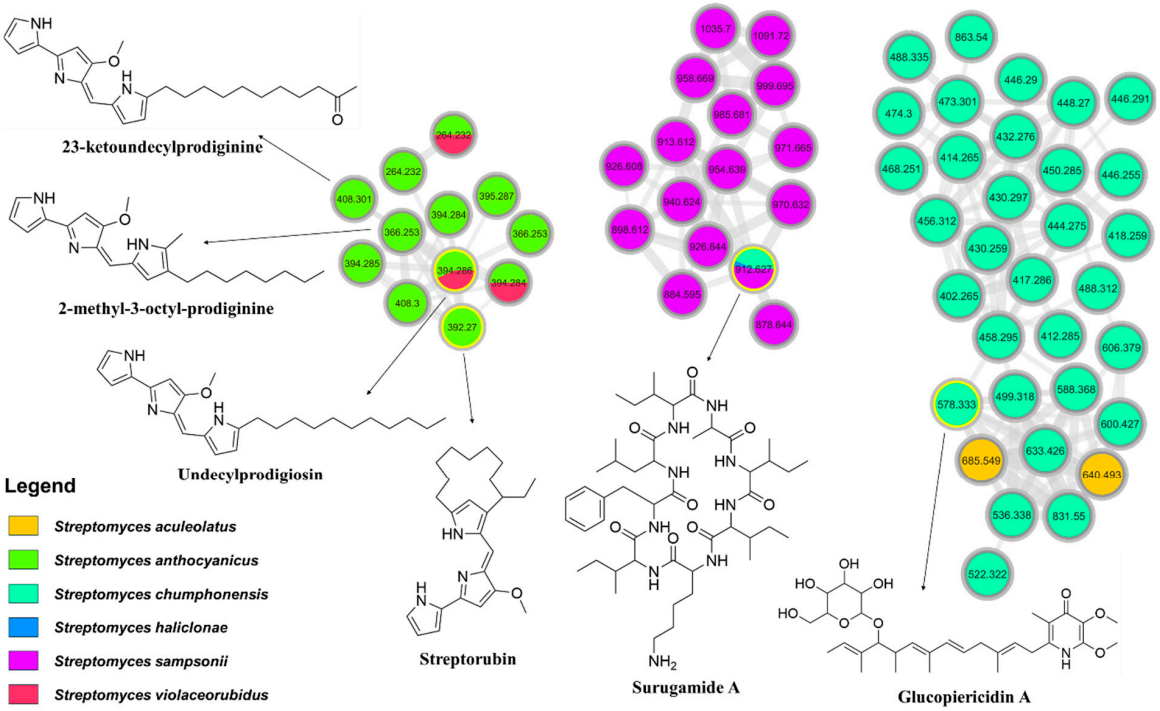

Figure 5. Cluster from streptorubins, surugamides and glucopiericidins produced by the Lisbon-Setúbal coastal strains. Node colors represent the extracts according to the legend. Nodes represent parent ions, and edge strength indicates the chemical similarity between the MS/MS spectra. Nodes highlighted in yellow circles presented a spectral match with the library.

Several compounds were shared between the two studies, antimycins, surugamides, and cyclo-dipeptides such as cyclo(phe-pro) and cyclo(leu-pro), as well as N-(2-phenylethyl) acetamide and indole-3-acetic acid. Fatty acid derivatives, including linoleic acid and 12-hydroxy-octadecadienoic acid, were also found in both datasets.

In contrast, the Alcochete study reported several compounds distinct from the Lisbon-Setúbal dataset. These included alteramide A, antibiotic X 14952B, and tubercidin, as well as germicidins, limazepines, virginiamycins, and phenolic compounds such as 2-butyl-5-propylresorcinol, nucleoside and amphiphilic derivatives, including N,N-dimethyladenosine and N-myristylamidopropyl-N,N-dimethylbetaine [29].

These comparative studies of actinomycetes isolated from geographically close sedimentary environments have shown that strains with similar taxonomic affiliations can nonetheless yield markedly different secondary metabolite profiles, highlighting the limitations of taxonomic proximity alone as a predictor of chemical output. For example, metabolomic analyses of marine actinomycetes from adjacent sediment samples revealed considerable heterogeneity in chemical signatures among isolates, even within the same genus [71]. In addition, environmental structure and local physicochemical gradients have been shown to influence both microbial community composition and secondary metabolite richness, suggesting that abiotic context plays an important role in shaping metabolic repertoires [72]. Studies of actinobacterial responses to varied ecological stimuli further support the idea that environmental cues can activate distinct suites of secondary metabolites, demonstrating the plasticity of metabolic expression in response to local ecological pressures [73,74]. Together, these findings underscore that exhaustive sampling of marine sediment bacterial communities, even at nearby geographic locations, is essential for uncovering unique metabolite chemistry with potential biotechnological relevance.

3. Materials and Methods

3.1. Marine Sediments Collection

A total of 22 marine sediment samples were analyzed in this study. Sixteen of these samples were collected from 15 stations off the Tagus river mouth with a box-corer or a multicorer at depths between 35 to 128 m between 21 and 25 March 2021 (Figure 1) aboard of the NRP Almirante Gago Coutinho research vessel. These samples mainly consisted of fine mud and sandy grains. Additional six samples from the Setúbal Peninsula were collected with snorkel from a single station at 5 m depths in the Sado river mouth, off the Portinho da Arrábida, on 12 September 2011 (Figure 1), containing sand, small rocks and shell fragments. Each sample was placed in a sterile bag, kept on ice during transport to the lab, and stored at −20 °C for long-term preservation.

3.2. Actinomycetes Isolation

To isolate marine actinomycetes, 22 sediment samples were processed using two distinct methods, designated as P1 and P2, following previously described approaches [20,21].

All sediment samples were cultured on four media formulations: (i) A1, (ii) $^{1}/_{2}$ A1, (iii) 10% A1, and (iv) SWA. Each medium was prepared using a mixture of filtered natural seawater and deionized water in a 75:25 ratio, supplemented with 100 µg/mL of cycloheximide to inhibit fungal growth.

The composition of each growth medium per liter was as follows: (i) A1: 18 g agar, 10 g starch, 4 g yeast extract, 2 g peptone; (ii) $^{1}/_{2}$ A1: 18 g agar, 5 g starch, 2 g yeast extract, 1 g peptone, (iii) 10% A1: 18 g agar, 1 g starch, 0.5 g yeast extract, 0.2 g peptone; and (iv) SWA: 18 g agar.

In the P1 method, 0.5 g of wet sediment was suspended in 2 mL of sterile seawater, vortexed thoroughly, and subjected to heat treatment at 55 °C for five minutes. Following

this, 100 µL of the resulting supernatant was plated on the surface of the four different agar media. The P2 method involved drying the sediment samples overnight in a laminar flow hood. Once dried, an autoclaved plug (1–2 cm in diameter) was pressed onto the sediment surface and then gently rolled across the surface of the four media agar plates, creating a serial dilution effect through physical transfer.

The inoculated plates were incubated at room temperature (approximately 25–28 °C) for a period of six months, during which they were regularly monitored for the emergence of actinomycete colonies. Identification of actinomycetes was based on distinct morphological traits, including the development of filamentous hyphae and the appearance of powdery or leathery colony textures. On each plate, colonies exhibiting actinomycete-like morphology were counted, and representative colonies were repeatedly subcultured onto fresh A1 agar plates to ensure purity. This process resulted in the successful isolation of 142 pure strains.

3.3. Actinomycetes Cryopreservation

All purified strains were subsequently cultivated in liquid A1 medium under agitation at 150 rpm and maintained at 25 °C for 3 to 7 days. Strains with slower growth rates were incubated up to 14 days to allow full development. Following cultivation, each strain was cryopreserved with 15% glycerol and stored at −80 °C for long-term preservation.

3.4. Marine Obligate Actinomycetes Evaluation

To evaluate the seawater dependency of the 142 pure actinomycete cultures, each strain was tested for its ability to grow in a modified A1 medium in which seawater was replaced with deionized water.[18] For this assessment, cells from a single colony were transferred onto fresh A1 agar plates prepared without seawater. The inoculated plates were incubated at room temperature and monitored over a six-month period for signs of growth.

3.5. DNA Extraction and Quantification

All 142 strains were cultured in liquid A1 medium, at 150 rpm and 25 °C for 3 to 7 days. Some strains exhibiting slower growth were maintained for up to 14 days.

DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the protocol for isolating genomic DNA from Gram-positive bacteria with minor modifications.

The concentration and quality of the extracted DNA was performed using a NanoDrop spectrophotometer (Thermo Scientific ND-1000; Wilmington, DE, USA).

3.6. 16S rRNA Gene Amplification and Sequencing

The 16S rRNA gene was amplified by PCR using the primers 27F (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) [75].

The PCR reaction was performed with 1X reaction buffer, 0.08 mM dNTPs mix, 2.5 mM MgCl2, 0.56 µM 27F primer, 0.56 µM 1492R primer, and 0.02 U/µL of NZYTaq II, with DNA template concentrations varying by sample. The PCR program was as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min.

To confirm the amplification of the 16S rRNA gene, the PCR products were analyzed using 0.8% (*w*/*v*) agarose gel electrophoresis. The PCR products were purified using the NZYGelpure kit, (Lisbon, Portugal), sequenced at STAB VIDA (https://www.stabvida.com/pt; accessed on 5 May 2025) using the 27F and 1492R primers. The strains were registered in the NCBI platform under accession numbers PX484717–PX484858.

3.7. Taxonomic Identification and Phylogenetic Analysis

The sequencing of the 16S rRNA gene involved two Sanger reactions, yielding a forward and a reverse sequence. The two sequencing chromatograms were analyzed individually using BioEdit 7.2 software (https://bioedit.software.informer.com/7.2/; accessed on 5 May 2025). The two complementary sequences were then aligned and assembled into a single consensus sequence [24].

Each consensus sequence was compared to the NCBI GenBank (rRNA) database using the BLASTn algorithm (accessed 22 July 2025), retrieving the nearest neighbor 16S rRNA sequence [76]. The corresponding 16S rRNA sequence of that best-hit species type strain was also obtained from the EzBioCloud database. When the NCBI best hit corresponded to a single species, the isolated strain was taxonomically classified based on the 99% sequence identity threshold for species identification. If NCBI identified multiple nearest neighbors with the same score, up to three hits were selected, and their type strains were retrieved from EzBioCloud. To further analyze the phylogenetic relationships between the isolates and clarify species identification for those with multiple best hits in the NCBI rRNA database, phylogenetic trees were constructed. All phylogenetic trees presented in the manuscript, including those in the Supplementary Materials, were constructed as follows: the consensus sequence of each isolated strain, along with the sequences of the NCBI best hits and EzBioCloud type strains, were aligned using MAFFT v.7.305b [77]. The alignment was submitted to JModelTest v.2.1.10 [78], which determined that GTR + G + I was the most appropriate substitution model. The phylogenetic estimation used MrBayes v3.2.3 with default parameters [79]. The phylogenetic tree was visualized and edited with iTOL v4 [80]. *Bacillus methanolicus* PB1 (AFEU010000002) served as the outgroup.

3.8. Antimicrobial Evaluation: Growth Inhibition Halo Assay

The antimicrobial activity of 142 actinomycete strains was screened using a growth inhibition halo assay against the Gram-positive MRSA COL strain and the Gram-negative *E. coli* K12 strain, gifted by Prof. Hermínia de Lencastre from Rockefeller University, USA and ITQB, NOVA University of Lisbon, Portugal.

The actinomycete strains were inoculated as a single spot in A1 agar plates. Liquid overnight cultures of COL and K12 strains were prepared in Tryptic Soy Broth (TSB, Difco, Detroit, MI, USA) and Lysogeny Broth (LB; NZYtech, Lisbon, Portugal), respectively, and incubated at 37 °C with shaking.

A volume of 200 µL from each culture was inoculated into 5 mL of soft-agar TSB and LB with 0.8% (*w*/*v*) agar, homogenised and poured over the actinomycete plates. After incubation at 37 °C overnight, the actinomycete strains exhibiting antimicrobial activity inhibited the growth of the test bacteria, resulting in a halo of growth inhibition. The inhibition halos diameters were measured in cm.

3.9. Actinomycetes Crude Extracts Preparation

Actinomycete strains that displayed a growth inhibition halo against at least one of the tested bacterial strains, MRSA COL or *E. coli* K12, were grown in 20 mL of A1 liquid medium, at 150 rpm and 25 °C for 7 days. Afterwards, 10 mL of each culture was transferred to 1 L of A1 medium and incubated at 100 rpm and 30 °C for 15 days. At the end of the incubation period, the culture broth was extracted with ethyl acetate (3 × 500 mL). The solvent was evaporated to dryness under vacuum.

3.10. Antimicrobial Evaluation: Minimum Inhibitory Concentration (MIC) Determination

The 22 crude extracts were tested against the test strains that were inhibited in the primary screening of inhibition (see Section 3.8).

The overnight cultures of the test strains were diluted to an optical density ($OD_{600\,nm}$) of 0.04–0.06 and incubated statically in 96-well polystyrene flat-bottom microplates, with varying concentrations of each crude extract. The cultures were serially diluted two-fold, yielding final extract concentrations ranging from 250 to 1.95 µg/mL. After 24 h of incubation at 37 °C, the minimal inhibitory concentration (MIC) was determined through visual inspection, as the lowest concentration that results in the absence of turbidity. Results were compared with positive controls, vancomycin for MRSA COL and tetracycline for *E. coli* K12, and a negative control, DMSO, all tested over the same concentration range of 250 to 1.95 µg/mL. Each crude extract was tested in triplicate and active extracts were re-tested for confirmation.

3.11. Anticancer Activity Evaluation

The anticancer activity of 22 crude extracts was tested against human colorectal adenocarcinoma HCT116 cells (ECACC 91091005, Porton Down, UK), as previously described [20,21,81,82]. Briefly, HCT116 cells were cultured in McCoy's 5A medium, with 10% fetal bovine serum and 1% (*v*/*v*) antibiotic/antimycotic supplementations (all from Gibco, Thermo Fisher Scientific, Paisley, UK) and maintained at 37 °C under 5% CO_2. Cells were seeded in 96-well plates (5000 cells per well), and 24 h later were treated with crude extracts (concentrations ranging from 100 to 0.0051 µg/mL), vehicle control (DMSO 1%, Sigma-Aldrich, St. Louis, MO, USA), or positive control (10 µM 5-fluorouracil, Sigma-Aldrich). After 72 h of incubation, cell viability was evaluated through MTS metabolism, using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Absorbance signal at 490 nm was recorded using a Multiskan™ microplate photometer (Thermo Scientific). Dose–response curves were created and IC_{50} best-fit values determined using the log-(inhibitor) vs. response–variable slope (four parameters) using GraphPad Prism 9 software (La Jolla, CA, USA).

3.12. LC-MS/MS Analysis

Actinomycete crude extracts were diluted in methanol (1.0 mg/mL) and analyzed by HPLC-MS. The analyses were carried out on an ELUTE + UHPLC (Bruker, Berlin, Germany) coupled to an Impact II QqTOF mass spectrometer with electrospray ionization (ESI+), using positive ionization mode. Chromatographic separation was performed on an Intensity Solo 2 C18 column (5 µm, 100 × 2.1 mm) at 40 °C. Water and acetonitrile were used as mobile phases A and B, respectively, both containing 0.1% formic acid. A gradient was applied from 5% to 1 min, then 95% until 12 min, returning to 5% to complete a 14-min chromatographic run with an injection volume of 1 µL. Ion source parameters were: endplate: 500 V, capillary voltage: 4500 V, drying temperature: 220 °C, drying gas: 10.0 L/min, *m/z* range: 50–1500, gas pressure: 40 psi. An untargeted method was used in the spectrometer in which ions of higher intensity were selected for fragmentation. A ramp from 20 to 50 eV was used for collision energy.

3.13. Molecular Networking

A molecular network was created using the online workflow (https://wang-bioinformatics-lab.github.io/GNPS2_Documentation/; accessed on 15 October 2025) on the GNPS2 website (https://gnps2.org/homepage) [83]. The data was filtered by removing all MS/MS fragment ions within +/− 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 fragment ions in the +/− 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.65 and more than 4 matched peaks. Further, edges

between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS2′ spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.65 and at least 4 matched peaks.

4. Conclusions

This study revealed that the marine sediments from the Lisbon and Setúbal coast harbor a highly diverse community of actinomycetes, predominantly belonging to the genera *Streptomyces* and *Micromonospora*. From 142 isolated strains, representatives closely related to 47 distinct species were identified, including several putative novel taxa and seawater-dependent isolates. These findings underscore the ecological and evolutionary distinctiveness of this coastal microbiome. Moreover, differences in actinomycete diversity compared with previous studies from nearby regions highlight the importance of thorough continuous sampling to uncover the potential of these sediments.

Bioactivity assays demonstrated significant antimicrobial activity, with 22 strains inhibiting MRSA and 6 inhibiting *E. coli*. Among them, *Streptomyces chumphonensis* PTS-083 showed the strongest activity (MIC = 1.95 μg/mL) and genetic divergence, suggesting a new species. Eight strains also displayed notable cytotoxicity against HCT-116 colon adenocarcinoma cells, particularly PTS-001 (*S. chumphonensis*; IC_{50} >0.005 μg/mL) and PTS-008 (*S. sundarbansensis*; IC_{50} = 0.58 μg/mL).

Untargeted LC-MS/MS metabolomic profiling further revealed a rich chemical landscape dominated by macrolides, siderophores, fatty acids, and cyclic peptides, many associated with antimicrobial, cytotoxic, or signalling functions. Comparison with other Portuguese marine environments confirmed both shared biosynthetic capacities and unique local chemotypes, emphasizing the impact of environmental factors on secondary metabolism.

These findings reinforce the value of Portugal's coastal microbiomes as reservoirs of untapped phylogenetic and chemical diversity. This study expands the catalogue of marine actinomycetes and lays the groundwork for future bioprospecting efforts to discover new antibiotics and anticancer agents. Continued exploration of these dynamic ecosystems is essential if we are to unlock their full biotechnological potential.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md24020068/s1, Table S1: Actinomycete species identified among the 142 cultivable Lisbon-Setúbal coastal strains; Figure S1: (A) Relationship between coastline distance and the number of isolated strains from the Lisbon-Setúbal coastal strains. (B) Non-metric multidimensional scaling (NMDS) plot based on Jaccard distance for the 142 culturable actinomycetes isolated from marine sediments; Figure S2: Phylogeny of the 73 actinomycete strains belonging to *Streptomyces* genus isolated from Lisbon and Setúbal coast, the BLASTn top-hit sequences from NCBI (GenBank) rRNA database and their type-strain sequences; Figure S3: Phylogeny of the 67 actinomycete strains belonging to genus *Micromonospora* isolated from the Lisbon and Setúbal coast, the BLASTn top-hit sequences from NCBI (GenBank) rRNA database and their type-strain sequences; Figure S4: Phylogeny of 59 isolated actinomycetes (one representative per species from three genera) from Lisbon-Setúbal collection, the BLASTn top-hit sequences from NCBI (GenBank) rRNA database and their type-strain sequences retrieved from EzBioCloud, and strains previously isolated from the Alcochete by dos Santos et al. (2024) [29]; Table S2: Marine-derived actinomycetes that displayed growth inhibition halo against at least one of the tested pathogens, methicillin-resistant *Staphylococcus*

aureus (MRSA, strain COL) and *Escherichia coli* (strain K12); Table S3: Chemical compounds annotated by the GNPS, corresponding species, sample code, bioactivity and other sources.

Author Contributions: M.P.C.: Investigation, Formal Analysis, Visualization, Writing—original draft, Writing—review and editing; P.S.-M.: Data curation, Formal Analysis, Software, Visualization, Writing—review and editing; M.R.: Investigation, Formal Analysis, Software, Visualization, Writing—review and editing; A.O.G.M.: Investigation, Formal Analysis, Software, Visualization, Writing—review and editing; V.M.: Investigation, Data curation, Formal Analysis, Visualization, Writing—original draft, Writing—review and editing. S.M.: Investigation, Visualization, Writing—review and editing; M.M.-H.: Methodology, Resources, Visualization, Writing—review and editing; C.M.P.R.: Methodology, Visualization, Writing—review and editing, Supervision; A.P.-D.: Methodology, Data curation, Formal Analysis, Software, Visualization, Writing—review and editing, Supervision; A.B.: Methodology, Data curation, Formal Analysis, Software, Visualization, Writing—review and editing, Supervision; R.G.S.: Methodology, Supervision, Data curation, Formal Analysis, Visualization, Visualization, Writing—review and editing; S.P.G.: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—original draft, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that supports the findings of this study are available on request and at the NCBI platform under accession numbers PX484717- PX484858, available at NCBI.

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Conflicts of Interest: The authors declare no conflicts of interest.

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