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Biorefineries from deep-sea environments: mining deep-sea actinobacteria for the production of novel compounds with biotechnological applications

Inês Ribeiro

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BIOREFINERIES FROM DEEP-SEA ENVIRONMENTS: MINING DEEP-SEA ACTINOBACTERIA FOR THE PRODUCTION OF NOVEL COMPOUNDS WITH BIOTECHNOLOGICAL APPLICATIONS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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In the scope of this PhD Thesis, one scientific paper was published in an international journal.

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Part of the content of the Chapter 1 was published in the book chapter:

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† Both authors contributed equally to this work

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Abstract

There is an urgent need to discover new pharmaceutical compounds to fight important diseases of modern societies, like infections caused by antibiotic-resistant microorganisms, cancer, obesity and chronic inflammation.

Actinobacteria are well known for their excellent capacity to produce secondary metabolites with a wide range of bioactive properties, but their intensive exploration from terrestrial environments has led to a slowdown in the discovery of new molecules produced by these microorganisms. With the eyes of the scientific community set on the oceans, the deep-sea emerges has one of the last frontiers for the exploration of bioactive natural products, being home to a large diversity of actinobacteria, many of them waiting to be discovered.

In this context, the present thesis aimed to investigate and explore actinobacteria inhabiting deep-sea environments of the Arctic and Atlantic (Azores and Madeira archipelagos) oceans, in terms of their phylogenies and potential to produce novel bioactive metabolites with biotechnological applications. The first work presented in this thesis focused on the study of the actinobacterial communities of deep-sea sediments from the Arctic (Arctic-Mid-Ocean Ridge) and Atlantic (Azores and Madeira) oceans, using both culture-dependent and -independent approaches, and on the subsequent investigation of the biosynthetic potential of the cultivable actinobacteria. The results showed that the actinobacterial communities varied between the studied regions, with higher abundance in the Arctic but higher diversity in the Atlantic. Forty-four actinobacterial strains were isolated and phylogenetically identified, revealing to be mainly affiliated with the genera *Brachybacterium*, *Microbacterium* and *Brevibacterium*, but also including novel taxa. Bioactivity screening of these isolates showed two actinobacterial extracts with significant activity against *Candida albicans* and 8 extracts active against the cancer cell lines HepG2 and T-47D. Furthermore, 15 actinobacterial extracts exhibited anti-inflammatory activity in the RAW 264.4 cell line, with no concomitant cytotoxicity. Dereplication and molecular networking analysis of the bioactive actinobacterial extracts showed the presence of some metabolites associated with known natural products, but one cluster associated to strain *Microbacterium* sp. DS3_6.1 (major compound m/z 694.878) did not show any matches with natural products present in the databases, probably corresponding to a new natural product.

One of the microorganisms isolated in this study from the Madeira archipelago, and that showed the potential to be a new species, was subjected to a deeper characterisation with the objective of validating its taxonomy (Chapter 3). For this, a

polyphasic approach was employed, which integrated morphological, biochemical, chemotaxonomic and genomic analysis. The results allowed to identify this novel species as *Streptomyces profundis* sp. nov..

The third study included in this thesis aimed to investigate the biodiversity of cultivable actinobacteria associated with 66 deep-sea samples of sediments, sponges and corals from the Madeira and Azores archipelagos, and screen their potential to produce new molecules with antimicrobial, anticancer, anti-inflammatory and anti-obesity activity. A total of 276 actinobacterial strains distributed by 20 genera were isolated from the analysed samples, with the largest fraction belonging to the genera *Brevibacterium* and *Microbacterium*. The results of the bioactivity assays allowed identifying 16 organic extracts with antimicrobial activity (against *C. albicans, B. subtilis and/or S. aureus*), 23 extracts capable of reducing the cellular viability of the cancer cell lines T-47D and/or HepG2, and 28 extracts with anti-inflammatory activity. Anti-obesity activity was tested for only 30 extracts, with six exhibiting significant neutral lipid-reducing activity after 48 h of exposure. The metabolic profile of the active extracts suggested the presence of both known and possibly new bioactive molecules.

The last study of this thesis focused on the investigation of the metabolic profile of the deep-sea coral-associated *Streptomyces chumphonensis* strain C_003 1.18, isolated from Madeira Archipelago in Chapter 4. This strain was selected due to its relevant anti-obesity activity and also due to the fact that no compound that could justify this activity was identified in the dereplication analysis. Large-scale culturing of this strain was performed, but the resultant extract became toxic to zebrafish larvae. In view of this result, the analysis of the metabolic profile of strain C_003 1.18 changed to a mass-guided isolation, targeting other promising masses presented in the extract. With mass-guided isolation, four compounds were isolated using high performance liquid chromatography (HPLC), including the well-known Piericidin A1 and three novel compounds.

Overall, the work present in this thesis illustrates the potential of bioprospecting actinobacteria in underexplored environments, such as the deep-sea, as a viable approach for uncovering new actinobacterial phylogenies and novel molecules with pharmaceutical applications. This thesis is an important contribution to enlarge the meagre knowledge still available on the diversity and biosynthetic potential of actinobacteria inhabiting deep-sea environments and highlight the importance that these microorganisms might have for the discovery of future drug leads.

Resumo

Existe uma necessidade urgente de descobrir novos compostos farmacêuticos para combater doenças importantes das sociedades modernas, como infeções causadas por microrganismos resistentes a antibióticos, cancro, obesidade e inflamação crónica. As actinobactérias são bastante conhecidas pela sua excelente capacidade de produzir metabolitos secundários com uma ampla gama de propriedades bioativas, mas a sua intensa exploração em ambientes terrestres tem levado a uma desaceleração na descoberta de novas moléculas produzidas por estes microrganismos. Com os olhos da comunidade científica postos nos oceanos, o mar profundo surge como uma das últimas fronteiras para a exploração de produtos naturais bioativos, albergando uma grande diversidade de actinobactérias, muitas delas ainda por descobrir.

Neste contexto, a presente tese teve como objetivo investigar e explorar actinobactérias que habitam o mar profundo dos oceanos Ártico e Atlântico (arquipélagos dos Açores e da Madeira), em termos das suas filogenias e potencial para produzir novos metabolitos bioativos com aplicações biotecnológicas. O primeiro trabalho apresentado nesta tese centrou-se no estudo das comunidades de actinobactérias de sedimentos de águas profundas dos oceanos Ártico (Arctic-Mid-Ocean Ridge) e Atlântico (Açores e Madeira), usando abordagens dependentes e independentes de cultivo, e na subsequente investigação do potencial biossintético das actinobactérias cultiváveis. Os resultados mostraram que as comunidades de actinobactérias variaram entre as regiões estudadas, com maior abundância no Ártico, mas maior diversidade no Atlântico. Quarenta e quatro estirpes de actinobactérias foram isoladas e identificadas filogeneticamente, estando principalmente afiliadas aos géneros *Brachybacterium*, *Microbacterium* e *Brevibacterium*, mas também incluindo novas taxonomias. A triagem de bioatividade desses isolados mostrou dois extratos de actinobactérias com atividade significativa contra *Candida albicans* e 8 extratos ativos contra as linhas celulares cancerígenas HepG2 e T-47D. Além disso, 15 extratos de actinobactérias exibiram atividade anti-inflamatória na linha celular RAW 264.4, sem citotoxicidade concomitante. A desreplicação e a análise de rede molecular dos extratos bioativos de actinobactérias mostraram a presença de alguns metabolitos associados a produtos naturais conhecidos, mas um cluster associado à estirpe *Microbacterium* sp. DS3_6.1 (composto principal m/z 694.878) não apresentou nenhuma correspondência com produtos naturais presentes nas bases de dados, provavelmente correspondendo a um novo produto natural.

Um dos microrganismos isolados neste estudo do arquipélago da Madeira, e que apresentou potencial de ser uma nova espécie, foi sujeito a uma caracterização mais aprofundada com o objetivo de validar a sua taxonomia (Capítulo 3). Para isso, foi realizada uma abordagem polifásica, que integrou uma análise morfológica, bioquímica, quimiotaxonómica e genómica. Os resultados permitiram identificar esta nova espécie como *Streptomyces profundis* sp. nov..

O terceiro estudo incluído nesta tese teve como objetivo investigar a biodiversidade de actinobactérias cultiváveis associadas a 66 amostras de mar profundo, incluindo esponjas e corais dos arquipélagos da Madeira e dos Açores, e rastrear o seu potencial para produzir novas moléculas com propriedades antimicrobiana, anticancerígena, anti-obesidade e anti-inflamatória. Das amostras analisadas foram isoladas 276 estirpes de actinobactérias distribuídas por 20 géneros, com a maior fração pertencendo aos géneros *Brevibacterium* e *Microbacterium*. Os resultados dos ensaios de bioatividade permitiram identificar 16 extratos orgânicos com atividade antimicrobiana (contra *C. albicans*, *B. subtilis* e/ou *S. aureus*), 23 extratos capazes de reduzir a viabilidade celular das linhas célulares cancerígenas T-47D e/ou HepG2, e 28 extratos com atividade anti-inflamatória. A atividade anti-obesidade foi testada para apenas 30 extratos, tendo seis exibido atividade significativa redutora de lipídos neutros, após 48 h de exposição. O perfil metabólico dos extratos ativos sugeriu a presença de moléculas bioativas conhecidas e possivelmente novas.

O último estudo desta tese centrou-se na investigação do perfil metabólico da estirpe *Streptomyces chumphonensis* C_003 1.18 associada a um coral de mar profundo, recolhido no Arquipélago da Madeira, descrito no Capítulo 4. Esta estirpe foi selecionada devido à sua relevante atividade anti-obesidade e também pelo facto de que nenhum composto capaz de justificar essa atividade foi identificado na análise de desreplicação. O cultivo em larga escala desta estirpe foi realizado, mas o extrato resultante tornou-se tóxico para larvas de peixe-zebra. Face a este resultado, a análise do perfil metabólico da estirpe C_003 1.18 foi alterada para um isolamento guiado por massa, visando outras massas promissoras presentes no extrato. Através do isolamento guiado por massa, quatro compostos foram isolados usando a cromatografia líquida de alta eficiência (HPLC), incluindo o composto bem conhecida Piericidina A1 e três novos compostos.

No geral, o trabalho apresentado nesta tese ilustra o potencial da bioprospecção de actinobactérias em ambientes pouco explorados, como o mar profundo, como uma abordagem viável para descobrir novas filogenias de actinobactérias e novas moléculas com aplicações farmacêuticas. Esta tese é uma importante contribuição

para ampliar o escasso conhecimento ainda disponível sobre a diversidade e o potencial biossintético de actinobactérias que habitam ambientes de mar profundo e destacar a importância que esses microrganismos podem ter para a descoberta de futuros novos fármacos.

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List of Abbreviations

°C – Celsius Degree
ACN(aq) - Acetonitrile aqueous
ACS - American Chemical Society
ANI - Average nucleotide identity
ANOVA - Analysis of variance
ASVs - Amplicon Sequence Variants
BGC - Biosynthetic gene clusters
BLAST - Basic Local Alignment Search Tool
bp - base pairs
CEMUP - Materials Center of the University of Porto
Cryo-SEM - Cryo-scanning electron microscope
d.w. - Dry weight
DCM – Dichloromethane
DMEM - Dubelco's modified eagle medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DNP - Dictionary of natural products
DPG - Diphosphatidylglycerol
EIC - Extracted ion chromatogram
EMEPC - Extension of the Continental Shelf
ENA - European Nucleotide Archive
EtOAc/n-hex - Ethyl acetate/n-hexane
G+C - Guanine + Citosine
GNPS - Global Natural Products Social Molecular Networking
GYM 50SW agar - Glucose-Yeast-Malt agar with 50% of seawater
h – Hour
HIV - Human Immunodeficiency Virus
HKY+G+I - Hasegawa-Kishino-Yano
HPLC - High performance liquid chromatography
I3S - Instituto de Investigação e Inovação em Saúde
LC-HRESIMS/MS - Liquid chromatography-high resolution electrospray ionization tandem mass spectrometry
LPS – Lipopolysaccharide
LPSZ - List of Prokaryotic names with Standing in Nomenclature

MEGA - Molecular Evolutionary Genetics Analysis

MeOH – Methanol

mg L^{-1} – Milligram per Liter

mg mL^{-1} - Miligram per milliliter

MH - Mueller-Hinton agar

MIC - Minimum inhibitory concentration

min – Minutes

MS - Mass spectrometry

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,+A2:A335-diphenyltetrazolium bromide

NCBI - National Center for Biotechnology Information

nm – Nanometres

NMR - Nuclear Magnetic Resonance

NO - Nitric Oxide

NPA - Natural products Atlas

NPS - Nutrient-poor sediment extract

NRPS - Nonribosomal peptide synthetases

OD - Optical Density

PCR - Polymerase Chain Reaction

PE - Phosphatidylethanolamine

PG - Glycophospholipids

PI – Phosphatidylinositol

PIM - Phosphatidylinositolmanosides

PKS - Polyketide synthases

PL - Phospholipids

RDP - Ribosomal Database Project

RH - Raffinose-Histidine

ROV - Remotely operated underwater vehicles

rRNA - Ribosomal ribonucleic acid

SCN - Starch-casein-nitrate agar

SD - Sabouraud dextrose agar

UV - Ultraviolet

v/v - Volume per volume

VLC - Vacuum Liquid Chromatography

w/v - Weight per volume

WHO - World Health Organization

µg mL^{-1} - Microgram per milliliter

CHAPTER 1

Introduction

Part of the content of this chapter was published in the book chapter: Girão, M[†], Ribeiro, I[†], & Carvalho, MF. (2022). Actinobacteria from Marine Environments: A Unique Source of Natural Products. In *Natural Products from Actinomycetes: Diversity, Ecology and Drug Discovery* (pp. 1-45). Singapore: Springer Singapore. Doi:10.1007/978-981-16-6132-7_1

[†] Both authors contributed equally to this work

1.1. Introduction

The extensive use of antibiotics, microbial evolution and gene transfer between species have been named as the main causes responsible for the increase in antibiotic-resistant microorganisms that we have been assisting today (Berdy, 2012; Duncan et al., 2015). In 2019, it was estimated that the infections caused by antibiotic-resistant microorganisms directly contributed to 1.27 million deaths, killing more people than human immunodeficiency virus (HIV) or malaria (Thompson, 2022). This worldwide problem compromises the efficacy of currently available antibiotics, creating an urgent need to discover new therapeutic agents (Undabarrena et al., 2016). According to the World Health Organization (WHO), in 2020, cancer was one of the leading causes of death worldwide, with nearly 10 million deaths, and obesity has reached epidemic proportions globally, with at least 2.8 million people dying each year (https://www.who.int/news-room/fact-sheets/detail/cancer and https://www.who.int/news-room/facts-in-pictures/detail/6-facts-on-obesity, accessed on 7 May of 2023). Chronic inflammatory diseases have also a serious impact in human health, being responsible for over 50% of all deaths caused by diseases with an inflammatory genesis, such as ischemic heart disease, stroke, cancer, diabetes mellitus, etc. (Furman et al., 2019). All of these diseases still lack effective or less aggressive treatments, demanding the discovery of pharmaceutical compounds more successful for their treatment. Despite the large number of natural products that have already been described and characterized, the search for novel bioactive compounds active against these diseases remains a major scientific objective.

The phylum Actinomycetota covers a large group of morphologically and physiologically diverse Gram-positive bacteria and is widely recognised for its remarkable capacity to produce secondary metabolites with a broad range of bioactive properties (e.g., antibiotic, anticancer, anti-inflammatory, antiviral, etc.) (Dharmaraj, 2010; Olano et al., 2009; Subramani et al., 2019). Microorganisms belonging to this phylum are responsible for the production of *ca.* 45% (around 10 000) of the bioactive microbial metabolites currently known with a natural origin. The genus *Streptomyces*, by itself, is responsible for the production of more than 70% of these bioactive compounds (Berdy, 2005). Due to their prodigious bioactive metabolism, these microorganisms have been explored for decades, traditionally from terrestrial sources. However, years of intensive exploration of terrestrial actinobacteria have led to the frequent re-isolation of known compounds, which has become a major bottleneck in the field of drug discovery, slowing down the discovery of new molecules (Andersson et al., 2020). This challenge has prompted the

scientific community to look at less explored sources, such as the marine environment (Bister et al., 2004). In fact, the bioprospecting of oceans has opened up new avenues for discovering novel drugs and other chemical structures, due to the vast unexplored biodiversity found in marine environments (Siro et al., 2023). Among the various environments that oceans offer, the deep-sea is considered one of the most promising and attractive for the discovery of new bioactive molecules (Ruth, 2006). It is a unique environment characterized by extreme conditions such as low nutrient availability, high pressure, low temperature, lack of light, high salinity and variable oxygen concentrations (Bredholt et al., 2008; Subramani et al., 2012). These peculiar conditions are a trigger for the development of new metabolic and genetic traits in deep-sea dwellers, from a macro to a micro scale, which may translate in the production of novel secondary metabolites (Bull et al., 2000; Manivasagan et al., 2013b). Members of the *Actinomycetota* phylum are successful colonizers of several extreme environments, including the deep-sea, and studies indicate that this phylum is one of the most abundant one in deep-sea environments (Chen et al., 2016; Yilmaz et al., 2016). Nonetheless, deep-sea actinobacteria are still largely unexplored, both in terms of new phylogenies and of their biosynthetic potential (Mora et al., 2011; Sayed et al., 2020). Thus, the unique characteristics of deep-sea environments associated with their underexplored and largely unknown microbial diversity make these ecosystems an attractive and promising source of novel microorganisms and bioactive compounds.

1.2. Phylum Actinomycetota

The phylum *Actinomycetota* is a major taxonomic unit within the domain Bacteria and is one of the largest lineages currently recognized (Ludwig et al., 2012). According to the List of Prokaryotic names with Standing in Nomenclature (LPSZ), the phylum comprises 9 classes, Acidimicrobiia, Actinomycetes, "*Candidatus* Aquicultoria", Coriobacteriia, "*Candidatus* Geothermincolia", "*Candidatus* Humimicrobiia", Nitriliruptoria, Rubrobacteria and Thermoleophilia (https://lpsn.dsmz.de/phylum/actinomycetota, accessed on 5 May of 2023). The class Actinomycetes is the most attractive one in terms of microorganisms capable of producing secondary metabolites (Simeis et al., 2021) and includes 21 orders, Acidothermales, Actinomycetales, Bifidobacteriales, Catenulisporales, Cryptosporangiales, Frankiales, Geodermatophilales, Glycomycetales, Jatrophihabitantales, Jiangellales, Kineosporiales, Kitasatosporales, Micrococcales, Micromonosporales, Motilibacterales, Mycobacteriales, Nakamurellales,

Propionibacteriales, Pseudonocardiales, Sporichthyales and Streptosporangiales. In this thesis, the term actinobacteria will be used as a synonym of the class Actinomycetes.

The taxonomy of actinobacteria at the genus and species levels is primarily determined by genome sequencing, microscopic morphology and chemotaxonomy (Barka et al., 2016). The latter is related to the composition of the cell wall and the distribution of whole-cell sugars, although phospholipid composition and menaquinone type may also be considered for taxonomic level classification (Labeda, 1987).

Actinobacteria are Gram-positive bacteria, mostly filamentous and with a high G+C content in their genomic DNA. These microorganisms exhibit a diverse array of morphologies, which may vary in terms of presence or absence of substrate or aerial mycelium, colour of the mycelium, production of diffusible melanoid pigments, and structure and morphology of their spores (Barka et al., 2016). In terms of reproduction, the most common is through asexual spores produced by actinobacteria with primarily mycelial lifestyles. Mycelium can differentiate into specialized structures, such as aerial mycelium, which carry spores. In other cases, spores can be produced in the substrate mycelium or inside special vesicles called sporangia. Mycelial fragmentation is another form of vegetative reproduction observed in some actinobacteria, in which portions of the mycelium break off and develop into new colonies (Barka et al., 2016).

Most actinobacteria are aerobic, though some genera can be microaerophilic or anaerobic (Ventura et al., 2007). Metabolically, these microorganisms can be heterotrophs or chemoautotrophs, being able to use a variety of nutritional sources, including complex polysaccharides (e.g., chitin, lignin, and cellulose) (Barka et al., 2016; Bull et al., 2005; Manivasagan et al., 2013b). Actinobacteria are capable of surviving in many different environments and are commonly distributed both in terrestrial and aquatic ecosystems, including marine environments (Barka et al., 2016; Subramani et al., 2012).

1.3. Biosynthesis of secondary Metabolites by Actinobacteria

Bacterial metabolism is divided into two categories: (i) Primary metabolism, that occurs during the exponential growth phase and where sets of molecules are produced to obtain energy and allow growth and proliferation of the microorganism; and (ii) secondary metabolism, that occurs during the late exponential and/or stationary phases, where extracellular molecules of low molecular weight are predominantly produced, enabling the interaction of the microorganism with its environment (Seyedsayamdost, 2019).

The regulation of the biosynthesis of secondary metabolites in actinobacteria is a complex process that involves various factors and is controlled by both global and

pathway-specific regulators. Several parameters that directly affect microbial growth and influence the biosynthesis of secondary metabolites have been identified, including nutrient and carbon availability, nitrogen or phosphorus concentration in the culture medium, pH, temperature, oxygen partial pressure, agitation, mineral salts and metal ions, as well as the presence of precursors, inducers or inhibitors (Manivasagan et al., 2013b; Wohlleben et al., 2017).

Actinobacteria hold in their genomes a rich diversity of biosynthetic gene clusters (BGC), co-located genes that work together to build a molecule, with the most common structural classes of BGC being nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS) and terpenes (Ayuso-Sacido et al., 2005; Cragg et al., 2013; Xu et al., 2018c). PKS synthesize polyketides by sequentially condensing small acetate building blocks or other small carboxylic acids through decarboxylative reactions. Each PKS cluster consists of one or more functional modules, including β-ketoacyl synthase, acyltransferase and acyl carrier proteins (Du et al., 2010; Khan et al., 2014). NRPS are multifunctional enzymes that synthesize peptides in a modular structure through domains such as adenylation, condensation and peptidyl carrier proteins (Du et al., 2010). The characteristic domains of NRPS and PKS can be used to identify novel gene clusters, and their abundance can serve as a proxy for biosynthetic potential (Reddy et al., 2012). The biosynthetic process of terpenes follows a distinct logic compared to other secondary metabolite biosynthetic pathways. Five-carbon named isoprene units, are combined in a single enzyme-catalyzed step. The enzyme responsible for this process is known as terpene cyclase, which holds the linear methyl-branched polyene in a defined conformation, initiating a series of carbocation-driven cyclizations and rearrangements that create the fundamental hydrocarbon skeleton of a terpene (Helfrich et al., 2019).

1.4. Marine Actinobacteria

The marine environment is the largest on the planet and is home to a remarkable and underexplored microbial diversity, including actinobacteria (Bhatnagar et al., 2010). Marine actinobacteria can be found in both pelagic and benthic ecosystems, also establishing symbiotic associations with a wide range of marine organisms, such as sponges, corals, macroalgae, among others (Nathani et al., 2020; Penesyan et al., 2010). Furthermore, these microorganisms have also been identified in various extreme marine ecosystems, including deep-sea regions, as the Mariana Trench, hydrothermal

vents and polar marine waters (Abdel-Mageed et al., 2020; Bull et al., 2019; Sivasankar et al., 2018; Zhang et al., 2020b).

Only recently actinobacteria have been recognized as “true” inhabitants of the marine realm (Ward & Bora, 2006). Their presence in this environment was originally attributed to the spread of resistant spores from terrestrial actinobacteria, as these microorganisms were considered exclusively soil-dwelling microorganisms (Jensen et al., 1991).

Several new species, genera and even families of actinobacteria have been isolated from marine sediments, seawater and marine fauna (Subramani et al., 2013; Subramani et al., 2019), and 6 genera of obligate marine actinobacteria have been identified: *Euzebya*, *Marihabitans*, *Marinitenerispora*, *Paraoerskovia*, *Salinispora* and *Spinactinospora* (Chang et al., 2011; Kageyama et al., 2008; Khan et al., 2009; Kurahashi et al., 2010; Maldonado et al., 2005a; Ng et al., 2019). In addition, it is noteworthy the discovery of a new order "*Candidatus* Actinomarinales", exclusively characterized by marine obligate actinobacteria from the epipelagic zone (López-Pérez et al., 2020).

Marine actinobacteria have been of great interest to the scientific community because they have proved to produce several bioactive compounds not found in their terrestrial counterparts (Jensen et al., 2005). Examples include: Abyssomicins, a family of polycyclic macrolactones with antibiotic properties, isolated from the marine actinobacteria *Verrucosispora maris* AB-18-032 obtained from a sediment collected in the Sea of Japan (Riedlinger et al., 2004); Bahamaolides A-B, antifungal polyene polyol macrolides produced by the marine *Streptomyces* sp. CNQ343 isolated from a sediment collected at North Cat Cay in the Bahamas (Kim et al., 2012); Salinosporamide A, a β-lactone proteasome inhibitor, produced by the *Salinispora tropica* CNB-392 strain isolated from a marine sediment, which is currently undergoing clinical phase III trials for the treatment of multiple myeloma, solid tumors and lymphoma (Feling et al., 2003; Marx et al., 2018). The latter is a prominent example of a natural product with pharmaceutical application produced by a marine actinobacteria. Examples of additional bioactive secondary metabolites produced by marine actinobacteria isolated from sediments, corals and sponges are described in Table 1. While many of these compounds fall under the polyketide antibiotics class, a wide range of chemical structures, including macrolactams, peptides and alkaloids, have also been reported to be associated with cytotoxic, antiviral, and antifungal activities.

Table 1 – Examples of new natural products produced by marine actinobacteria isolated from sediments, corals and sponges, since 2018

Source	Compound	Chemical class	Bioactivity	Microorganism	Reference
Sediments	2-Epi-anthracimycin	Polyketide	Cytotoxic activity	*Streptomyces* sp.	(Fukuda et al., 2020)
	Ala-geninthiocin	Thiopeptide	Antibacterial and cytotoxic activity	*Streptomyces* sp.	(Iniyan et al., 2019)
	Ashimides A, B	Cyclopeptides	Cytotoxic activity	*Streptomyces* sp.	(Shi et al., 2019)
	Cebulantin	Lanthipeptide	Antibacterial activity	*Saccharopolyspora cebuensis*	(Moon et al., 2019)
	Dionemycin	Alkaloid	Antibacterial and cytotoxic activity	*Streptomyces* sp.	(Song et al., 2020a)
	Fluvirucin B6	Macrolactam	Antibacterial activity	*Nocardiospis* sp.	(Leutou et al., 2018)
	Hexaricins	Polyketide	Antioxidant activity	*Streptosporangium* sp.	(Gao et al., 2018)
	Lakyricidins A–D	Piericidin	Antiproliferative activity	*Streptomyces iakyrus*	(Li et al., 2019)
	Isomethoxyneihumicin	Diketopiperazine	Cytotoxic activity	*Nocardiopsis alba*	(Fukuda et al., 2017)
	Kendomycins B–D	Polyketide	Antibacterial and cytotoxic activity	*Verrucosispora* sp.	(Zhang et al., 2019)
	Litoralimycins A, B	Thiopeptide	Cytotoxic activity	*Streptomonospora* sp.	(Khodamoradi et al., 2020)
	Merochlorins E, F Meroindenon	Meroterpenoids	Antibacterial activity	*Streptomyces* sp.	(Ryu et al., 2019)
	Neoabyssomicins	Polyketide	Antibacterial, antiviral	*Streptomyces koyangensis*	(Huang et al., 2018)
	Neothioviridamide	Polythioamide	Cytotoxic activity	*Streptomyces* sp.	(Kawahara et al., 2018)
	Nivelactam B	Macrolactam	Cytotoxic and antifungal activity	*Streptomyces* sp.	(Chen et al., 2018a)
	Nocarterphenyls A–C	p-Terphenyls	Cytotoxic activity	*Nocardiopsis* sp.	(Wang et al., 2019a)
	Phocoenamicins	Spirotetronate	Antibacterial and antituberculosis activity	*Micromonospora* sp.	(Pérez-Bonilla et al., 2018)
	Rakicidins G – I	Depsipeptide	Antibacterial and cytotoxic activity	*Micromonospora chalcea*	(Chen et al., 2018b)
	Saccharoquinoline	Alkaloid	Cytotoxic activity	*Saccharomonospora* sp.	(Le et al., 2019)
	Salinaphthoquinones A-E	Macrolide	Antibacterial activity	*Salinispora arenicola*	(da Silva et al., 2019)
	Taromycin A, B	Lipopeptide	Antibacterial activity	*Saccharomonospora* sp.	(Reynolds et al., 2018)

Corals	Anthracimycin B	Polyketide	Antibacterial activity	*Streptomyces cyaneofuscatus*	(Rodríguez et al., 2018)
	Aranciamycin K	Anthracycline	Cytotoxic activity	*Streptomyces* sp.	(Cong et al., 2019)
	Iseolides A–C	Macrolide	Antifungal activity	*Streptomyces* sp.	(Zhang et al., 2020c)
	Isotirandamycin B	Polyketide	Antibacterial activity	*Streptomyces* sp.	(Cong et al., 2019)
	Nesteretal A	Polyketide	Anticancer activity	*Nesterenkonia halobia*	(Xie et al., 2019)
	Nocarimidazoles C, D	Alkanoylimidazole	Antimicrobial activity	*Kocuria* sp.	(Karim et al., 2020)
Sponges	Actinozine A	Diketopiperazine	Antimicrobia activity l	*Streptomyces* sp.	(Shaala et al., 2019)
	Fridamycins H, I	Hydroxyquinones	Anti-parasitic activity	*Actinokineospora spheciospongiae*	(Tawfike et al., 2019)
	Heliomycin	Anthraquinone	Anticancer activity	*Streptomyces* sp.	(Abdelfattah et al., 2018)
	Madurastatin D1, D2	Siderophore	Antimicrobial activity	*Actinomadura* sp.	(Yan et al., 2019)
	Nocardiopsistins A-C	Polyketide	Antibacterial activity	*Nocardiosis* sp.	(Xu et al., 2018b)
	Nocardiotide A	Peptide	Cytotoxic activity	*Nocardiopsis* sp.	(Ibrahim et al., 2018)
	Yangpumicins F, G	Enediyne	Antibacterial and cytotoxic activity	*Micromonospora yangpuensis*	(Wang et al., 2019b)

1.5. Deep-sea actinobacteria

Oceans cover 70% of the Earth's surface and most of this area is below 1000 m deep. The deep-sea is divided into three zones: (i) bathyal – with depths between 200 and 2000 m; (ii) abyssal – with depths between 2000 and 6000 m; and (iii) hadal – with depths below 6000 m (Harino et al., 2009). Under sea level, pressure increases with depth, with values reaching more than 1000 atm in the deepest areas, and temperature, oxygen level and light intensity decrease exponentially. Thus, life in the deep-sea must have morphological and biochemical processes adapted to survive under these conditions (Thistle, 2003). Metagenomics studies on deep-sea microbial diversity have shown that the deep-sea is home to a large number of novel species that have not yet been isolated under laboratory conditions, which makes deep-sea environments an attractive source of novel microorganisms and, consequently, of novel secondary metabolites (Kamjam et al., 2017; Thistle, 2003). This is also true for actinobacteria, with several studies showing a high diversity of these bacteria in deep sea environments (Chen et al., 2016; Ribeiro et al., 2023; Salazar et al., 2016) and the occurrence of novel phylogenies (Table 2). However, comparing to other environments, the diversity of deep-sea actinobacteria continues to be largely underexplored. This is mainly due to the technological barriers associated with collection of deep-sea samples and microbial cultivation under laboratory conditions. In the last years, we have witnessed some advances in deep-sea sampling, with new technical devices emerging in the market, such as multi-core samplers, untethered coring devices, remotely operated underwater vehicles (ROV) and manned submarine vehicle (Fenical et al., 2006; Pathom-Aree et al., 2006c; Prieto-Davó et al., 2013). On the other hand, isolation strategies to cultivate deep-sea Actinobacteria need to be refined to mimic the conditions found at deep-sea environments and promote the recovery of novel phylogenies. Therefore, it is important to consider oligotrophic culture media and growth at low temperatures, as these are natural characteristics of this ecosystem (Kamjam et al., 2017).

In the deep-sea, actinobacteria can inhabit a variety of ecological niches, being also found as symbionts of various invertebrates, like corals and sponges, and of other marine organisms. According to the literature, these microorganisms are more predominant in deep-sea sediments and their diversity seems to increase with depth, being greater at depths higher than 1000 m (Chen et al., 2016; Fan et al., 2022; Kamjam et al., 2018). In addition, actinobacteria play a key role in deep-sea environments as ecosystem engineers, contributing to several biogeochemical cycles (Jagannathan et al., 2021; Siro

et al., 2022) in the water column, ocean floor and hydrothermal vents, having also an important role in the degradation of complex molecules.

Table 2 – New species of deep-sea actinobacteria reported between 2018 and 2023.

Species	Family	Source	Geographic localization	Depth	Reference
Actinomarinicola tropica SCSIO 58843^T (=KCTC 49408^T=CGMCC 1.17503^T)	*Iamiaceae*	Deep-sea sediment	South China Sea	460 m	(He et al., 2020)
Brevibacterium profundi O1^T (=JCM 33845^T=MCCC 1A16744^T)	*Brevibacteriaceae*	Deep-sea sediment	Western Pacific Ocean	7068 m	(Pei et al., 2020)
Chryseoglobus indicus CTD02-10-2^T (=JCM 33842^T=MCCC 1A16619^T)	*Microbacteriaceae*	Deep-sea water	Indian Ocean	3456 m	(Pei et al., 2021)
Microcella flavibacter WY83^T (=KCTC 39637^T=MCCC 1A07099^T)	*Microbacteriaceae*	Deep-sea sediment	Indian Ocean	3039 m	(Xie et al., 2021a)
Micromonospora ferrugínea 28ISP2-46^T (=NCTC 14469^T=DSMZ 111791^T)	*Micromonosporaceae*	Deep-sea sponge (unidentified)	Atlantic Ocean	971 m	(Back et al., 2021)
Micromonospora pelagivivens KJ-029^T (=NBRC 113519^T=TBRC 9233^T)	*Micromonosporaceae*	Deep-sea sediment	Kagoshima, Japan.	260 m	(Intra et al., 2020)
Micromonospora provocatoris MT25^T (=NCIMB 15245^T=TISTR 2834^T)	*Micromonosporaceae*	Deep-sea sediment	Mariana Trench	10898 m	(Abdel-Mageed et al., 2021)
Miltoncostaea marina SCSIO 60955^T (DSM 110281^T= CGMCC 1.18757^T) and *Miltoncostaea oceani* SCSIO 61214^T (=KCTC 49527^T=CGMCC 1.18758^T)	*Miltoncostaeaceae*	Deep-sea sediment	South China Sea	460 m	(Li et al., 2021)
Mycetocola spongiae MSC19^T (=MCCC 1K06265^T=KCTC 49701^T)	*Microbacteriaceae*	Deep-sea sponge, *Cacospongia mycofijiensis*	Mariana Trench	2681 m	(Chen et al., 2022c)
Nesterenkonia sedimenti MY13^T (=LMG 28111^T=MCCC 1A09979^T=JCM 19767^T=CGMCC 1.12784^T)	*Micrococcaceae*	Deep-sea sediment	Western Pacific Ocean	4302 m	(Xie et al., 2021b)

Nesterenkonia salmonea GY074^T (=KCTC 39639^T=MCCC 1A11256^T) and *Nesterenkonia sphaerica* GY239^T (=KCTC 39640^T=MCCC 1A10688^T)	*Micrococcaceae*	Deep-sea sediment	Southern Atlantic Ocean	3223 m	(Zhang et al., 2020a)
Pseudonocardia abyssalis KRD168^T (=DSM 111918^T=NCIMB 15270^T) and *Pseudonocardia oceani* KRD185^T (=DSM 111919^T=CIMB 15269^T)	*Pseudonocardiaceae*	Deep-sea sediment	Antarctic Ocean	4539 m and 4060 m	(Parra et al., 2021)
Rubrobacter indicoceani SCSIO 08198^T (=DSM 105148^T=CGMCC 1.16398^T)	*Rubrobacteraceae*	Deep-sea sediment	Indian Ocean	4602 m	(Chen et al., 2018c)
Rubrobacter tropicus SCSIO 52909^T (=KCTC 49412^T=CGMCC 1.13853^T) and *Rubrobacter marinus* SCSIO 52915^T (=KCTC 49411^T=CGMCC 1.13852^T)	*Rubrobacteraceae*	Deep-sea sediment	South China Sea	3448 m	(Chen et al., 2020)
Streptomyces bathyalis ASO4wet^T (=DSM 106605^T=NCCB 100657^T)	*Streptomycetaceae*	Deep-sea sponge (unidentified)	Madeira island, North Atlantic Ocean	1092 m	(Risdian et al., 2021)
Streptomyces spiramenti 5675061^T (=LMG 31896^T=DSM 111793^T)	*Streptomycetaceae*	Deep-sea hydrothermal vents	Axial Seamount caldera on the Juan de Fuca Ridge (NE Pacific Ocean)	2190 m	(Loughran et al., 2022)

1.6. Natural products from deep-sea Actinobacteria

Actinobacteria isolated from deep-sea environments have also proven to be a source of natural products, many of them with new chemical structures. In fact, the number of novel actinobacterial metabolites isolated from deep-sea environments, mainly from deep-sea sediments, has been increasing in the literature (Kamjam et al., 2017; Puttaswamygowda et al., 2019; Subramani et al., 2019). Examples of these natural products include: Dermacozines A-G, a new phezanine family produced by *Dermacoccus abyssi*, with moderate cytotoxic activity against the leukemia cell line K562 (Abdel-Mageed et al., 2010); Microbacterins A-B, two new peptaibols isolated from *Microbacterium sediminis*, presenting significant inhibitory effect against a panel of human cancer cells, including human hepatoma cell line and human gastric cancer cell line (Liu et al., 2015); Pseudonocardians A-C, three new diazaanthraquinone derivatives with anticancer and antimicrobial activity, recovered from a *Pseudonocardia* strain (Li et al., 2011); Diketopirazines, Ammosamides A-B and Dehydroxyaquayamycin, secondary metabolites with antifouling, anticancer and antibacterial activity, respectively (Gaudêncio et al., 2008; Li et al., 2006; Song et al., 2015). Additional examples are indicated in Table 3. New compounds from actinobacteria are waiting to be discovered as research in this environment expands.

Table 3 – New natural products produced by deep-sea actinobacteria in the period between 2018 and 2023

Compound	Class	Microorganism	Geographic localization	Depth	Bioactivity	Reference
3-hydroxyquinaldic acid derivates 1-3	Chromodepsipeptides	*Streptomyces cyaneofuscatus* M-157	Cantabrian Sea	2000 m	Weak cytotoxic activity	(Ortiz-López et al., 2018)
3R-OH-1,6-diene-cyclohexylacetic acid, linear tetradepsipeptide, N1,N5-di-p-(EE)-coumaroyl-N10-acetylspermidine and furan fatty acid	Cyclohexylacetic acid, depsipeptide, spermidine, and fatty acid	*Agrococcus* sp. SCSIO 52902	South China Sea	2061 m	No activity	(Ding et al., 2022)
Aclidinomycins C–K	Tetrahydroisoquinolines	*Streptomyces niveus* SCSIO 3406	South China Sea	3536 m	Antibacterial activity	(Yang et al., 2021)
Actinopyrones E-G	Pyrone polyketide	*Streptomyces* sp. SCSIO ZS0520	Okinawa Trough	1039 m	No activity	(Zhang et al., 2022b)
Albisporachelin	Hydroxamatet-ype siderophore	*Mycolatopsis albisporachelin* WP1T	Indian Ocean	2945 m	No activity	(Wu et al., 2018)
Amycolachromes A-F	Chromone derivatives	*Amycolatopsis* sp. WP1	Southwest Indian Ocean	2945 m	No activity	(Chen et al., 2022a)
Dermacozine N	Phenazine	*Dermacoccus abyssi* MT1.1	Mariana Trench	10898 m	No activity	(Juhasz et al., 2021)
Dionemycin	*bis*-indole alkaloids	*Streptomyces* sp. SCSIO 11791	South China Sea	1765 m	Antibacterial and cytotoxic activity	(Song et al., 2020b)
Filipins VI-XIV	Polyene macrolides	*Streptomyces antibioticus* OUCT16-23	Indian Ocean	4495 m	Antifungal activity	(Bao et al., 2022)
Georgenione A	Piperazinedion	*Georgenia* sp. 40DY18	Pacific Ocean	5591 m	Anti-tyrosinase activity	(Chen et al., 2022b)
Glaciapyrrole D-E	Pyrrolosesquiterpene	*Streptomyces* sp. GGS5	East Sea of Kore	2163 m	Antiviral activit	(Ko et al., 2022)
Grincamycin L	Angucycline	*Streptomyces lusitanus* OUCT16-27	Indian Ocean	4495 m	Antibacterial activity	(Yang et al., 2020)
Kumemicinones A–G	Angucyclinones	*Actinomadura* sp. KD439	Kumejima Island, Okinawa, Japan	612 m	Cytotoxic activity	(Zhang et al., 2022c)
Lobophorin L-M	Spirotetronate	*Streptomyces* sp. 4506	South China Sea	3565 m	Antibacterial activity	(Luo et al., 2021)

Nenestatin B	Benzofluorene-containing angucyclines	*Micromonospora echinospora* SCSIO 04089	Norrn South China Sea	3025 m	No activity	(Jiang et al., 2021)
Nocardiopsistin A-C	Angucyclines	*Nocardiopsis* sp. HB-J378;	Not designed	Not designed	Antimicrobial activity	(Xu et al., 2018b)
Olimycins A and B	Naphthoquinone macrolides	*Streptomyces olivaceus* SCSIO T05	Indian Ocean	4617 m	No activity	(Sun et al., 2018)
Rausuquinone	Pluramycin-class polyketid	*Rhodococcus* sp. RD01514	Not designed	Not designed	Antimicrobial activity	(Harunari et al., 2022)
Saccharopolytide A	Cyclic tetrapeptide	*Saccharopolyspora cebuensis* MCCC 1A09850	Atlantic Ocean	2875 m	weak anti-allergic and anti-proliferate activity	(Xie et al., 2018)
Sealutomicins A	Enediyne	*Nonomuraea* sp. MM565M-173N2	Japan Trench	329 m	Antibacterial activity	(Igarashi et al., 2021)
Seco-salinomycin A-E	Salinomycin and α-pyron	*Streptomyces* sp. SCSIO ZS0520	Okinawa Trough	1039 m	No activity	(Zhang et al., 2022a)
Somamycin A	Polycyclic tetramate macrolactams	*Streptomyces somaliensis* SCSIO ZH66	South China Sea	3536 m	Antifungal activity	(Hou et al., 2020)
Streptolactams A–C	Macrolactams	*Streptomyces* sp. OUCMDZ-3159	South Mid-Atlantic Ridge	2782 m	Streptolactam A and C display antifungal activity	(Wang et al., 2020b)
Streptonaphthyridine A	Naphthyridine	*Streptomyces* sp. SY2111	Mariana Trench	11000	Antiproliferative activity	(Qin et al., 2023)
Streptothiazolidine A, Streptodiketopiperazines A-B, and (S)-1-(3-ethylphenyl)-1, 2-ethanediol	Salicylamide, diketopiperazine, and phenylethanediol	*Streptomyces* sp. SY1965	Mariana Trench	11000 m	Antifungal activity	(Yi et al., 2020)
β, γ-butenoate derivative phenylbutenote and α-pyrone nocapyrone T	β,γ-butanoate and α-pyrone	*Nocardiopsis* sp. HDN 17-237	Mariana Trench	4448 m	No activity	(Wang et al., 2020a)

1.7. Best approaches to isolate actinobacteria from marine environments

Marine actinobacteria require different growth conditions when compared to their terrestrial counterparts (Claverias et al., 2015; Hames-Kocabas et al., 2012; Zotchev, 2012). A high number of bacteria cannot be cultivated through conventional isolation methods (Overmann et al., 2016). This has been clearly shown by culture-independent studies, and because of the low success of culture-dependent approaches, molecular techniques are being increasingly used to study the diversity and function of microbial communities in environments (Jung et al., 2021; Subramani et al., 2019). These techniques are allowing the discovery of many functional characteristics of actinobacteria and are contributing to the improvement of isolation strategies, growth conditions and culture media formulation to recover previously uncultivable actinobacteria (Kaeberlein et al., 2002; Stewart, 2012; Subramani et al., 2019).

The cultivation and isolation of marine actinobacteria are key steps for bioprospection purposes and for the discovery of novel natural products (Sekurova et al., 2019). To maximize the success in cultivating these microorganisms, it is important to know the environmental factors that can influence their isolation, such as pH, temperature, oxygen, availability of nutrients, among others (Jiang et al., 2016). Since actinobacteria from marine habitats are often salt-dependent, culture media containing different concentrations of seawater, artificial seawater or deionized water supplemented with marine salts have been successfully employed for their cultivation (Hames-Kocabas et al., 2012; Maldonado et al., 2005b). Different carbon (chitin, dextrose, glucose, glycerol, maltose, mannitol, soluble starch and oatmeal) and nitrogen sources (casein, histidine, L-argine, malt extract, meat extract, peptone, tryptone and yeast extract) are widely used as components of selective media for the isolation of marine actinobacteria (Axenov-Gribanov et al., 2020; Ribeiro et al., 2020; Shamikh et al., 2020; Sharma et al., 2021). Extracts of sediments or marine organisms, like sponges, and seawater have been used to mimic the natural conditions present in the marine environment (Jensen et al., 2005; Maldonado et al., 2005b; Rego et al., 2019). In addition, due to the natural oligotrophy of marine environments, many microorganisms only grow under low nutrient conditions (Hames-Kocabas et al., 2012; Jensen et al., 2005).

Several pre-treatments can be applied to the samples for selecting slow-growing marine Actinobacteria, including physical (dry heat, desiccation, freezing and radiation) (Bredholt et al., 2008; Jensen et al., 2005; Mincer et al., 2002; Naikpatil et al., 2011), mechanical (gradient centrifugation and shake with glass beads) (Bredholt et al., 2008;

Maldonado et al., 2005a) and chemical (chemical compounds and antibiotics) (Bredholt et al., 2008; Ribeiro et al., 2020). Desiccation and heating can be used for selecting sporulating actinobacteria and inhibiting other Gram-positive bacteria (Fenical et al., 2006). Treatment with several chemical compounds, such as benzethonium chloride, chloramine-T, phenol and sodium dodecyl sulfate, have been shown to be very successful in selecting marine actinobacteria (Bredholt et al., 2008; Hong et al., 2009). Furthermore, the addition of antibacterial and antifungal antibiotics, such as cycloheximide, gentamicin, kanamycin, nalidixic acid, novobiocin, nystatin, penicillin, rifampicin, streptomycin, tunicamycin and vancomycin, to the culture medium has been also shown very relevant for selecting different genera of marine actinobacteria (Amin et al., 2020; Bredholt et al., 2008; Claverias et al., 2015; Hong et al., 2009).

In terms of isolation of deep-sea actinobacteria, besides special nutritional requirements, like the presence of salts or oligotrophic conditions, there may also be physical constraints, such as the requirement for low temperatures and high pressure (Manivasagan et al., 2013a). Some studies reported that a long cultivation time and a low incubation temperature were productive in the isolation of deep-sea actinobacteria (Bhadra et al., 2008; Kamjam et al., 2017; Pathom-Aree et al., 2006b).

In general, the process of isolating piezophilic microorganisms involves a combination of conventional methods and high-pressure techniques. The use of pressure vessels to maintain *in situ* pressure during the isolation and cultivation process is one of the techniques developed. However, as these equipments are very expensive, it is more common to apply pressure conditions only after the microorganism has been isolated and test after its ability to grow under pressure conditions (Zhang et al., 2018). For example, the piezotolerant actinobacteria obtained from Mariana Trench sediment, *Dermacoccus abyssi* strain MT1.1^{T}, was isolated under traditional conditions (at atmospheric pressure), but was found to grow better under pressure conditions (up to 40 MPa) (Pathom-Aree et al., 2006a). In addition, several studies do not apply pressure conditions both during the isolation and cultivation of deep-sea microorganisms, essentially targeting piezotolerant microorganisms (Ribeiro et al., 2023; Xu et al., 2018a; Zhang et al., 2018)

1.8. Aim and outline of this thesis

The quest for new drugs to treat diseases that lack effective treatments is a pressing issue. In this regard, natural products derived from marine actinobacteria have been explored to address the urgent need for novel drugs. Deep-sea environments, in particular, are considered one of the last frontiers for the bioprospection of bioactive molecules.

This thesis aims to investigate and explore actinobacteria inhabiting deep-sea environments of Artic and Atlantic oceans in terms of their phylogenies and potential to produce novel bioactive metabolites with biotechnological applications. The success in finding novel pharmaceuticals is highly conditioned by this type of research. In this context, the specific objectives of this thesis are:

1. Isolation and identification of actinobacteria from underexplored deep-sea environments;

2. Investigation of the capacity of isolated actinobacteria to produce bioactive compounds with antimicrobial, anticancer, anti-inflammatory and anti-obesity activities;

3. Chemical isolation and characterization of novel bioactive metabolites produced by deep-sea actinobacteria;

4. Characterization of novel actinobacterial phylogenies obtained from deep-sea environments.

In this context, the present thesis is organized in 6 chapters. Chapter 1 consists in a general introduction to the theme of the thesis, where several aspects related with actinobacteria are described, mainly focusing in marine and deep-sea actinobacteria, including their environmental distribution, novel species of deep-sea actinobacteria, best approaches for their isolation/cultivation and main metabolites produced with biotechnological applications. Chapter 2 describes a study on the actinobacterial diversity of deep-sea sediments from the Arctic (Arctic-Mid-Ocean Ridge) and Atlantic (Azores and Madeira) oceans, by simultaneously employing culture-dependent and -independent methods, and also evaluated the bioactive potential of the actinobacterial strains isolated from those samples. Chapter 3 focus on the characterization of a novel deep-sea *Streptomyces* species, isolated in the study described in Chapter 2, using a polyphasic approach that combined morphological, biochemical, chemotaxonomic and genomic analysis, having in mind the taxonomic validation of this new species. Chapter 4 comprises a study that explored the biodiversity of cultivable actinobacteria from 66

deep-sea samples collected in the Portuguese archipelagos of Madeira and Azores (depths between 150 and 3000 m) and their capacity to produce new molecules with antimicrobial, anticancer, anti-inflammatory and anti-obesity activities. Chapter 5 focus on the metabolomic profile of *Streptomyces chumphonensis* strain C_003 1.18, isolated in the study presented in Chapter 4. Finally, Chapter 6 consists in a general discussion and main conclusions of the work presented in this thesis.

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CHAPTER 2

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Actinobacteria from Arctic and Atlantic deep-sea sediments – biodiversity and bioactive potential

Ribeiro I†, Antunes JT†, Alexandrino DAM, Tomasino MP, Almeida E, Hilário A, Urbatzka R, Leão PN, Mucha AP, Carvalho MF. (2023). Actinobacteria from Arctic and Atlantic deep-sea sediments-Biodiversity and bioactive potential. *Frontiers in Microbiology*, 14. Doi:10.3389/fmicb.2023.1158441

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2.1. Abstract

The deep-sea covers over 70% of the Earth's surface and harbours predominantly uncharacterized bacterial communities. Actinobacteria are the major prokaryotic source of bioactive natural products that find their way into drug discovery programs, and the deep-sea is a promising source of biotechnologically relevant actinobacteria. Previous studies on actinobacteria in deep-sea sediments were either regionally restricted or did not combine a community characterization with the analysis of their bioactive potential. Here we characterized the actinobacterial communities of upper layers of deep-sea sediments from the Arctic and the Atlantic (Azores and Madeira) ocean basins, employing 16S rRNA metabarcoding, and studied the biosynthetic potential of cultivable actinobacteria retrieved from those samples. Metabarcoding analysis showed that the actinobacterial composition varied between the sampled regions, with higher abundance in the Arctic samples but higher diversity in the Atlantic ones. Twenty actinobacterial genera were detected using metabarcoding, as a culture-independent method, while culture-dependent methods only allowed the identification of nine genera. Isolation of actinobacteria resulted on the retrieval of 44 isolates, mainly associated with *Brachybacterium*, *Microbacterium* and *Brevibacterium* genera. Some of these isolates were only identified on a specific sampled region. Chemical extracts of the actinobacterial isolates were subsequently screened for their antimicrobial, anticancer and anti-inflammatory activities. Extracts from two *Streptomyces* strains demonstrated activity against *Candida albicans*. Additionally, eight extracts (obtained from *Brachybacterium*, *Brevibacterium*, *Microbacterium*, *Rhodococcus* and *Streptomyces* isolates) showed significant activity against at least one of the tested cancer cell lines (HepG2 and T-47D). Furthermore, fifteen actinobacterial extracts showed anti-inflammatory potential in the RAW 264.4 cell model assay, with no concomitant cytotoxic response. Dereplication and molecular networking analysis of the bioactive actinobacterial extracts showed the presence of some metabolites associated with known natural products, but one of the analysed clusters did not show any match with the natural products described as responsible for these bioactivities. Overall, we were able to recover taxonomically diverse actinobacteria with different bioactivities from the studied deep-sea samples. The conjugation of culture-dependent and -independent methods allows a better understanding of the actinobacterial diversity of deep-sea environments, which is important for the optimization of approaches to obtain novel chemically-rich isolates.

2.2. Introduction

The deep-sea is one of the most extensive habitat on earth, harbouring highly diverse bacterial communities (Paulus, 2021). Deep-sea sediments are characterized by a variety of geophysical parameters that affect the associated microbial life. To face deep-sea conditions, microorganisms had to develop adaptation strategies, including the evolution of unique metabolic processes, to survive in this, often extreme, environment (Kamjam et al., 2017; Kerou et al., 2021). Due to the fact that the deep-sea is very vast and of difficult access, their bacterial communities remain broadly unfamiliar (Chen et al., 2016; Roman et al., 2019). Among the prokaryotic groups present in marine sediments, actinobacteria are often reported as one of the most predominant (Chen et al., 2016; Jose & Jha, 2017; Pathom-Aree et al., 2006). These microorganisms have also been shown to have high diversity in deep-sea sediments, of which a great share is predicted to be associated to novel species and genera (Bull et al., 2005).

Marine actinobacteria have revealed to have unique metabolic and physiological capabilities, translating into a high potential for the biosynthesis of novel metabolites (Yang & Song, 2018; Yang et al., 2020). Compounds synthesized by these microorganisms were shown to exhibit various bioactivities, such as antibacterial (Bull et al., 2005), antifungal, (Qi et al., 2019) anticancer (Silva et al., 2020), antioxidant (Dholakiya et al., 2017; Kamjam et al., 2017), among others.

Until now, the diversity of actinobacteria associated with marine sediments, including deep-sea sediments, has been mostly investigated by culture-dependent methods. Limited efforts have been spent on the study of actinobacterial community composition of such habitats by conjointly employing culture-independent methods, such as high-throughput sequencing approaches (Chen et al., 2016; Jose & Jha, 2017), which is essential to accurately elucidate the diversity of these microorganisms. Previous culture-independent studies of deep-sea sediments from unexplored regions, such as the Arctic, have already demonstrated a prevalence of actinobacteria in these samples (Fang et al., 2019), however few studies have focused on the concomitant actinobacterial isolation and analysis of their bioactive potential. On the other hand, the actinobacterial diversity associated with deep-sea sediments of the Atlantic Macaronesia region is scarcely explored by culture independent-methods, although actinobacteria isolated from deep-sea sediments have already shown interesting bioactive potential (Chen et al., 2016; Prieto-Davó et al., 2016; Siro et al., 2023).

In this context, we combined culture-dependent and -independent methods to investigate the actinobacterial diversity of deep-sea sediments from two underexplored regions: the Macaronesia region of the Atlantic Ocean, within the Portuguese Continental Shelf, which

includes, among others, the archipelagos of Azores and Madeira, and the Mohn's Treasure region, an inactive sulfide mound in the Arctic Mid-Ocean Ridge. In addition, the actinobacteria isolated from these samples were investigated in terms of their bioactivity, by screening their antimicrobial, anticancer and anti-inflammatory potential. To the best of our knowledge, this is the first study of actinobacteria diversity of deep-sea sediments from two distinct oceanic regions, coupling culture-dependent and -independent approaches with investigation of the respective bioactive potential.

2.3. Material and methods

2.3.1. Sampling

Deep-sea sediment samples were obtained from two geographical locations, Atlantic and Arctic, and were acquired from the upper layers of different bathyal zones (Table 4) using a remotely operated vehicle (ROV). The Atlantic samples were obtained from two regions of the Portuguese Continental Shelf (Azores and Madeira). For the Azores, samples were collected between 1072 m and 1167 m depth, in September 2016, during the oceanographic campaign EMEPC\PEPC\Luso\2016 in the Mid-Atlantic Ridge, North Atlantic Ocean. Sampling was carried out by the ROV 'LUSO' of the Portuguese Task Group for the Extension of the Continental Shelf (EMEPC), which collected three sediment samples (CMA_L1, CMA_L2 and CMA_L4) with a mini-corer (upper 5 cm depth) and one composite sediment through suction from different points on the sediment surface (CMA_L3). For Madeira, samples (M_E147 and M_MA3) were collected between 2300 m and 3199 m depth, using a box-corer, during the mission SEDMAR 1/2017 carried out by the Portuguese Hydrographic Institute in June 2017. The Arctic samples were obtained from Mohn' Treasure, an inactive sulfide mound on the Arctic Mid-Ocean Ridge that is covered by a thick layer of fine sediments (Ramirez-Llodra *et al.*, 2020). Sampling was conducted in the framework of the MarMine campaign, in the summer of 2016. Six samples (A_136, A_78, A_79, A_80, A_81, A_82) of the top sediment layer were collected with push corers using a Triton work-class ROV at depths between 2682 and 2826 m.

For each site, two sediment samples were collected and stored under different conditions. One was stored at -80 °C for genomic analysis and the other was preserved with LifeGuard™ Soil Preservation Solution (MO BIO Laboratories; Carlsbad, CA USA), under refrigerated conditions, for bioprospection of deep-sea actinobacteria.

Table 4 – Site description of the deep-sea sediment samples used in this study

Site	Site description	Sample Code	Sample property	Depth (m)	Latitude	Longitude
Atlantic - Azores	Portuguese Continental Platform	CMA_L1	Sediment corer	1167	33.9175	-37.5053
		CMA_L2	Sediment corer	1072	33.9230	-37.5103
		CMA_L3	Composite sediment	1065 to 1073	33.92297 start 33.91748 end	−37.5103 start −37.5053 end
		CMA_L4	Sediment corer	1167	33.9175	-37.5054
Atlantic - Madeira	Portuguese Continental Platform	M_E147	Box-corer	3199	30.2208	-16.1032
		M_MA3	Box-corer	2300	32.5218	-16.9683
Arctic	Mohn's Treasure Ridge	A_78	Layer 1-3 cm	2682	81.56960	63.3248
		A_79	Layer 1-3 cm	2682	81.56960	63.3248
		A_80	Layer 1-3 cm	2822	81.56960	63.3248
		A_81	Layer 1- 3 cm	2683	81.56913	63.3277
		A_82	Layer 10-15 cm	2683	81.56915	63.3283
		A_136	Layer 0-1 cm	2826	81.56505	63.3945

2.3.2. DNA library preparation and amplicon sequencing

DNA of the sediment samples was extracted from 0.5 g of sediment using the Power Soil DNA Isolation Kit (MO BIO Laboratories; Carlsbad, CA USA), following the manufacturer's instructions. The extracted DNA samples were quantified and sent for sequencing at Genoinseq (Cantanhede, Portugal) company. The hypervariable V4-V5 regions of the 16S rRNA gene were amplified using the universal primers 515YF (5-GTGYCAGCMGCCGCGGTAA-3) and Y926R (5-CCGYCAATTYMTTTRAGTTT-3), developed by Parada et al. (2016). Pair-end sequencing with MiSeq® V3 chemistry was performed according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Raw reads extracted from Illumina MiSeq ® System were demultiplexed and pre-processed. Sequences were quality-filtered with PRINSEQ software and merged by using the AdapterRemoval v2.1.5 software (Schubert et al., 2016) with default parameters. Full description of the protocol is reported in (Bragança et al., 2019). Raw Illumina fastq files obtained in this study were deposited in the European Nucleotide Archive (ENA) database under the accession number PRJEB59507.

2.3.3. Bioinformatics analysis

Raw reads provided by the Genoinseq sequencing company were then processed in the present work by Cutadapt plugin for primer removal (Martin, 2011). Later, amplicon sequences were submitted to the open-source software Quantitative Insights into Microbial Ecology (QIIME 2; v.2019.7; (Bolyen et al., 2019) for the upstream analysis. Dada2 algorithm within

QIIME2 was used to merge paired-end reads (Callahan et al., 2016), denoise, dereplicate sequences, remove chimeras and identify Amplicon Sequence Variants (ASVs) with default settings (Callahan et al., 2016), providing as output a ASV abundance table.
Taxonomy assignation of the ASV representative sequences was carried out against SILVA v 132 database (Quast et al., 2012) trained with a naïve Bayes classifier against the same the V4-V5 region of 16S rRNA gene target for sequencing. ASV assigned to non-target sequences (e.g “Eukarya”, Choloroplasts”, “Mitochondria”) and not classified at Kingdom level were removed and excluded from the analysis. Rarefaction curves were obtained through QIIME 2 core diversity metrics to exhibit the sequencing depth of samples. Taxonomy plots were created using the ggplot2´ package in R environment (version 3.2.2. Copyright 2015 The R Foundation for Statistical Computing).

2.3.4. Actinobacteria isolation and phylogenetic identification

For the isolation of actinobacteria from the deep-sea sediment samples, each sediment was initially shaken for 30 min at 150 rpm to homogenize the sample. One gram of each sediment sample was transferred to an eppendorf and suspended in 1 ml of sterile seawater. Pre-treatments were applied to all samples except for the Azores sediments (Table 2). For the Madeira samples the following pre-treatments were applied: (i) incubation in a water bath at 60 °C for 30 min (wet heat) (Terahara et al., 2013); (ii) incubation at 120 °C for 60 min (dry heat) (Bredholt et al., 2008) and (iii) incubation in a microwave at 120 W for 3 min (Bredholdt et al., 2007). The Arctic sediment samples were processed using both a wet heat pre-treatment and no pre-treatment. After applying the pre-treatment(s) (when applicable), samples were vortexed at maximum speed for 5 min, to dissociate the bacteria from the sediments and transfer them to the sterile seawater, after which ten-fold dilutions (until 10^{-4}) were prepared. An aliquot of 100 µl of each dilution was spread over the surface of different isolation media, according to the information indicated in Table 5. The media used were: Gauze's Synthetic Medium No. 1 (per litre, with a ratio of seawater: deionized water of 70:30): 20 g of soluble starch, 1 g of KNO_3, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 0.05 g of $FeSO_4.7H_20$ and 17 g of agar; Starch-casein-nitrate agar (SNC) (per litre, with a ratio of seawater: deionized water of 70:30): 10 g of soluble starch, 0.3 g of casein, 2 g of K_2HPO_4, 2 g of KNO_3, 2 g of NaCl, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_20$ and 17 g of agar; M1 agar (per litre of seawater): 10 g of soluble starch, 4 g of yeast extract, 2 g of peptone and 17 g of agar; Nutrient-poor sediment extract (NPS) (per litre of seawater): 100 mL of marine sediment extract (obtained by washing 900 mL of sediments with 500 mL of seawater) and 17 g of agar; and Raffinose-Histidine (RH) (per litre, with a ratio of seawater:

deionized water of 70:30): 10 g of raffinose, 1 g of L-histidine, 1 g of KH_2PO_4 , 0.5 g of $MgSO_4.7H_2O$, 0.01 g of $FeSO_4.7H_20$ and 17 g of agar.

All media were supplemented with cycloheximide (50 mg L^{-1}), nystatin (50 mg L^{-1}) and nalidixic acid (50 mg L^{-1}) to reduce the growth of fungi and Gram-negative bacteria. The plates were incubated for a period of up to 6 months at 28 °C. Along the incubation period, plates were regularly inspected and colonies with different morphological characteristics were picked and streaked on new agar plates until obtainment of pure colonies.

Table 5 – Pre-treatments and culture media used for the isolation of actinobacteria from the deep-sea sediments

Site	Site description	Sample Code	Pre-treatments	Culture media
Atlantic - Azores	Portuguese Continental Platform	CMA_L1	Not applied	Gauze, SCN and RH
		CMA_L4		
		CMA_L3		
		CMA_L4		
Atlantic - Madeira	Portuguese Continental Platform	M_E147	(i)Wet heat (ii)Dry heat (iii)Microwave	SCN, NPS and M1
		M_MA3		
Arctic	Mohn's Treasure Ridge	A_78	Not applied (i)Wet heat	SCN, NPS and M1
		A_79		
		A_80		
		A_81		
		A_82		
		A_136		

Biomass for cryopreservation and DNA extraction was obtained by growing each isolate in the corresponding isolation liquid medium, with the exception of those obtained from the NPS medium that were grown in marine broth (Laboratorios Conda, Madrid, Spain). Each pure isolate was cryopreserved at -80 ºC in 30% (v/v) glycerol (Passari et al., 2018). For DNA extraction, 1 mL of liquid culture was centrifuged for 5 min at 7000 g and the pellet was stored at -20 °C. The E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek, GA, USA) was used for DNA extraction, following the instructions of the manufacturer, with a few modification steps: (i) before starting the extraction protocol, samples were incubated at 95 °C for 10 min, followed by incubation on ice for 10 min; (ii) in the step of lysozyme addition, the samples were incubated at 37 °C for 30 min instead of 10 min as described in the protocol; (iii) in the optional step used for Gram-positive bacteria, two Zirconia beads (2.3 mm diameter) were added together with the glass beads and the samples were vortexed for 10 min; (iv) incubation with proteinase K was performed by addition of a concentrated stock (10 mg mL^{-1}) instead of the solution provided in the kit and was extended up to 2 h (v) the centrifugation speeds described in the kit protocol were increased in all steps from 10,000 g to 13,000. 16S rRNA gene was

amplified by Polymerase Chain Reaction (PCR) using the universal primers 1492R (5'-GGTTACCTTGTTACGACTT-3') and 27F (5'-GAGTTTGATCCTGGCTCAG-3'). The PCR mixture (total volume of 10 µL) contained 5 µL of Taq PCR Master Mix (Qiagen, Valencia, CA, USA), 1 µL of primer 27F (2 µM), 1 µL of primer 1492R (2 µM) and 3 µL of DNA template. PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 48°C for 90 s, 72 °C for 90 s and a final extension at 72 °C for 10 min. PCR products were separated on a 1.5% agarose gel containing SYBR Safe (ThermoFisher Scientific, NY, USA), at 150 V for 30 min. Purification and sequencing of the amplified fragments were performed at GenCore, i3S (Instituto de Investigação e Inovação em Saúde, Portugal). The sequences were analysed using the Geneious software package (version 11.1.4) and the resulting consensus sequences were compared against the databases: 16S ribosomal RNA (Bacteria and Archaea), nucleotide collection (nr/nt), both from NCBI BLAST, using the blastn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the 16S-based ID tool from EZBioCloud (https://www.ezbiocloud.net/). The 16S rRNA gene sequences of the isolated actinobacterial strains were deposited in GenBank® (NCBI, Maryland, USA) with the accession numbers indicated in Table S1 (Appendix I).

2.3.5. Preparation of actinobacterial crude extracts

For the preparation of crude extracts, the actinobacterial isolates cryopreserved at -80 °C were first grown in the respective agar medium in which they were isolated. A loopful of each isolate was used to prepare a pre-inoculum in falcon tubes containing 5 mL of the respective culture medium (with the same composition of the isolation medium, but without the addition of antibiotics), which was after used to inoculate 100 mL Erlenmeyer flasks containing 30 mL of culture medium. The flasks were incubated at 28 °C, 100 rpm, in the dark for 3-5 days (depending on their growth rate) in a rotatory incubator (Model 210, Comecta SA, Barcelona, Spain), after which 0.5 g of Amberlite® XAD16N resin (Sigma-Aldrich, MO, USA) was added to the cultures (to adsorb compounds produced by the actinobacteria and eventually released to the culture medium) and left to incubate for an additional period of 2-3 days (Ribeiro et al., 2020). The biomass and resin were centrifuged at 3600 g for 10 min, washed twice with dH2O and freeze-dried. The freeze-dried biomass and resin were then extracted with a 1:1 (v/v) mixture of acetone and methanol. The biomass was initially immersed in the mixture (30 mL) for 30 min with constant agitation, then centrifuged (4500 g for 10 min) and the liquid phase was collected in round bottom flasks after passage through a Whatman No1 filter paper. The process was repeated twice, and the extract was dried in a rotary evaporator and transferred to a vial. The organic extract was then dissolved in dimethyl sulfoxide (DMSO ≥ 99.9%, Sigma-

Aldrich, MO, USA) to obtain stock solutions with final concentrations of 10 mg L^{-1} and 1 mg L^{-1}.

2.3.6 Bioactivity assays

2.3.6.1 Antimicrobial Bioactivity Screening

All the actinobacterial extracts were tested for antimicrobial activity through the agar-based disk diffusion method. In this assay, five reference microorganisms were tested: two Gram-positive bacteria - *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 29213); two Gram-negative bacteria - *Escherichia coli* (ATCC 25922) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 25241); and one Yeast - *Candida albicans* (ATCC 10231). The bacterial strains were grown in Mueller-Hinton agar (MH) (Liofilchem, Roseto d. Abruzzi, Italy) and the yeast strain was grown in Sabouraud dextrose agar (SD) (Liofilchem, Roseto d. Abruzzi, Italy).

For each strain, an inoculum was prepared in the respective culture broth and the turbidity of the cultures was adjusted to 0.5 McFarland standard scale (OD_{625} = 0.08-0.13). These cultures were used to seed MH (for the bacterial strains) or SD (for the yeast strain) agar plates by uniformly streaking the plates with a swab. Sterile blank paper discs (6 mm in diameter; Oxoid Limited, Hampshire, UK) were placed on the top of the inoculated plates and loaded with 15 µL of each actinobacterial extract at a concentration of 1.0 mg L^{-1}. For the negative controls, 15 µL of molecular biology grade DMSO (Scharlab, Barcelona, Spain) were loaded on the blank paper discs, while for the positive controls the paper disks were loaded with 15 µL of enrofloxacin (1.0 mg L^{-1}; Sigma-Aldrich, MO, USA) for the bacterial strains and 15 µL of nystatin (1.0 mg L^{-1}; Sigma-Aldrich, MO, USA) for the yeast. Agar plates were incubated at 37 °C for 24 h, and the diameter of halos corresponding to a zone of growth inhibition was measured. Each extract was tested in two independent assays.

Minimum inhibitory concentration (MIC) of the bioactive crude extracts was also determined using *Candida albicans* as inoculum. For each extract, solutions were prepared at 1000 mg L^{-1} in SD broth. Two-fold dilutions in the same culture medium were performed to obtain extracts with concentrations ranging from 1000 to 0.5 mg L^{-1}. In 96-well plates, 50 µL of *C. albicans* inoculum (diluted 1:100) and 50 µL of each extract dilution were added to each well. The MIC values were established by spectrophotometry at 625 nm (model V-1200, VWR, PA, United States), after 18 h of incubation at 37 °C. The controls consisted of: (i) negative control - 100 µl of broth medium; (ii) positive control - 50 µl of yeast inoculum and 50 µl of broth medium; and (iii) solvent control - 50 µl of yeast inoculum and 50 µl of DMSO (DMSO ≥ 99.9%, Sigma-Aldrich, MO, USA). Assays were performed in triplicate and in two independent experiments.

2.3.6.2 Anticancer Bioactivity Screening

The screening of anticancer activity in the actinobacterial extracts was performed in two cancer cell lines: breast ductal carcinoma (T-47D) and liver hepatocellular carcinoma (HepG2), both from Sigma-Aldrich (St. Louis, MO, USA), as well as in a non-cancer cell line hCMEC/D3 (human brain capillary endothelial cells), kindly donated by Dr. P. O. Courad, (INSERM, France), which was used to access general toxicity. Dubelco's modified eagle medium (DMEM; Gibco, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 1% (v/v) penicillin/streptomycin (Biochrom, Berlin, Germany) at 100 IU/mL and 10 mg L^{-1}, respectively, and 0.1% (v/v) amphotericin (GE Healthcare, Little Chafont, UK) was used for the growth and maintenance of the cell lines. The cells were incubated at 37 °C in a humidified atmosphere containing 5% of CO_2 (Biosystem w/o Controller, Cimarec., Thermo Fischer Scientific, MA, USA). Cellular viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. The cells were seeded in 96-well plates at a density of $6.6x10^4$ cells mL^{-1}. After 24 h, the cells were exposed to the actinobacterial extracts at a final concentration of 15 µg/mL, during 48 h. After this period, the cells were incubated with MTT at a final concentration of 0.2 mg mL^{-1} (Sigma-Aldrich, MO, USA) for 3-4 h at 37 °C. The medium was then removed and 100 µL of DMSO were added at each well to dissolve formazan crystals. Absorbance was read at 570 nm on a multi-detection microplate reader (Synergy HT, Biotek, Bart Frederick Shahr, Germany). Staurosporine (at a final concentration of 15 µg mL^{-1}) and 0.5% DMSO (same concentration as actinobacterial extracts) were used as a positive and solvent controls, respectively. Cellular viability was calculated as a percentage relative to the solvent control. The extracts were tested in triplicate in each two independent assays (n=6).

2.3.6.3 Anti-inflammatory Bioactivity Screening

The mouse macrophage cell line (RAW264.7) was used for the study of anti-inflammatory activity. This cell line was kept at 37 °C in a 5% CO_2 incubator and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 1% (v/v) penicillin/streptomycin (Biochrom, Berlin, Germany) at 100 IU/mL and 10 mg L^{-1}, respectively, and 0.1% (v/v) amphotericin (GE Healthcare, Little Chafont, UK). For the seeding of the cells, confluent macrophage cells ($3.5x10^5$ cells) were added to 96 well plates and incubated at 37 °C for 24 h. In the next day, the medium was renewed, and the cells were exposed to the actinobacterial extracts at a final concentration of 15 µg mL^{-1}, for 24 h. In the anti-inflammatory assay, the cells were pre-treated with LPS

(lipopolysaccharide, Sigma-Aldrich) at a final concentration of 200 µg mL^{-1}, while in the pro-inflammation assay no LPS was added to the wells. Solvent control consisted of 0.5% DMSO and the positive control of LPS at 200 mg L^{-1}. Inflammation was analysed by Nitric Oxide (NO) level using the Griess reaction. Seventy-five µl of the supernatant of the cell culture were transferred to a new 96 well plate and NO was quantified by adding 75 µl Griess reagent (1% Sulfanilamide (w/v) in 2% phosphoric acid, 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride). The plates were incubated for 15 min in the dark and the level of NO was determined by measuring the absorbance at 562 nm. Additionally, the MTT assay was conducted to determine the cell viability in each condition by adding MTT to the original 96 well plates at a final concentration of 0.5 mg L^{-1} (Sigma-Aldrich, MO, USA) and incubating the plates for 45 min at 37 ºC. The medium was then removed and 100 µL of DMSO were added at each well, after which the absorbance was read at 510 nm (Synergy HTX, Biotek, Winooski, VT, USA). The extracts were tested in triplicate in each two independent assays (n=6).

2.3.6.4 Statistical Analysis

Results from the anticancer and anti-inflammatory assays were statistically analyzed by comparing the results obtained in the extracts and in the solvent control. Firstly, the Kolmogorov Smirnov test was used to verify normality distribution of each data, and Bartlett's test for equal variances. For parametric data, one-way ANOVA (Analysis of variance) followed by Dunnett's post hoc test was applied. For nonparametric data, the Kruskal-Wallis test was used followed by Dunn's multiple comparison test. The significance level established for all tests was at $p < 0.05$.

2.3.6.5 Dereplication and Molecular networking analysis

The actinobacterial extracts that showed bioactivity in the performed bioassays were selected for mass spectrometry (MS)-based dereplication and molecular networking analysis. Initially, the bioactive extracts were resuspended in methanol (at a final concentration of 2 mg L^{-1}) and analysed by liquid chromatography-high resolution electrospray ionization tandem mass spectrometry (LC-HRESIMS/MS) to identify crude extracts with potentially new bioactive compounds. This analysis was performed on a Dionex Ultimate 3000 HPLC connected to a qExactive focus mass spectrometer controlled by XCalibur 4.1 software (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic step was carried out in an ACE UltraCore 2.5 Super C18 column (75 mm _ 2.1 mm, Advanced Chromatography Technologies, Aberdeen, United Kingdom). Five microliters of each extract were injected and

separated using a gradient from 99.5% eluent A (95% water, 5% methanol, 0.1% formic acid, v/v) and 0.5% eluent B (95% isopropanol, 5% methanol, 0.1% formic acid, v/v) to 10% eluent A and 90 % eluent B, over 9.5 min and then maintained for six min before returning to initial conditions. The UV absorbance of the eluate was measured at 254 nm and full MS scans, at a resolution of 70,000 full width at half maximum (FWHM) (range of 150–2000 m/z), followed by data dependent MS2 (ddMS2, Discovery mode) scans at the resolution of 17,500 FWHM (isolation window used was 3.0 amu and normalized collision energy was 35) were carried out. The resulting raw data from MS-based dereplication was converted to the mzML format and submitted to the Global Natural Products Social Molecular Networking (GNPS) platform to analyse if the extracts showed significant hits that could explain the activities observed (Wang et al., 2016). Three dereplication analyses were used: Insilico Peptidic Natural Product Dereplicator, Dereplicator VarQuest and Dereplicator+, with default parameters, excluding ion mass precursor tolerance and fragment ion mass tolerance (set to 0.005 Da). In addition, a molecular network was constructed using the GNPS data analysis workflow, applying default parameters (except precursor ion mass tolerance and fragment ion mass tolerance which were set to 0.02 Da). The molecular network was loaded into Cytoscape v3.8.2 to visualize the sets of spectra from related molecules and the distribution of m/z clusters among actinobacterial extracts. For this analysis we only focused on unique single-strain m/z clusters and manually checked the corresponding LC-HRESIMS chromatograms to determine the mass of each target metabolite. The deduced masses from m/z values for major compounds were submitted to two databases of natural products, the Dictionary of natural products (DNP) (version 27.1, CRC Press, Abingdon, UK) and the natural products atlas (NPA) database (van Santen et al., 2022), to check if they corresponded to compounds previously reported from actinobacteria.

2.4 Results

2.4.1 16S rDNA metabarcoding analysis of deep-sea sediments

In order to survey the abundance and diversity of actinobacteria in the different deep-sea sediments, a culture-independent approach based on the high-throughput sequencing of 16S rRNA gene amplicons was implemented. The sequencing effort produced a total of 769,127 sequences of V4-V5 16S rRNA gene with an average sequencing depth of ~64k reads per sample. Rarefaction curves of the 12 sequenced samples reached a horizontal asymptote, indicating that an adequate sequencing depth was achieved for all samples (Fig. S1, appendix I).

Results showed that the Actinomycetota phylum was particularly abundant in the Arctic deep-sea sediments, in some cases ranking as the first or second most dominant phylum, ranging from 3 to 31 %, but showed a lower representation in the Madeira and Azores samples, with relative abundances never exceeding 7% (Fig. 1).

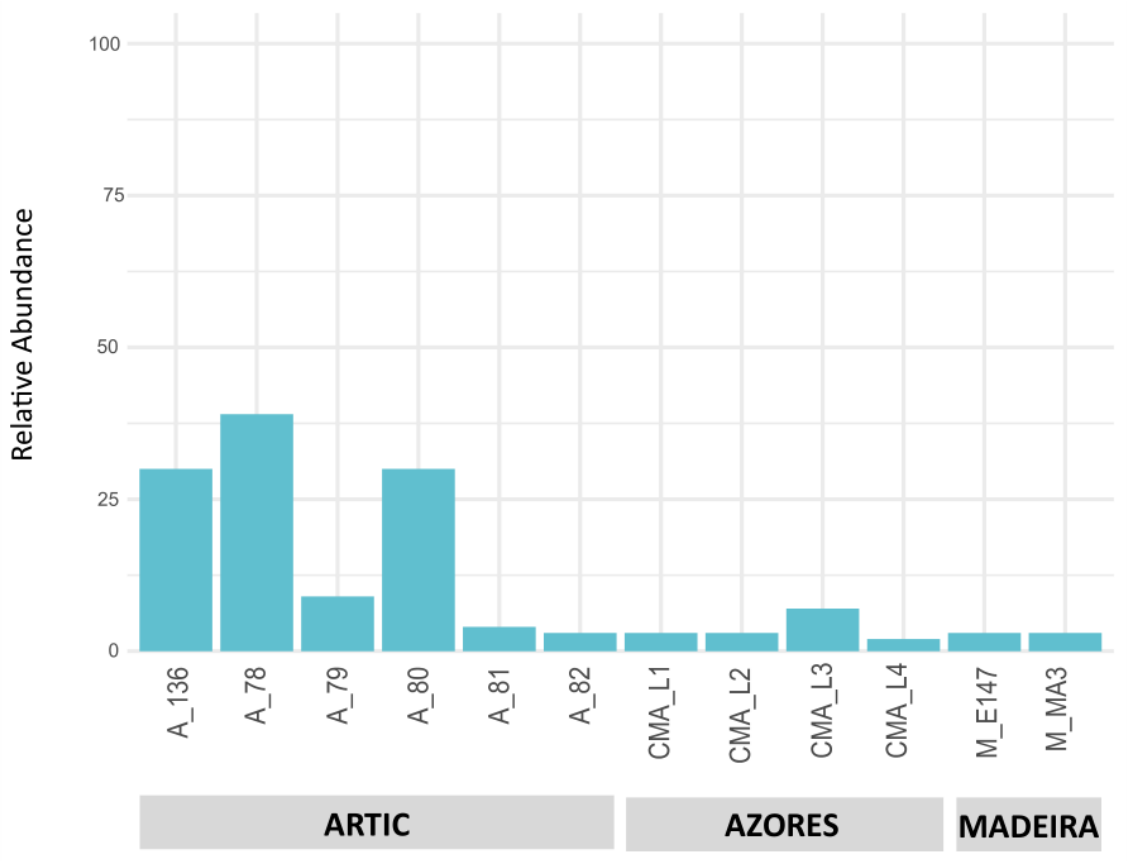

Figure 1 – Relative abundance of the Actinomycetota phylum from the deep-sea sediment samples.

A deeper look into the actinobacterial community of these samples reveals interesting features. On the one hand, the high dominance of the Actinomycetota phylum in the Arctic deep-sea sediments was apparently driven by the high abundances of only a handful of actinobacterial taxa, particularly those accommodated in the *Actinomarinales* order as well as in the *Ilumatobactereaceae* and *Microtrichaceae* actinobacterial families (Fig. 2).

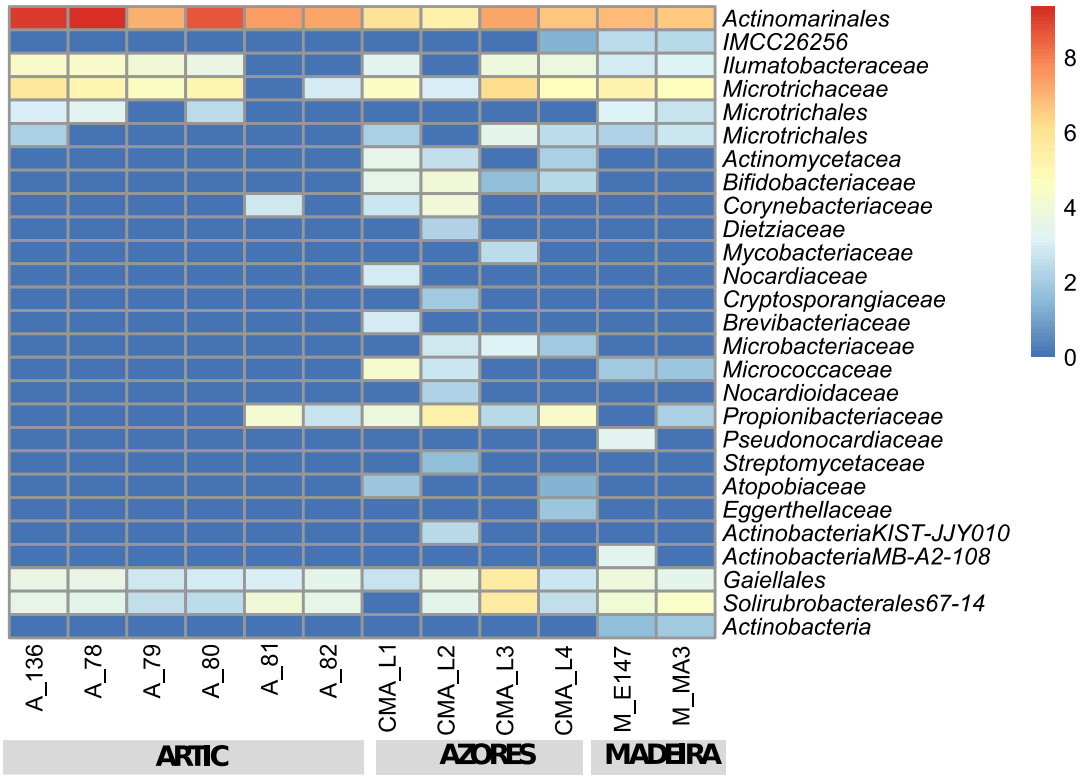

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Figure 2 – Heatmap with log transformation of the ASV abundance of actinobacteria at order and family taxonomic levels in all deep-sea samples.

The most prominent actinobacterial genera accommodated in these taxa was *Illumatobacter* and those affiliated with the *Sva0996 marine group* (Fig. 3). On the other hand, the Madeira and Azores deep-sea sediments revealed a higher diversity of actinobacterial families (13 and 22, respectively) (Fig. 3), despite the lower overall prevalence of the Actinomycetota phylum in these samples. At the genus level, however, the Azores deep-sea samples stood out, as they exhibited a much higher number of different actinobacterial genera (19 in total) than the Madeira samples, which showcased a total of only 4 different actinobacterial genera (Fig. 3).

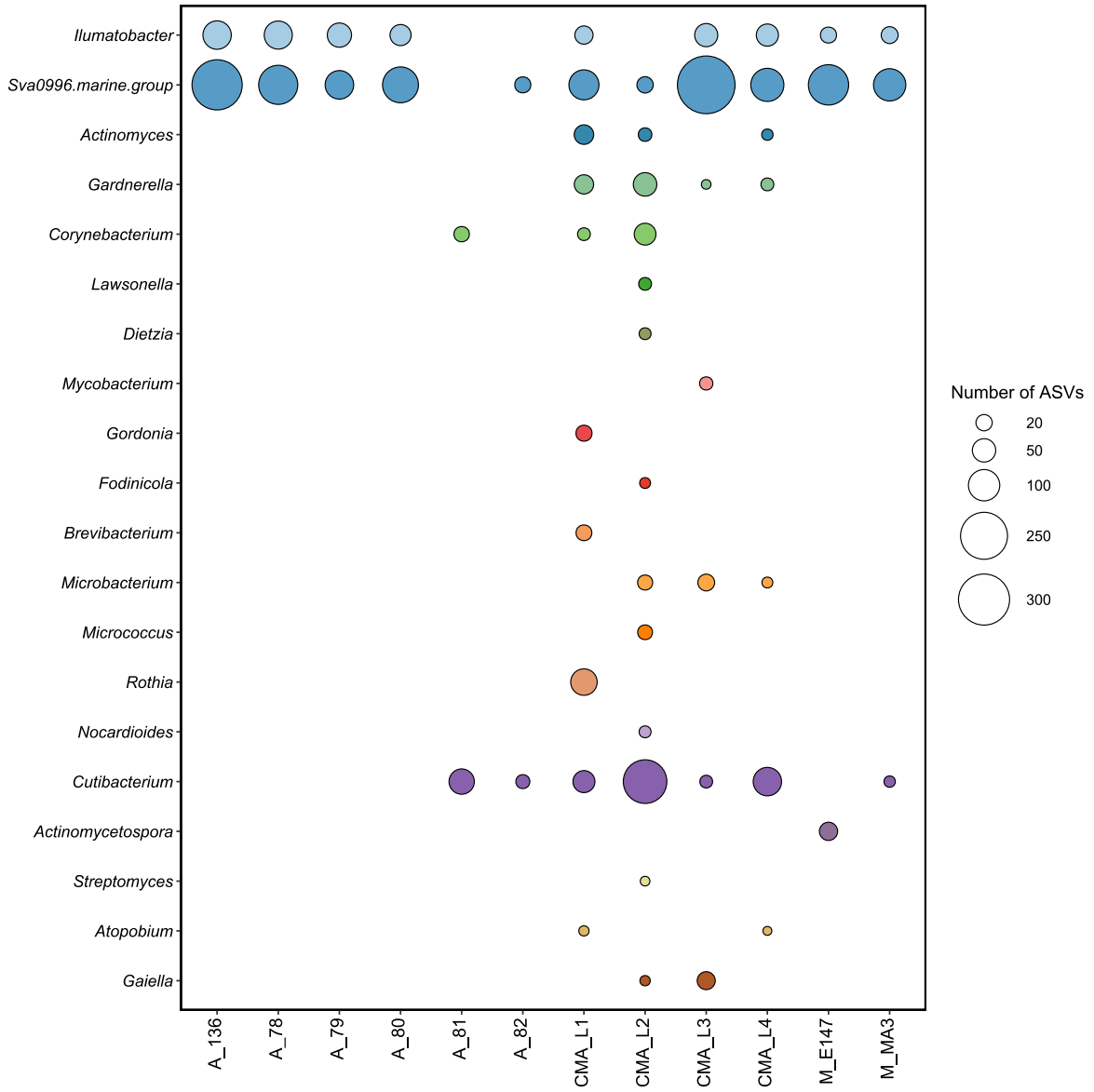

Figure 3 – Absolute abundances (as number of ASVs) of the actinobacterial genera found across the deep-sea samples.

2.4.2 Actinobacteria isolated from the deep-sea sediments

A total of 44 actinobacterial isolates were obtained from the 12 deep-sea sediment samples analysed. These isolates were distributed by nine genera: *Actinotalea*, *Brachybacterium*, *Brevibacterium*, *Dietzia*, *Leucobacter*, *Microbacterium*, *Micrococcus*, *Rhodococcus* and *Streptomyces.* The largest fraction was associated with *Brachybacterium* (11 isolates), *Microbacterium* (11 isolates) *and Brevibacterium* and (10 isolates) genera. Regarding the distribution of the isolates per region, actinobacterial strains isolated from the Arctic and Madeira samples were mostly associated with the *Brachybacterium* and *Brevibacterium* genera, while isolates from Azores sediments were predominantly affiliated with the genus

Microbacterium. Among the less abundant genera, *Actinotalea* and *Dietzia* were only retrieved from the Arctic sediments, *Rhodococcus* was obtained only from the Azorean sediments and *Leucobacter* was solely isolated from the Madeira sediments.

The pre-treatments applied to the samples from the Arctic and Madeira regions did not produce a significant effect on the number of actinobacteria recovered from each sample. However, the genera *Actinotalea* and *Dietzia* were only retrieved from the Arctic samples subjected to the wet heat pre-treatment, while this same pre-treatment allowed the recovery of the genera *Streptomyces* and *Leucobacter* in the samples from Madeira. No actinobacterial growth was obtained in the Madeira samples exposed to the pre-treatment dry heat, indicating that this treatment was overly selective.

Taxonomic analysis of some of the retrieved isolates indicate that they may constitute new species, in the light of the similarity threshold for a new species of 98.7% (Stackebrandt, 2006). Of these, *Streptomyces* strain MA3_2.13 was already proven to be a new species (Albuquerque et al., 2021).

2.4.3 Bioactivity screening of the actinobacterial extracts

The crude extracts from all deep-sea actinobacterial isolates were tested for their antimicrobial, anticancer and anti-inflammatory activities. Two actinobacterial crude extracts, obtained from the *Streptomyces* strains MA3_2.14 and 82_2.13, isolated from the Madeira and Arctic regions, respectively, exhibited antimicrobial activity against *C. albicans* (Table 6). The actinobacteria isolated from the Azores sediments did not show antibacterial activity against any of the pathogenic strains used.

Table 6 – Actinobacterial crude extracts with antimicrobial activity. MIC: Minimal inhibitory concentration.

Site	Strain	Closest taxonomic identification	Disk diffusion method	
			C. albicans (inhibition halo mm)	MIC (µg mL^{-1})
Madeira	MA3 2.14	*Streptomyces* sp.	21	7.81
Arctic	82_2.13	*Streptomyces* sp.	16	15.62

Eight actinobacterial crude extracts demonstrated significant cytotoxic activity against one or both cancer cell lines tested (HepG2 and T-47D). Actinobacterial strains belonging to the genera *Microbacterium* (DS1_10.4), *Rhodococcus* (DS4_20), *Streptomyces* (MA3_2.13 and MA3_2.14), *Brevibacterium* (79_1.12) and *Brachybacterium* (80_1.4) reduced the cellular viability of the T47-D line in more than 30% (Fig. 4). The extracts from the strains *Brevibacterium* 136.30 and *Brachybacterium* E147_1.8 reduced the viability of the HepG2 cancer line in more than 35%. Curiously, crude extracts from strains DS1_10.4, 80 1.4, 136.30

and E147_1.8 showed activity only in cancer cells lines and not in the non-tumour cell line hCMEC/D3 (Fig. 4). On the other hand, the extracts DS4_20, MA3 2.13 and 79_1.12 showed a generalized toxicity, reducing the viability of the hCMEC/D3 cell line by more than 30%. The extract MA3_2.14 was the only one that showed activity in the three cell lines tested. Regarding to the described cytotoxicity profile, the extract E147_1.8 seems the most promising, reducing by 30% specifically the HepG2 cells, but not T47D and the non-carcinogenic cells.

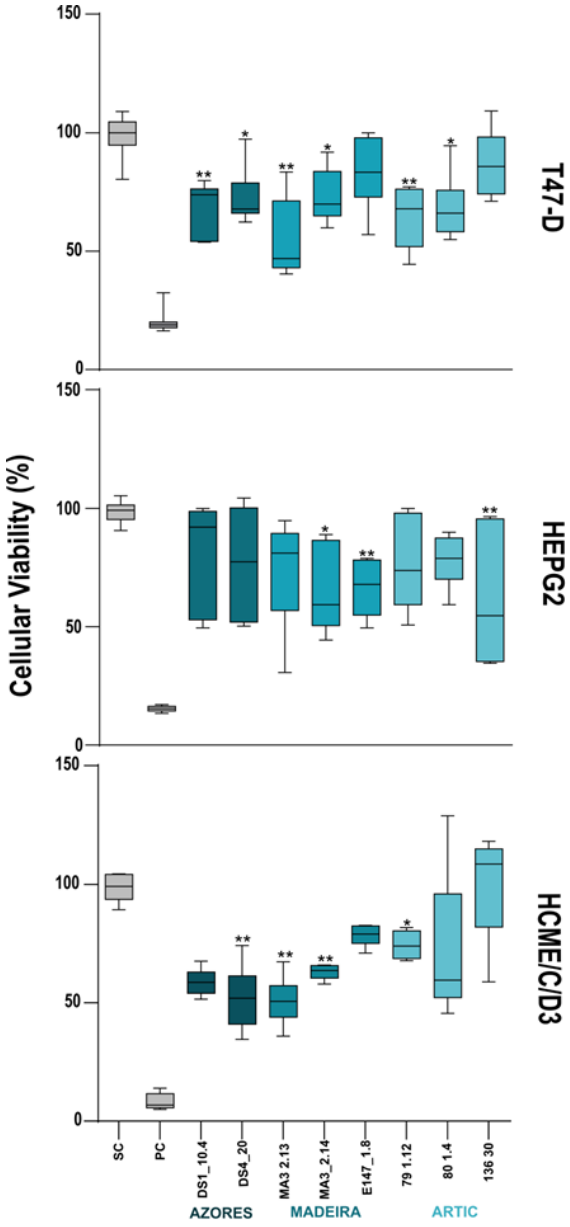

Figure 4 – Cytotoxic activity of the deep-sea actinobacterial crude extracts tested at 15 µg mL^{-1}. The effects on the viability in the two cancer cell lines: breast ductal carcinoma (T-47D) and liver hepatocellular carcinoma (HepG2), as well as in the non-cancer cell line hCMEC/D3 (human brain capillary endothelial cells) are shown after 48 h of exposure. Only the extracts with statistically significant differences are indicated on the graphs. PC and SC mean positive and solvent controls, respectively. Values are represented as interval displaying the data distribution through their quartiles with 5-95% confidence from two independent assays conducted in triplicate and line within the box-whisker represents the median. Significant differences compared to the solvent control are annotated with asterisks in the graphs (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$).

Regarding anti-inflammatory activity, 17 extracts decreased significantly the inflammatory response quantified by NO production. However, two of these extracts also reduced the viability of the macrophage cell line RAW264.7, indicating that the reduction of NO production in the presence of these extracts is a result of the decrease of cellular viability rather than of an anti-inflammatory effect (Fig. 5). Fifteen actinobacterial extracts, derived from the *Brachybacterium* strains 78.3, 79_1.12A, 79_1.6 and 79.4, the *Brevibacterium* strains 80_1.6 and 136.30, the *Microbacterium* strains DS1_1.6, DS1_10.2, DS3_6.1, DS4_17.1 and DS4_2.1, the *Rhodococcus* strain DS4_3, the *Dietzia* stain 136.2, the *Leucobacter* strain MA3_2.6 and the *Streptomyces* strain MA3_2.14 had the ability to reduce the inflammatory response by more than 60%, without showing associated cytotoxicity (Fig. 5). In terms of geographic location, these actinobacteria were isolated from the three regions targeted in this work. Importantly, all the extracts with anti-inflammatory activity did not show pro-inflammatory properties. In addition, it should be emphasized that the extract obtained from the *Brevibacterium* strain 136.30 demonstrated both anti-inflammatory (70% reduction) and anticancer (33% inhibition of viability of the HepG2 cancer line) activities, while no activity was observed against the non-cancer cell line (hCMEC/D3). On the other hand, the *Streptomyces* strain MA3_2.14 showed a cytotoxic activity against all cell lines tested (including cancer and non-cancer cell lines) and also reduced inflammation without affecting the viability of the macrophage cell line (Figs. 4 and 5).

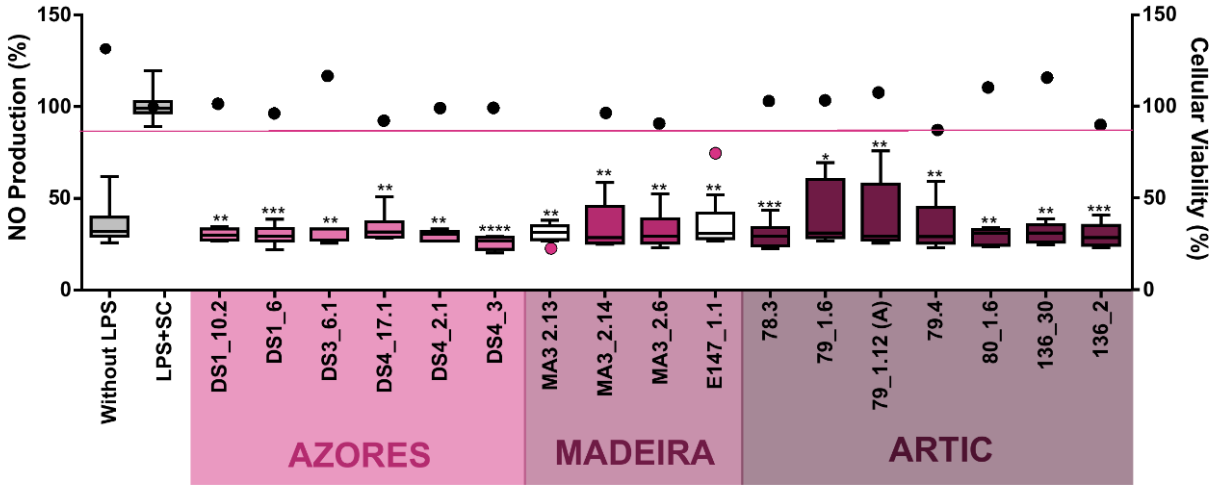

Figure 5 – Anti-inflammatory activity of the deep-sea actinobacterial crude extracts (15 µg mL^{-1}.) after inflammation induced by LPS exposure (200 µg mL^{-1}.). The inflammatory effect on the RAW 264.4 cell line as measured by NO production (box-whisker graph) and the effect on the viability of the same cellular line (plot graph) are shown after 48 h of exposure. Only the extracts with statistically significant differences are indicated in the graphs. All plot values represented below the pink line indicate that the extracts significantly reduce cell line viability in addition to having anti-inflammatory activity. Without LPS and LPS+SC denote positive and solvent with lipopolysaccharide controls, respectively. Values are demonstrated as interval displaying the data distribution through their quartiles with 5-95% confidence from two independent assays conducted in triplicate and line within the box-whisker represents the median. Significant differences compared to the solvent control are annotated with asterisks in the graphs (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$).

2.4.4 Dereplication and molecular networking analysis

The metabolic profile of the actinobacterial crude extracts that showed bioactivity was studied to understand if these bioactivities could be a result of known or new secondary metabolites. A dereplication analysis was performed on 22 extracts using Insilico Peptidic Natural Products tools available in the GNPS Platform. For analysis of the results, a threshold p-value ≤ 10^{-10} for DEREPLICATOR (Mohimani et al., 2017) and DEREPLICATOR VarQuest (Gurevich et al., 2018), and a very strict score value of 15 on DEREPLICATOR+ were selected to reduce false matches (Mohimani et al., 2018). After this, all matches for compounds not produced by actinobacteria were discarded.

For the DEREPLICATOR, the results showed the presence of the peptide Surugamide A in the extract of the *Streptomyces* strain 82_2.13, obtained from Arctic sediments, and Surugamides B and D in the extract of the *Streptomyces* strain MA3_2.14, derived from Madeira sediments. When using DEREPLICATOR with the VarQuest algorithm, 23 matches were obtained for only eight actinobacterial extracts, five derived from strains of the Arctic region (*Brachybacterium* sp. strain 78.3 and strain 79.4, *Brevibacterium* sp. strain 80_1.6, *Dietizia* sp. strain 136_2 and *Streptomyces* sp. strain 82_2.13), two from strains isolated from the Azores archipelago (*Microbacterium* sp. strains DS1_10.4 and DS3_6.1) and one from a *Streptomyces* sp. strain MA3 2.14 from Madeira. This analysis allowed the annotation of analogues of anticancer compounds and lipopeptides antibiotics. DEREPLICATOR+ in silico tool allowed the annotation of different secondary metabolites in 13 actinobacterial extracts derived from strains from all the regions studied. However, it is important to mention that extracts belonging to *Brachybacterium* sp. strains 80_1.4 and 79_1.6, both from the Arctic region, *Microbacterium* sp. strain DS1_1.6, DS1_10.2, DS4_17.1 and DS4_2.1 obtained from the Azores region, and *Leucobacter* sp. strain MA3_2.6 from the Madeira region, did not show matches with any secondary metabolite that could explain the anticancer and anti-inflammatory activities observed in these extracts (Table S2).

To complement these data, a molecular network was additionally constructed using GNPS. We found a total of 16 clusters that are shared by two or more actinobacterial extracts and nine belonging only to a single extract, which we specified as the main criterion selected to increase the possibility that a cluster corresponds to an unknown compound and, therefore, to undergo more dereplication analysis using more comprehensive, non-MS/MS based databases. Concerning the single-strain clusters, four were discovered in the extract obtained from *Streptomyces* sp. MA3_2.13 and two others in the extract from *Brachybacterium* sp. E147_1.18, both derived from the Madeira region. Three additional single-strain clusters were found in the extract of *Microbacterium* sp. DS3_6.1 isolated from Azores sediments.

The extracted ion chromatogram (EIC) from each m/z value was analysed for all single-strain clusters, to understand the relative abundance and look for the presence of common adduct ions (such as hydrogen, sodium, and ammonia). After this analysis, only four clusters were selected and the most abundant m/z values in each cluster were used to calculate an accurate mass to be used as query in the Dictionary of NP (version 27.1) and NP atlas databases. For three clusters (major compound *m/z* 297.17, *m/z* 387.180 and m/z 543.369), hits from compounds produced by actinobacteria were obtained that correspond to the exact masses of known natural products (Methyl 2-(3,5-dimethyl-2-oxocyclohexyl)tetrahydro-6-oxo-2H-pyran-4-acetate, Furaquinocin H and Milbemycin β2, respectively). For the cluster from the extract of *Microbacterium* sp. DS3_6.1 (major compound *m/z* 694.878), no hits were found in the dereplication, indicating that this cluster may be a new metabolite. The chromatographic peak corresponding to the most abundant m/z value in the cluster revealed that it will probably be produced in sufficient quantities, under the employed culture conditions, to allow for its isolation after scaling-up (Fig. 6 and Fig. S2 (Appendix I)).

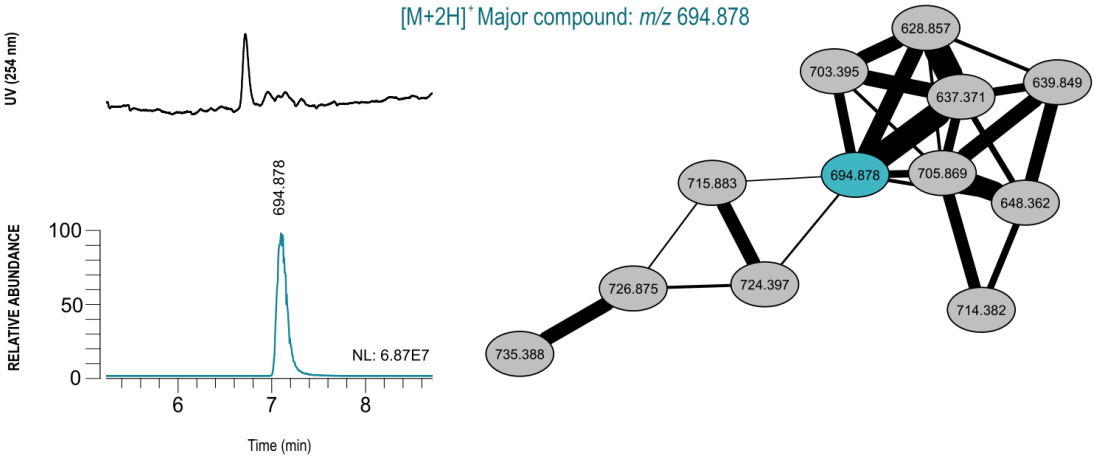

Figure 6 – GNPS-based LC-HRESIMS/MS molecular networking analysis of the actinobacterial crude extract from *Microbacterium* sp. DS3_6.1, retrieved from the Arctic region, which probably contains news natural products. Unique molecular cluster represented by connected ellipses and marked with the corresponding *m/z* value. The major compound is estimated from the UV chromatogram and EICs, and shown in a different colour from the remaining cluster nodes. The NL (normalization level) value corresponds to the base peak intensity of the m/z value when compared with the mass composition of the actinobacterial crude extract.

2.5 Discussion

The development of new technology, mainly involving unmanned underwater platforms, such as remote operated vehicles, has allowed a rapid progress in deep-sea research that is accompanied by an increase for the exploration and exploitation of deep-sea resources (Ramirez-Llodra et al., 2011). However, our capability to develop robust management measures that are essential to balance resource use and ecosystem conservation, is hindered by our limited understanding of the composition, diversity and functioning of many deep-sea ecosystems (Howell et al., 2021). In this study, we explored the biodiversity of actinobacteria associated with deep-sea sediments from the Azores and Madeira regions, within the limits of the Portuguese Continental Shelf, and the Arctic Mid-Ocean Ridge, through culture-dependent and -independent methods. In parallel to this, we also studied their bioactivities and potential to produce new natural products.

The deep-sea sediments analysed in our study showed that the Actinomycetota phylum has the highest representation in the Arctic deep-sea sediments, which is in line with other deep-sea biodiversity studies performed in this region (Fang et al., 2019; Hoffmann et al., 2017), including deep-sea sediments from the Mohn's Ridge, from where our samples were collected (Lysnes et al., 2004). As for the deep-sea sediments from the Madeira archipelago, where the lowest proportion of the Actinomycetota phylum was observed, no studies on the prokaryotic diversity of deep-sea sediments from this region have been reported.

The abundance and diversity of actinobacteria in the deep-sea sediments seemed to be negatively correlated. For instance, most of the dominance of the Actinomycetota phylum in the Arctic deep-sea samples was driven by the predominance of *Actinomarinales*, *Ilumatobacteraceae* and *Microtrichaceae*, which represented on average 97% of the Actinomycetota phylum, while in the Azores and Madeira deep-sea sediments it was noted a higher diversity of actinobacterial taxa (including the dominance of *Actinomarinales*), despite their lower overall relative abundances in the prokaryotic microbiomes of these sediments. Still, the high prevalence of *Actinomarinales* in the deep-sea sediments across the different regions studied is likely correlated with the cosmopolitan distribution of this taxon across the marine environment (Ghai et al., 2013), having also been observed in deep-sea sediments from São Paulo Plateau, in the Atlantic Ocean (Queiroz et al., 2020). Yet, this abundance-diversity dichotomy is less expressive at the genus level, probably due to a low resolution of taxonomic classifications achieved in this study. At this taxonomic level, *Illumatobacter* and *Sva0996 marine group* were the most abundant and more frequently detected actinobacterial genera across all samples analysed, representing the major genera identified in the Arctic and Madeira. For the Azores deep-sea samples, however, up to five actinobacterial genera were

detected in at least half of the samples analysed, alongside *Illumatobacter* and *Sva0996 marine group*. The actinobacterial *Sva0996 marine group* has been detected in various marine sediments, including shelf sediments, tidal sediments (Miksch et al., 2021) and deep-sea sediments (Raggi et al., 2020). Similarly, the genus *Ilumatobacter* was described to be predominant in coastal surface sediments of the Mediterranean Sea (Duran et al., 2015) and of the Pacific Ocean (Choi et al., 2016), and it was also reported as one of the main taxa in deep-sea sediments of the Indic Ocean (Chen et al., 2016). On the other hand, many of the actinobacterial genera exclusively detected in the Azores deep-sea sediments (e.g., *Cutibacterium, Garnerella*) have never been described so far as being associated to deep-sea sediments.

The culture-dependent methods employed in our study led to the isolation of 44 actinobacterial strains, which were retrieved from deep-sea samples that suffered different pre-treatment schemes. As the deep-sea samples collected in the different geographical regions were not processed in the same time period, the distinct pre-treatments applied were a reflection of the experience acquired during their analysis, having in mind the limitation of non-actinobacterial growth and the maximisation of growth of actinobacterial strains.

The retrieved actinobacterial isolates were affiliated with nine genera. Among the most common genera identified in our work, *Brachybacterium*, *Microbacterium* and *Brevibacterium* were also previously isolated from deep-sea sediments (Prieto-Davó et al., 2016; Zeng et al., 2010; Zhang et al., 2014). The genus *Micrococcus*, isolated only from the Arctic samples, was also retrieved by Zhang et al. (2014) from marine sediments from the same region. Other studies report the isolation of members of this genus from Mediterranean deep-sea sediments (Jroundi et al., 2020) and from the Mariana Trench in the Pacific (Pathom-Aree et al., 2006). Regarding the genera *Leucobacter*, *Dietzia* and Rhodococcus, detected in less abundance in our samples, these have not been reported so far in deep-sea sediments, although they have been detected in shallow marine sedimentary deposits (Hackbusch et al., 2020; Saimmai et al., 2012).

In our study, nine actinobacterial genera were identified using culture-dependent methods, while metabarcoding analysis, as a culture-independent method, led to the identification of 20 genera. Overall, only five genera were retrieved by both methods (*Brevibacterium*, *Dietzia*, *Microbacterium*, *Micrococcus* and *Streptomyces*). Curiously, according to our metabarcoding data, the genera *Streptomyces*, *Dietzia* and *Brevibacterium* were only detected in the Azores sediments, while these genera were isolated from the Arctic and Madeira sediments (A81, A82 and MA3). One possible explanation for this could lie in the fact that these genera were present in low abundance in the libraries constructed for metabarcoding analysis, but the culture conditions used in our study were suitable for their isolation. In addition, the DNA

extraction methodology and biased primer amplification can have also caused differences in abundances, resulting in the underrepresentation of some genotypes in the environmental sample (Zhang et al., 2014). The fact that our rarefaction curve reached saturation suggests that there was a high recovery of bacterial diversity through the culture-independent approach. This diversity was not observed through culture-dependent method, which is in agreement with the well-known difficulty in isolating some genera under laboratory conditions. The combined use of culture-dependent and -independent approaches was essential to provide a better characterization of the actinobacterial community of our deep-sea sediment samples (Anguita-Maeso et al., 2020; Prieto-Davó et al., 2016).

Antibiotic resistance is a growing concern worldwide and, in this respect, screening microorganisms from unexplored regions, like the deep-sea, is highly important to boost the discovery of new antimicrobial compounds (Bredholt et al., 2008; Fiedler et al., 2005). In our study, the antimicrobial screening conducted with the extracts obtained from the isolated actinobacterial strains revealed two *Streptomyces*-derived extracts active against *C. albicans*. Some compounds exhibiting antifungal activity against *C. albicans* were already isolated from deep-sea sediments-associated *Streptomyces*, like Streptolactams A and C, polyene macrolactams with MIC values of 10.4 and 16.1 μM, respectively (Wang et al., 2020), and Tunicamycin E, an antibiotic with moderate antifungal activity, with MIC values between 2 and 32 μg mL^{-1} (S. Zhang et al., 2020). Comparing the MIC values of our extracts (7.81 and 15.62 μg mL^{-1}, respectively) with those of the previously mentioned compounds, suggests that our extracts exhibit potent antifungal activity.

Microorganisms have been the source of many chemotherapeutics used in cancer treatment, and amongst them, actinobacteria are one of the most promising sources (Manivasagan et al., 2014). Our results showed eight actinobacterial extracts (derived from *Rhodococcus*, *Streptomyces*, *Brevibacterium*, *Microbacterium* and *Brachybacterium* strains, isolated from both the Arctic and Azores) with anticancer activity against at least of the tested cancer cell lines. Some of these extracts have the pArcticularity of inhibiting only the cancer cell lines tested and not the non-cancer cell line, suggesting a selective anticancer activity. The anticancer potential of deep-sea *Streptomyces* strains has been demonstrated in previous studies carried out in sediments from the Arctic and the Madeira Archipelago, in which strains of this genus exhibited activity against the human lung cancer cell line and the human colon carcinoma cell line (Dhaneesha et al., 2017; Prieto-Davó et al., 2016). A vast number of natural products with anticancer activity has been reported for *Streptomyces* strains isolated from deep-sea sediments, such as Grincamycins B–F, C-glycoside angucyclines with anticancer activity against a wide range of cancer cell lines (Huang et al., 2012), Ammosamides A-B, anticancer agents with capacity to decrease the viability of the colon carcinoma cancer cell

line HCT-116 (Hughes et al., 2009), and Spiroindimicins A–D, bisindole alkaloids with moderate cytotoxicity against various cancer cell lines. In terms of anticancer activity exhibited by deep-sea associated actinobacteria of the genus *Microbacterium*, only two compounds with cytotoxic activity have been described, the Microbacterins A and B that were isolated from the deep sea *Microbacterium sediminis* YLB-01 (Liu et al., 2015). As far as we know, no compounds with anticancer activity have been reported for deep-sea actinobacterial strains of the genera *Brevibacterium*, *Brachybacterium* and *Rhodococcus*.

Very few studies have targeted the potential of deep-sea actinobacteria to produce anti-inflammatory compounds (Manivasagan et al., 2014), in spite of actinobacteria isolated from marine intertidal sediments (Park et al., 2006), mangroves (Gomathi & Gothandam, 2019) and macroalgae (Braña et al., 2015) having demonstrated anti-inflammatory potential. In our study, fifteen actinobacterial extracts exhibited promising anti-inflammatory activity, with some of these extracts being derived from strains affiliated with genera that previously demonstrated this activity, like *Brevibacterium* (Srilekha et al., 2017) and *Brachybacterium* (Al-Halbosiy et al., 2018). On the other hand, we could not find studies reporting anti-inflammatory activity in actinobacteria associated to the genera *Microbacterium* and *Rhodococus* for any kind of environment, so, to the best of our knowledge, our study is the first one linking these genera to anti-inflammatory activity. Taking into account the scarcity of studies on the anti-inflammatory potential of actinobacteria and associated secondary metabolites, the study of the metabolic profile of each bioactive strain will be highly relevant for the discovery of new molecules with anti-inflammatory action. The fact that our extracts with anti-inflammatory activity are derived from actinobacteria isolated from extreme and underexplored environments further potentiates the chance of discovering new metabolites.

Metabolomic profiling offers a unique approach to find new natural products with biotechnological interest (Abdelrahman et al., 2022). In this study, we investigated the metabolic profile of all bioactive extracts obtained from the deep-sea actinobacterial strains. To obtain an overall knowledge of the metabolic profile, three dereplicator tools were employed. In a first analysis, using DEREPLICATOR, the compounds Surugamides A, B and D were found to be associated with only two extracts from *Streptomyces* isolated from the Madeira and Arctic regions. Surugamides are cyclic octapeptides, isolated from a marine-derived *Streptomyces* retrieved from deep-sea sediments collected at Kinko Bay, in Japan. These compounds were shown to have cytotoxic activity by inhibiting cathepsin B (Takada et al., 2013), however, this activity was not observed in our study, since the actinobacterial extracts did not show cytotoxic activity against the tested cancer cell lines. In addition, cyclic octapeptides are considered uncommon among bacterial secondary metabolism (Takada et al., 2013), nonetheless it has been described that the biosynthetic gene cluster responsible

for their production are widely distributed in marine *Streptomyces* (Almeida et al., 2019; Takada et al., 2013).

According to the second analysis, that employed DEREPLICATOR VarQuest, it was possible to match our MS/MS date with analogues of known peptidic natural products using GNPS. One of the matches obtained were Phepropeptins A and D, which were found in the crude extract of *Microbacterium* sp. DS1_10.4, from Azores. These compounds, isolated from *Streptomyces* sp. MK600-cF7, are cyclic hexapeptides exhibiting anticancer activity (Sekizawa et al., 2001) and may explain this same activity observed in our extract.

In the other bioactive actinobacterial crude extracts, analogues of known lipopeptide antibiotics, such as cyclic octapeptide (Champacyclin) and cyclic depsipeptides (Amphomycin, Antibiotic A54145, Antibiotic A0341A, Daptomycin, Enduracidin C, Glycinocin B, Skyllamycin A-B and Telomycin) were detected. All these lipopeptide antibiotics were isolated from several *Streptomyces* strains and are known to exhibit activity against Gram-positive bacteria (Boeck et al., 1990; Bracegirdle et al., 2021; Corcilius et al., 2017; Fu et al., 2015; Pesic et al., 2013; Robbel & Marahiel, 2010; Whaley et al., 1966; H.-J. Yang et al., 2014; Yin et al., 2010). However, the previously referred extracts did not exhibit antimicrobial activity that could be explained by the presence of these lipopeptide antibiotics.

The last analysis was conducted with DEREPLICATOR+, which generated the highest number of hits as this analysis, besides improving the annotation of peptide natural products, also allows the annotation of polyketides and terpenes (Mohimani et al., 2018). One of the hits of this analysis was the compound Mycolactone F, which was only found in the crude extract of *Streptomyces* sp. MA3_2.13 and may account for the observed anticancer activity. Mycolactone F is a polyketide-derived macrolide produced by the pathogen *Mycobacterium marinum* exhibiting cytotoxic, anti-inflammatory and immunosuppressive activities (Ranger et al., 2006). The other matches obtained with DEREPLICATOR+, included antifungal compounds (Dermostatin A-B, Monensin M1-M2, Flavofungin II, Vacidin A, Langkolide and Deferoxamine), antibiotics (Erythromycin, Landomycin A, Piericidins, A54556, H668, YL02107Q-A and Concanamycin B) and anticancer agents (Antimycin A, Norcadamine) (Table S2), which may explain the activities observed in our bioactive actinobacterial crude extracts.

The overall metabolomic profile of our marine actinobacterial extracts revealed a diversity of natural products similar to other metabolomic studies in deep-sea sediments (Abdelrahman et al., 2022; Tangerina et al., 2020). However, dereplication analysis showed matches to several natural products for which bioactivities were not observed in our extracts. This can be due to the relative amount of secondary metabolites present in the actinobacterial extracts. As mass spectrometry is a very accurate technique, even compounds with low abundances

can be detected and, consequently, noted as hits in dereplication (Tangerina et al., 2020). Thus, the lack of activity observed may be due to the low concentration of these compounds in the crude extracts. On the other hand, dereplication of the bioactive actinobacterial extracts showed that some of the observed activities may result from new compounds, since hits to natural products accounting for these bioactivities were not obtained.

As the GNPS platform provides annotation comparing fragmentation patterns, it is possible to detect not only compounds present in a sample and but also their analogues that have similar fragments in the mass spectrum (Tangerina et al., 2020). Therefore, the construction of a molecular network allows grouping together sets of spectra of related molecules to form clusters, even when the spectra do not correspond to any known compound (M. Wang et al., 2016). For the crude extract derived from *Streptomyces* sp. MA3_2.13, three different clusters with major compounds m/z 297.17, m/z 387.180 and m/z 543.369 were found, which were associated with Methyl 2-(3,5-dimethyl-2-oxocyclohexyl)tetrahydro-6-oxo-2H-pyran-4-acetate, Furaquinocin H and Milbemycin β2, respectively. Cycloheximide derivative, Methyl 2-(3,5-dimethyl-2-oxocyclohexyl)tetrahydro-6-oxo-2H-pyran-4-acetate, was isolated from the marine-derived *Streptomyces* sp. h-119 and does not show antifungal activity like Cycloheximide (Dou et al., 2015). The antibiotic Furaquinocin H was isolated from *Streptomyces* sp. KO-3988 and exhibits cytocidal activity against human cervical adenocarcinoma (HeLa S3) and murine melanoma (B16) (Ishibashi et al., 1991). Milbemycin β2 shows antibiotic and anthelmintic properties and is produced by *Streptomyces* sp. B-41-146 (Mishima et al., 1975).

The additional cluster found in the extract of *Microbacterium* sp. DS3_6.1 (major compound m/z 694.878), did not result in any matches with natural products present in the databases, so it probably corresponds to a new natural product. Furthermore, as only single-strain clusters were analysed in detail, there might be additional chemical novelty when analysing the set of crude extracts. These results show the importance of combining different techniques of metabolomic studies with bioactive assays for strain prioritization.

2.6 Conclusions

This study assessed the actinobacterial diversity of deep-sea sediments from the Arctic (Arctic-Mid-Ocean Ridge) and Atlantic (Azores and Madeira) oceans, by simultaneously employing culture-dependent and -independent methods, and evaluated the bioactive potential of the actinobacterial strains isolated from those samples. Our work constitutes one of the first integrated assessments of the diversity of actinobacteria and their biosynthetic potential in these underexplored regions. Culture-independent studies revealed that the Actinomycetota phylum was more abundant in deep-sea sediments from the Arctic when

compared to samples from the Atlantic Macaronesia region. This abundance was due to just a few taxa, namely belonging to the order *Actinomarinales* and to the families *Ilumatobactereaceae* and *Microtrichaceae*. On the other hand, deep-sea sediments from Madeira and Azores revealed a superior diversity of actinobacterial families, despite the lower overall incidence of the Actinomycetota phylum in these samples. Culture-dependent methodologies led to the recovery of a total of 44 actinobacterial isolates from the studied deep-sea sediment samples, which were distributed by nine genera. In terms of the bioactive potential of the isolated strains, our findings indicate that actinobacteria derived from deep-sea sediments are an important source of bioactive compounds. One of the highlights of our results was the significant anti-inflammatory activity of the tested actinobacterial extracts, which is particularly noteworthy since this bioactivity remains almost totally unreported for this phylum. The overall metabolomic profile of our deep-sea actinobacterial extracts revealed the presence of some metabolites associated to known natural products, but also possibly a new one since hits to natural products accounting for the observed bioactivity were not obtained. Our results thus demonstrate that bioprospecting actinobacteria in unexplored or underexplored environments, like the deep-sea, is a promising strategy for potentiating the discovery of new molecules with pharmaceutical applications. Further, characterizing the diversity of these poorly known sites is essential if robust resource management measures are to be developed and implemented.

2.7 References

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CHAPTER 3

This chapter will be submitted to International Journal of Systematic and Evolutionary Microbiology

Streptomyces profundis sp. nov., a novel marine actinobacterium isolated from deep-sea sediment of Madeira Archipelago, Portugal

Ribeiro I[†], Correia S[†], Blümel M, Albuquerque P, Gorb SN, Mendes MV, Tasdemir D, Mucha AP, Carvalho MF. "*Streptomyces profundis* sp. nov., a novel marine actinobacterium isolated from deep-sea sediment of Madeira Archipelago, Portugal". *In prep*

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3.1 Abstract

A novel strain, *Streptomyces* MA3_2.13^{T}, was isolated from a deep-sea sediment of Madeira Archipelago, in Portugal, and characterised using a polyphasic approach. This strain produced a brownish substrate mycelia and an aerial mycelium with white/beige spores, when grown on Glucose-Yeast-Malt agar with 50% seawater. Phylogenetic analysis of the 16S rRNA gene sequence showed that this strain was most closely related to *Streptomyces triticirhizae* NEAU-YY642^{T} (97.9%), *Streptomyces sedi* YIM 65188^{T} (97.4%), *Streptomyces mimosae* 3MP-10^{T} (97.3%) and *Streptomyces zhaozhouensis* NEAU-LZS-5^{T} (97.0%). The 16S rRNA-based phylogenetic analysis was corroborated by the calculation of the whole genome average nucleotide identity (ANI) between MA3_2.13^{T} and other type strains. Overall, the phylogenetic analysis indicates that strain MA3_2.13^{T} represents a novel *Streptomyces* species. The main respiratory quinones were MK-10(H4), MK-10(H6) and MK-10(H8). Diphosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids and two glycophospholipids were identified as the main phospholipids. The major cellular fatty acids were iso-$G_{16:1}$, iso-$C_{16:0}$, anteiso-$C_{17:1}$ and anteiso-$C_{17:0}$.

Based on these results, strain MA3_2.13^{T} (=DSM 115980^{T}=LMG 33094^{T}) is proposed as the type strain of a novel species of the genus *Streptomyces*, for which the name *Streptomyces profundis* sp. nov. is proposed.

3.2 Introduction

The genus *Streptomyces* was first described in 1943, by Waksman and Henrici (Waksman et al., 1943) and is considered the largest genus of the phylum Actinomycetota, with more than 800 taxa with validly published names (https://lpsn.dsmz.de/genus/streptomyces). Members of this genus typically include aerobic Gram-positive and spore-forming bacteria with high G+C content in their DNA (often more than 70%), that produce highly branched substrate and aerial mycelia and contain LL-diaminopimelic acid in their cell walls (Lee et al., 2020).

Species of this genus are known for producing a wide variety of bioactive natural products, including antibiotics, antifungals, antivirals, anticancer and immunosuppressants agents (Barka et al., 2016; Jose et al., 2021). *Streptomyces* spp. possess a well-established and complex life cycle (Jones et al., 2018) and have been described from all environments, including the deep-sea (Kamjam et al., 2017). Several *Streptomyces* spp. have been isolated from deep-sea sediments and their contribution to the production of new secondary metabolites has been notorious (Donald et al., 2022; Kamjam et al., 2017). During an event of isolation of Actinomycetota from deep-sea sediment samples collected in the Madeira archipelago (Portugal), a new *Streptomyces* strain, MA3_2.13^T, was obtained. In this paper, we characterize this strain using a polyphasic approach, aiming at its validation as a new species. The results showed that this represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces profundis* sp. nov., is proposed.

3.3 Strain isolation

Strain MA3_2.13^T was isolated from a deep-sea sediment sample collected at 2300 m depth in the Madeira Archipelago, Portugal (Lat 32.5218, Lon -16.9683), in June 2017, during the oceanographic campaign SEDMAR 1/2017, carried out by the Portuguese Hydrographic Institute. The isolation process was performed as described in Chapter 2. Briefly, one gram of sediment was suspended in 1 ml of sterile seawater and subjected to a pre-treatment consisting in the incubation of the suspension in a water bath at 57 °C, for 15 min. The suspension was ten-fold diluted until 10^{-3} and 100 µl of each dilution were plated on M1 agar medium (soluble starch, 10 g L^{-1}; yeast extract, 4 g L^{-1}; peptone, 2 g L^{-1}; agar, 17 g L^{-1}; seawater, 1 L) supplemented with cycloheximide (50 mg L^{-1}), nalidixic acid (50 mg L^{-1}) and nystatin (50 mg L^{-1}). The plates were incubated at 28 °C for 6 months. The purified strain was cryo-preserved at -80 °C in 30% (v/v) glycerol.

3.4 Morphology and Growth Conditions

Cell morphology of MA3_2.13^T colonies grown on GYM 50SW agar (per liter: 4 g Glucose; 4 g yeast extract; 10 g malt extract; 2 g $CaCO_3$; 12 g agar; 500 mL of seawater and 500 mL of deionized water), for 14 days, at 25 °C was observed using a light microscope (LEICA ICC 50 HD), coupled to LAS Core software, under maximum magnification (1000x). A cryo-scanning electron microscope (Cryo-SEM) (Hitachi S-4800, Hitachi Europe GmbH, Düsseldorf, Germany) equipped with the Gatan Alto 2500 cryo-preparation system was used to image cells in the native conditions. For the Cryo-SEM analysis, few cells were transferred from the culture to the sample holder of the SEM and frozen on the stage at -140°C, i.e., inside the cryo-preparation chamber of the SEM, sputter-coated with 10 nm thin layer of Au-Pa, and observed at the temperature -120°C and accelerating voltage of 3 kV.

Gram staining was carried out by using the standard Gram stain method. Catalase activity was determined with 3% H_2O_2, according to standard methods (Reiner, 2010) and oxidase activity was assessed with the commercial strips Bactident® Oxidase.

Strain MA3_2.13^T grown on GYM 50SW agar for 2 weeks at 25 °C revealed the production of soluble pigments that diffuse into the agar medium, giving the substrate mycelia a brownish colour. The aerial mycelium shows powdery consistency with white/beige spores. The aerial hyphae differentiated into long spiral spore chains with three to five turns of cylindrical and smooth surface spores with 0.8 – 1.2 µm (Fig. 7). Cells of strain MA3_2.13^T were Gram-positive, oxidase negative and catalase negative.

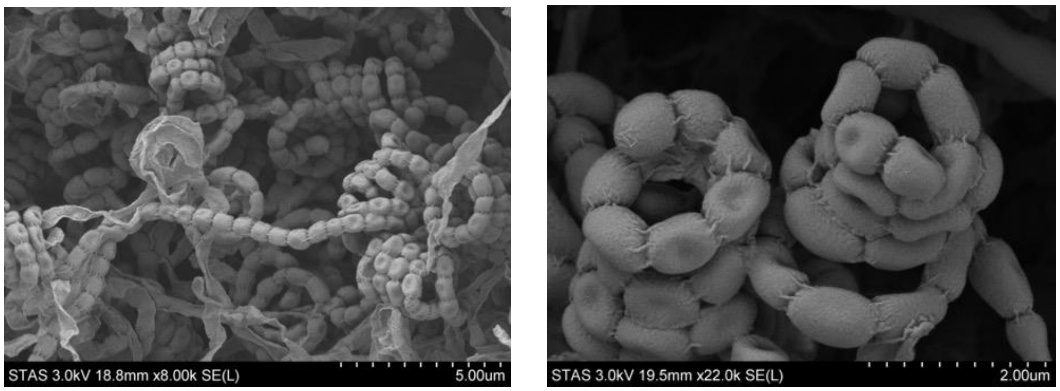

Figure 7 – Scanning electron microscopy of spore chains of strain MA3_2.13^T after growth on GYM 50SW agar medium at 25 °C for 14 days.

3.5 Physiology

Cultural characteristics were analysed on International Streptomyces Project (ISP) medium 2, 3, 4, 5 and 7 (Shirling and Gottlieb, 1966) and GYM agar after incubation at 28 °C for 14 days. Due to the fact that strain MA3_2.13^T has a marine origin, growth was tested in media prepared without seawater and with 50% of seawater. Growth temperature range (4, 12, 19, 25, 28, 35, 40 and 45°C) was evaluated on GYM 50SW agar after 14 days. pH range (pH values between 4.0 and 9.0, in intervals of 1.0 pH unit) was assessed in GYM 50SW agar, at 25 º C, using as buffer systems, acetate buffer (for pH between 4.0 and 5.5), phosphate buffer (for pH between 6.0 and 8.5) and Tris buffer (for pH 9.0). NaCl tolerance (0, 1, 3, 5, 7, 10, 15, 20 and 25%) was analysed after 14 days by observing growth in GYM 50SW agar, at 25 ºC.

This strain grew well on ISP4 agar (with 50% seawater) and GYM agar (with 50% seawater or 100% deionized water), in which sporulation occurred after one week of growth. Moderate growth was visible on ISP3 agar (with 50% seawater or 100% deionized water), while no growth was observed on ISP2, ISP5 and ISP7 agar. Growth of strain MA3_2.13 ^{T} was observed at 19-35 °C (optimal growth at 25 °C), pH 6.0-9.0 (optimum growth at pH 7.0) and in the presence of 0-7% of NaCl (optimum growth between 1-5% NaCl). Detailed growth characteristics of this strain are indicated in Table 7, together with a comparison to its closely related type strains.

Growth of strain MA3_2.13^T in different carbon sources was assessed on basal mineral salts agar medium, supplied with 1% (w/v) of L-arabinose, sucrose, D-fructose, myo-inositol, D-raffinose, D-mannitol, α-Cellulose and L-Xylose (Shirling et al., 1966). The positive control consisted in the basal mineral salts agar medium supplemented with 1% glucose (w/v), and the negative control consisted of the same medium without the addition of carbon sources. Nitrogen source utilization was analysed on basal agar medium with 0.1% (w/v) of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamic acid, glycine, L-serine, L-threonine and L-tyrosine (Williams et al., 1983). Basal agar medium complemented with either L-asparagine and without the addition of nitrogen sources were used as positive and negative controls, respectively. Enzyme activities were assessed using the API® ZYM system (bioMérieux) following the manufacturer's instructions and the results were observed after incubation for 4 h, at 25 ºC. The enzymes tested were alkaline phosphatase, esterase (C 4), lipase esterase (C 8), lipase (C 14), valine arilamidase, cystine arilamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-

glucuronidase, α-glucosidase, n-acetyl- β -glucosaminidase, α-mannosidase and α-fucosidase.

The carbon sources sucrose, myo-inositol and D-raffinose were found to support the growth of strain MA3_2.13 ^{T}, while D-mannitol, L-rhamnose and L-xylose yielded a weak growth and L-arabinose, D-fructose and α-cellulose led to no growth. Strain MA3_2.13^{T} was able to use L-alanine, L-arginine, L-asparagine, glycine, L-proline, L-serine, L-aspartic acid and creatine as nitrogen source. Growth with L-glutamic acid and L-threonine was moderate, and no growth was observed with L-tyrosine. This strain tested positive for the enzymes alkaline phosphatase, esterase (C 4), esterase lipase (C 8) and naphthol-AS-BI-phosphohydrolase and had a weak positive result for acid phosphatase. As shown in Table 7, strain MA3_2.13^{T} presented various differences in terms of carbon and nitrogen utilization profile when compared with its closest relatives, *Streptomyces zhaozhouensis* NEAU-LZS-5^{T}, *Streptomyces sedi* YIM 65188^{T}, *Streptomyces triticirhizae* NEAU-YY642^{T} and *Streptomyces mimosae* 3MP-10^{T}.

3.6 Chemotaxonomy

Analysis of polar lipids, respiratory quinones, cellular fatty acids and whole-cell sugars were carried out by the Identification Service of Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, Germany, using biomass harvested in the exponential phase from a culture of MA3_2.13^{T} grown in GYM 50SW agar medium.

The phospholipid profile of strain MA3_2.13 consisted of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), three unidentified phospholipids (PL) and two unidentified glycophospholipids (PG). Comparing this profile with the phospholipids of the genus *Streptomyces* in general, some differences can be verified, as these mainly comprise diphosphatidylglycerol (DPG) and phosphatidylethanolamine (PE), like our strain, but also include phosphatidylinositol (PI) and phosphatidylinositolmanosides (PIM), which were not identified in our strain (Kämpfer, 2006). According to Kämpfer (2006), the predominant menaquinones of members of the genus *Streptomyces* are MK-9(H6) and MK-9(H8). In light of this, strain MA3_2.13^{T} has an unusual respiratory quinones composition, with the main ones being MK-10 (H4) (20.8%), MK-10 (H6) (19.3%) and MK-10 (H8) (21.2%). The predominant cellular fatty acids (> 5%) found in strain MA3_2.13^{T} were iso-$G_{16:1}$ (11.1%), iso-$C_{16:0}$ (48.5%), anteiso-$C_{17:1}$ (6.0%) and anteiso-$C_{17:0}$ (12.8%).This profile is in line with the common fatty acid composition of the genus *Streptomyces*, which usually contain large amounts of saturated iso- and anteiso-

fatty acids (Kämpfer, 2006). Whole-cell sugars of strain MA3_2.13 consisted of galactose and ribose. While these sugars have been occasionally reported in small amounts in members of the genus *Streptomyces*, they are mainly found in other actinobacterial genera, which is another peculiar feature of our strain (Kämpfer, 2006).

3.7 16S rRNA gene phylogeny

Genomic DNA of strain MA3_2.13^T was extracted using the E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek, GA, USA), as previously described (Albuquerque et al., 2021). The 16S rRNA gene was amplified using the universal primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), as described in Chapter 2 and sequenced at GenCore platform (I3S—Instituto de Investigação e Inovação em Saúde, Porto, Portugal). The 16S rRNA gene sequence of strain MA3_2.13^T was analysed using the Geneious Prime (version 2022.2.1) and the identity of the consensus sequence (1415 bp) was confirmed by comparison to the full-length 16S rRNA sequence (1542 bp) extracted from the whole genome sequence. A phylogenetic tree was developed based on the ten closest 16S rRNA gene type strain sequences, according to the similarity percentage available in the 16S ribosomal RNA (Bacteria and Archaea) database from NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *Micromonospora aurantiaca* ATCC 27029^T (NR_074415.1) was used as an outgroup. The alignment was performed with MUSCLE alignment tool from the Geneious software. A maximum likelihood phylogenetic tree was constructed using the HKY+G+I model of nucleotide substitution and with bootstraps analysis based on 1000 replicates, to estimate the confidence of the branches. The phylogenetic tree was built using the MEGA program Version 11 (Tamura et al., 2021).

BLAST analysis of the 16S rRNA gene sequence of strain MA3_2.13 revealed that this microorganism is most closely related to *Streptomyces triticirhizae* NEAU-YY642^T (NR_180032.1; 97.9%), *Streptomyces sedi* YIM 65188^T (NR_044582.1; 97.4%), *Streptomyces mimosae* 3MP-10^T (NR_170412.1; 97.3%) and *Streptomyces zhaozhouensis* NEAU-LZS-5^T (NR_133874.1; 97.0%). In a maximum likelihood phylogenetic tree, strain MA3_2.13 was found to cluster together with these same strains (Fig. 8).

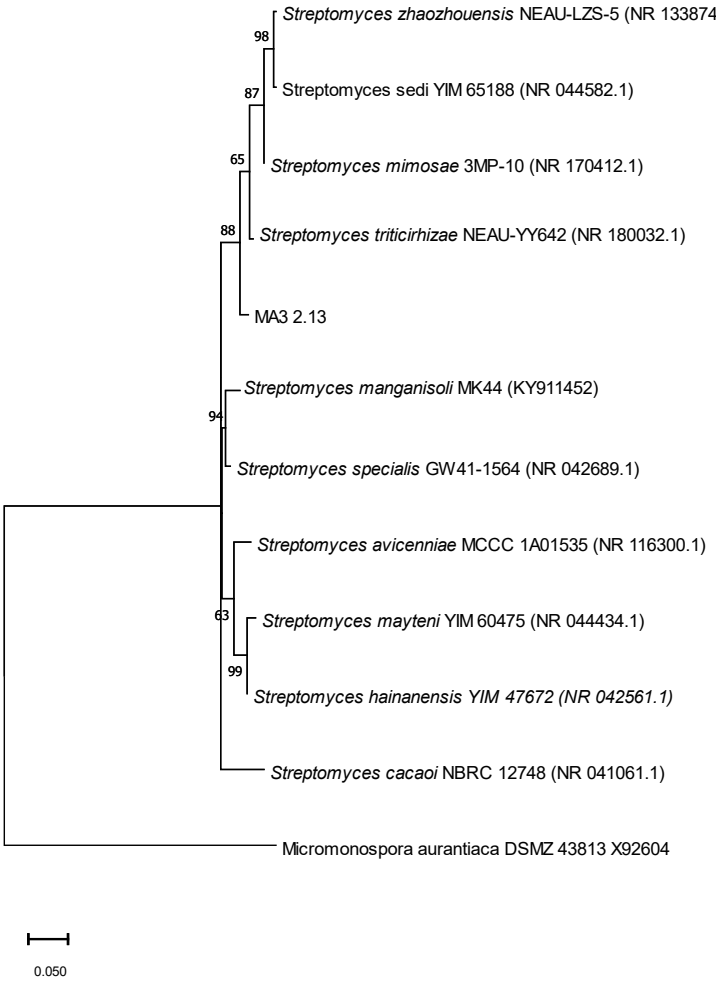

Figure 8 – Phylogenetic tree based on the ten type strains closest to MA3 2.13^T, according to NCBI BLAST analysis. Maximum likelihood phylogenetic tree was performed in MEGA 11.1 using 12 sequences with 1397 bp. The phylogeny test used was the bootstrap analysis based on 1000 replicates to estimate branching order of the tree. The GenBank accession numbers are indicated in parenthesis. *Micromonospora aurantiaca* ATCC 27029^T (NR_074415.1) was used as an outgroup. Scale bar corresponds to 0.05 substitutions per nucleotide position.

3.8 Genome features

For whole-genome sequencing, genomic DNA of strain MA3_2.13^T was isolated as previously described (Albuquerque et al., 2021). In brief, genomic DNA was obtained from cells grown in Tryptic Soy Broth (TSB) (30 °C, at 220 rpm, for 48 h) and extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Illumina and PacBio library preparation and sequencing were carried out at Novogene (Cambridge, UK). The *de novo* assembly of genome was generated using a hybrid approach implemented in Unicycler (v. 0.4.9b) that combines short-reads

(Illumina) and long-reads (PacBio) sequencing data. The genome assembly was deposited in the NCBI GenBank with accession number GCA_020740535.1.

The complete genome of strain MA3_2.13 was 7.7 Mbp, with an average G+C content of 72.1%. To complement the 16S rRNA-based taxonomic analysis, we calculated the whole genome average nucleotide identity (ANI) between strain MA3_2.13 and the type strains previously identified. The ANI values were determined using the ANIb method implemented in the Pyani algorithm v0.2.12 (Pritchard et al., 2016). The ANI values for all type strains ranged between 75.9 and 82.0% identity with an alignment coverage between 24.7 and 55.7% of the genome. In accordance with the 16S rRNA analysis, the best ANI matches were with *Streptomyces triticirhizae* NEAU-YY642^T (82% identity and 52.4% genome coverage) and *Streptomyces mimosae* 3MP-10^T (81.7% identity and 55.7% genome coverage). In both cases the ANI identity values are below the limit of 95-96% recommended as minimal standards for prokaryotic species definition (Chun et al., 2018; Richter et al., 2009).

Table 7 – Differential characteristics of strain MA3_2.13^T and its four closest related *Streptomyces* type strains.
Strains: 1, MA3_2.13^T; 2, *Streptomyces zhaozhouensis* NEAU-LZS-5^T; 3, *Streptomyces sedi* YIM 65188^T; 4, *Streptomyces triticirhizae* NEAU-YY642^T; 5, *Streptomyces mimosae* 3MP-10^T. All data are from this study except were indicated by a symbol (¤(He et al., 2014); §(Li et al., 2009); ¥(Han et al., 2020); ϱ(Klykleung et al., 2020)). +, Positive; −, negative; w, weakly positive; ND, not determined. Phospholipids profile: DPG, diphosphatidylglycerol; GPL, Glycophospholipid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL, unidentified phospholipids; L, unidentified lipids.

Characteristics	1	*2*	*3*	*4*	*5*
Growth Temperature range (°C)	19 – 35	15 - 35	15 - 37	15 – 45	20 - 37
Optimum growth temperature (°C)	25	28	28	28	28
Growth pH range	6.0 – 9.0	6.0 – 9.0	6.0 – 9.0	6.0 – 9.0	5.0 – 9.0
Optimum growth pH	6.0	7.0	8.0	6.0	6.0
NaCl tolerance range (%)	0 - 7	0 - 7	0 – 7	0 - 7	0 - 5
Optimum NaCl tolerance range (%)	3 - 5	1 - 5	1 - 3	0 - 3	0 - 1
Carbon source utilization:					
L-arabinose	-	w	-	-	+
Sucrose	+	+	+	w	+
D-fructose	-	-	-	-	w
Myo-inositol	w	w	+	+	+
D-Raffinose	w	+	+	+	+
D-mannitol	w	+	w	+	+
α-Cellulose	-	w	+	+	+
L-Xylose	w	-	-	-	w
Nitrogen source utilization:					
L-alanine	+	+	+	+	+
L-arginine	+	+	+	+	+
L-asparagine	+	+	+	+	+
L-aspartic acid	+	-	-	+	-
L-glutamic acid	w	-	w	+	-
Glycine	+	+	+	-	w
L-serine	+	+	+	+	+

L-threonine	w	+	+	+	+
L-tyrosine	-	w	+	-	+
Enzymatic activity:					
Alkaline phosphatase	+	+	+	+	w
Esterase (C 4)	+	+	+	+	+
Lipase esterase (C 8)	+	+	+	+	+
Lipase (C 14)	-	+	+	+	+
Valine arilamidase	-	+	+	+	+
Cystine arilamidase	-	+	+	+	+
Trypsin	-	-	+	+	+
α-chymotrypsin	-	-	-	w	+
Acid phosphatase	w	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+
Galactosidase	-	-	-	+	+
α- β-galactosidase	-	+	+	+	+
β-glucuronidase	-	-	-	-	-
α-glucosidase	-	+	+	+	+
N-acetyl- β -glucosaminidase	-	-	-	+	+
α-mannosidase	-	-	+	+	+
α-fucosidase	-	-	-	-	w
Catalase	-	+	+	+	+
Oxidase	-	-	-	-	-
Phospholipids profile	DPG, PE, 3PL, 2GPL	DPG, PE, 3PL, PI ¤	DPG, PE, PG, PI, 2PIM, 4PL §	DPG, 2L, PE, PL, 2PIM ¥	DPG, PIM, PI, PE, PG, PL ǫ
Respiratory quinones	MK-10(H4), MK-10(H6), MK-10(H8)	MK-9(H8), MK-9(H6) ¤	MK-11(H6), MK-10(H6), MK-10(H8), MK-11(H8) §	MK-10(H4), MK-10(H6), MK-9(H6), MK-10(H2), MK-9 (H8), MK-9(H4) ¥	MK-10(H8), MK-10(H6), MK-9(H8) ǫ
Cellular fatty acids composition	iso-$G_{16:1}$, iso-$C_{16:0}$, anteiso-$C_{17:1}$, anteiso-$C_{17:0}$	$C_{16:0}$, $C_{17:1}$ ω7c, $C_{15:0}$, iso-$C_{15:0}$, $C_{18:0}$, $C_{16:1}$ω7c, anteiso-$C_{17:0}$ ¤	ND	iso-$C_{16:0}$, anteiso-$C_{17:0}$, $C_{16:0}$ ¥	iso-$C_{16:0}$, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ ǫ
Whole-cell sugars	Galactose, ribose	Glucose ¤	glucose, galactose, madurose §	glucose, ribose ¥	glucose, ribose ǫ

3.9 Description of *Streptomyces profundis* sp. nov.

Streptomyces profundis sp. nov. (pro.fun'di. L. gen. neut. n. profundis, of the depths of the sea, of the deep-sea); referring to the origin of the strain from deep-sea sediment of Madeira Archipelago, Portugal.

Aerobic, Gram-positive and filamentous actinobacterium that forms a well-developed branched substrate and aerial mycelium. Soluble pigments that diffuse into the agar medium are produced, giving the substrate mycelia a brownish colour. The aerial mycelium shows powdery consistency with white/beige spores. Long spiral spore chains with three to five turns of cylindrical and smooth surface spores (0.8-1.2 µm) were observed in the branched aerial hyphae. Presents a good growth on ISP4 (with 50% or 70% seawater) and GYM agar (with 50% seawater or 100% deionized water), moderate growth on ISP3 agar (with 50% seawater or 100% deionized water) and no growth on ISP2, ISP5 and ISP7 agar. Grows in a temperature range between 19 and 35 ºC

(optimum 25 ºC), pH range between 6.0 and 9.0 (optimum pH 7.0) and in the presence of 0 - 7% NaCl (optimum between 1-5% NaCl). Sucrose, myo-inositol and D-raffinose are used as carbon sources, while growth is low with D-mannitol, L-rhamnose and L-xylose and L-arabinose, D-fructose and α-cellulose are not used as carbon sources. L-alanine, L-arginine, L-asparagine, glycine, L-proline, L-serine, L-aspartic acid, creatine, L-glutamic acid and L-threonine are used as nitrogen source, but not L-tyrosine. Positive for the enzymes alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. Catalase and oxidase negative. The main respiratory quinones are MK-10(H4), MK-10(H6) and MK-10(H8). Phospholipid profile consists of diphosphatidylglycerol, phosphatidylethanolamine, three unidentified phospholipids and two glycophospholipids. The major fatty acids are iso-$G_{16:1}$, iso-$C_{16:0}$, anteiso-$C_{17:1}$ and anteiso-$C_{17:0}$. Whole-cell sugars consist of galactose and ribose.

The type strain, MA3_2.13^T (=DSM 115980^T=LMG 33094^T) was isolated from a deep-sea sediment collected in Madeira Archipelago, Portugal. The genome size of strain MA3_2.13^T is 7.7 Mbp and the DNA G+C content is 72.1%. The NCBI GenBank accession number for the genome assembly is GCA_020740535.1 and the accession number of the 16S rRNA gene sequence is OQ363655.

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CHAPTER 4

This chapter will be submitted to Environmental Microbiology

Deep-sea Actinobacteria from Madeira and Azores Archipelagos: Isolation and Bioprospecting for New Natural Products

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4.1 Abstract

The deep-sea is characterized by extreme conditions and due to its difficult access, this environment is poorly investigated in terms of microbial diversity. Actinobacteria are widely distributed in this environment and are considered an excellent source of new bioactive compounds. In this study, we explored the biodiversity of cultivable actinobacteria in 66 deep-sea samples collected in the Portuguese archipelagos of Madeira and Azores (between 150 and 3000 m depth), and investigated their capacity to produce new molecules with pharmaceutical relevance. A total of 276 actinobacterial strains distributed by 20 genera were isolated from the analysed samples, included novel species. Organic extracts of these strains were screened for antimicrobial, anticancer, anti-inflammatory and anti-obesity activities. Results revealed 16 extracts active against one or more tested microorganisms (*Bacillus subtilis*, *Candida albicans* and *Staphylococcus aureus*), 23 extracts capable of reducing the cellular viability of the cancer cell lines T-47D (breast ductal carcinoma) and HepG2 (liver cancer), after 48 h of exposure, and 28 extracts with anti-inflammatory activity in the cell line RAW264.7. Regarding anti-obesity activity, of the 30 crude extracts tested, six exhibited significant neutral lipid-reducing activity after 48 h of exposure. Metabolic profile of active actinobacterial extracts revealed the presence of some metabolites associated to known natural products, but also indicated the presence of possible new bioactive molecules.
The bioactivities obtained and the potential to produce possible new metabolites prove that the deep-sea actinobacteria are prolific biosynthesizers, reinforcing the bioactive characteristics of this group.

4.2 Introduction

Natural products (NP) have become one the most valuable resources in modern pharmacology, for the development new lead compounds and scaffolds (Pilkington, 2019; Sorokina et al., 2020). Until 2019, about ca. 60% of new drugs had their origin or were inspired in NP or their derivatives (Newman et al., 2020). Several drugs used in the treatment of cancer and inflammatory diseases are often NP or its derivatives, and major classes of antibiotics and antifungals are based on NP obtained from microorganisms (Jenab et al., 2020; Sorokina et al., 2020).

The phylum Actinomycetota covers a large group of morphologically and physiologically diverse Gram-positive bacteria. Members of this phyla are the most economically and biotechnologically valuable prokaryotes for their remarkable capacity to produce secondary metabolites with a broad range of bioactive properties (Dharmaraj, 2010; Olano et al., 2009). Although most of these NP have terrestrial origin, there is currently an increasing interest in the discovery and production of new drugs from the marine realm (Altmann, 2017). This trend is largely due to the fact that marine NP have shown different chemical novelty when compared to their terrestrial counterparts, mainly because most of the structural scaffolds found in marine NP are unique and only found in marine life (Kong et al., 2010; Pilkington, 2019). Marine actinobacteria have already proven to be an abundant source of secondary metabolites and novel drugs. (Haefner, 2003; Lu et al., 2020). In addition, actinobacteria living in symbiosis with marine invertebrates (such as corals and sponges) demonstrated to produce a wide range of molecules with many biological activities that may be attributed to chemical defence functions they provide to their hosts (Atanasov et al., 2021).

Actinobacteria are successful colonizers of several marine environments, including the deep-sea where they constitute one of the most abundant phyla (Chen et al., 2016; Yilmaz et al., 2016). The deep-sea is a unique environment characterized by extreme conditions, such as low nutrient availability, high pressure, low temperature, lack of light, high salinity and variable oxygen concentrations (Bredholt et al., 2008; Subramani et al., 2012). Life in these environments had to evolve in order to adapt and survive to such harsh conditions, often leading to unique metabolisms that may translate into the production of new molecules with single biological properties (Bull et al., 2000; Manivasagan et al., 2013).

The Portuguese archipelagos of Madeira and Azores are located in the Atlantic region of Macaronesia and have a unique biogeography and biodiversity. The deep-sea of these

archipelagos is largely unexplored in terms of actinobacteria bioprospection, making these regions a very attractive source of new phylogenies and chemistry. In this context, this study had as objective to explore the diversity and bioactive potential of cultivable actinobacteria associated with 66 deep-sea samples (comprising sediments, corals and sponges) collected from poorly surveyed areas of Madeira and Azores archipelagos, with the ultimate goal of bioprospecting for novel NP with antimicrobial, anticancer, anti-inflammatory and anti-obesity activities.

4.3 Materials and methods

4.3.1 Samples collection

Sixty-six deep-sea samples were collected in the Madeira and Azores archipelagos (NE Atlantic Ocean), which included sediments (n=18), corals (n=29), sponges (n=18) and sea-lily (n=1) (Table 9). The samples from Madeira (n=32) were collected at depths between 430 m and 1980 m, with a remotely operated vehicle (ROV) during the mission OOM2018_LUSO (organized by Observatório Oceânico da Madeira), or with a manned submersible (Lula1000) under the mission Deep_Madeira 2019 (financed by Direção Regional do Ordenamento do Território-DROTA, Madeira). The Azores samples (n=34) were collected between 150 m and 3000 m depth using a ROV, during the oceanographic campaign M150 BIODIAZ (financed by DFG-Senatskommission für Ozeanographie). Deep-sea samples were collected aseptically, immediately frozen at -20 ºC, and transported and conserved at this temperature until its processing in the laboratory.

Table 9 – Information relative to the deep-sea samples collected in Madeira and Azores archipelagos and used in this study for the isolation of actinobacteria

Site	Site description	Sample code	Depth (m)	Sampling gear	Sample type	Pre-treatments applied	Culture media used for isolation of actinobacteria
Madeira	Canyon Ribeira Brava	OOM2018_002*	1980	Biobox	Coral	PT1 PT2	RH M4 NPS
	Canyon Ribeira Brava	OOM2018_003*	1980	Biobox	Coral	PT1 PT2	RH M4 NPS
	Canyon Ribeira Brava	OOM2018_006*	1965	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_010*	1980	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_012*	1637	Biobox	Coral	PT1	M1 NPS M4

	Canyon Ribeira Brava	OOM2018_013*	1637	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_015*	1637	Biobox	Crinoidea (Sea-lily)	PT1 PT2	RH M4 NPS
	Canyon Ribeira Brava	OOM2018_018*	1634	Biobox	Sponge	PT1 PT2	RH M4 NPS
	Canyon Ribeira Brava	OOM2018_020*	1634	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_030*	504	Corer	Sediment	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_033*	498	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_034*	498	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_039*	448	Biobox	Sponge	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_040*	446	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_041*	446	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_044*	431	Biobox	Sponge	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_057*	656	Corer	Sediment	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_058*	656	Corer	Sediment	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_059*	656	Corer	Sediment	PT1	M1 NPS M4
	Canyon Ribeira Brava	OOM2018_071*	650	Biobox	Sponge	PT1 PT2	RH M4 NPS
	Canyon Ribeira Brava	OOM2018_072*	650	Biobox	Sponge	PT1	M1 NPS M4
	Off Funchal	OOM2018_081*	600	Biobox	Coral	PT1	M1 NPS M4
	Off Funchal	OOM2018_084*	600	Biobox	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	#001†	747	Lula1000 manned submersible	Sponge	PT1	M1 NPS M4
	Canyon Ribeira Brava	#004†	747	Lula1000 manned submersible	Sediment	PT1	M1 NPS M4
	Canyon Ribeira Brava	#014†	804	Lula1000 manned submersible	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	#017†	804	Lula1000 manned submersible	Coral	PT1	M1 NPS M4
	Canyon Ribeira Brava	#020†	463	Lula1000 manned submersible	Sponge	PT1	M1 NPS M4
	Canyon Ribeira Brava	#021†	463	Lula1000 manned submersible	Sponge	PT1	M1 NPS M4

	Canyon Ribeira Brava	#023†	463	Lula1000 manned submersible	Sponge	PT1	M1 NPS M4
	Off Garajau/Lazareto	#025†	593	Lula1000 manned submersible	Coral	PT1	M1 NPS M4
	Off Garajau/Lazareto	#026†	865	Lula1000 manned submersible	Sponge	PT1	M1 NPS M4
Azores	Flores Island	M150-ABH_003§	151	Boxcorer	Sediment	PT1	M1 NPS M4
	Flores Island	M150-ABH_004§	306	Hanning grab	Sponge	PT1	M1 NPS M4
	Flores Island	M150-ABH_018§	515	Boxcorer	Sediment	PT1	M1 NPS M4
	Flores Island	M150-ABH_041§	426-564	Agassiz Trawl	Sponge	PT1	M1 NPS M4
	Flores Island	M150-ABH_042§	2076	Multicorer	Sediment	PT1	M1 NPS M4
	Flores Island	M150-ABH_044§	2061	Multicorer	Sediment	PT1	M1 NPS M4
	Flores Island	M150-ABH_051§	515-579	Rocky dredge	Coral	PT1	M1 NPS M4
	Flores Island	M150-ABH_052§	515-579	Rocky dredge	Sponge	PT1	M1 NPS M4
	Flores Island	M150-ABH_053§	515-579	Rocky dredge	Sponge	PT1	M1 NPS M4
	Flores Island	M150-ABH_053§	519-579	Rocky dredge	Coral	PT1	M1 NPS M4
	Flores Island	M150-ABH_063§	1501-1516	Agassiz Trawl	Sediment	PT1	M1 NPS M4
	Flores Island	M150-ABH_069§	1501-1516	Agassiz Trawl	Sponge	PT1	M1 NPS M4
	Flores Island	M150-ABH_077§	1501-1516	Agassiz Trawl	Coral	PT1	M1 NPS M4
	Flores Island	M150-ABH_083§	1501-1516	Agassiz Trawl	Coral	PT1	M1 NPS M4
	Flores Island	M150-ABH_086§	968	Agassiz Trawl	Sediment	PT1	M1 NPS M4
	Flores Island	M150-ABH_094§	892-995	Boxcorer	Coral	PT1	M1 NPS M4
	Flores Island	M150-ABH_107§	892-995	Agassiz Trawl	Sponge	PT1	M1 NPS M4
	Flores Island	M150-ABH_110§	892-995	Agassiz Trawl	Sponge	PT1	M1 NPS M4
	Flores Island	M150-ABH_113§	892-995	Agassiz Trawl	Coral	PT1	M1 NPS M4
	Flores Island	M150-ABH_114§	892-995	Agassiz Trawl	Coral	PT1	M1 NPS M4
	Princess Alice Bank	M150-ABH_124§	892-995	Agassiz Trawl	Coral	PT1	M1 NPS M4

	Princess Alice Bank	M150-ABH_139§	2062	Agassiz Trawl	Sediment	PT1	M1 NPS M4
	Terceira Island	M150-ABH_143§	2019	Multicorer	Sediment	PT1	M1 NPS M4
	Terceira Island	M150-ABH_174§	1864	Multicorer	Sediment	PT1	M1 NPS M4
	Terceira Island	M150-ABH_190§	1535-1557	Multicorer	Coral	PT1	M1 NPS M4
	Terceira Island	M150-ABH_192§	1535-1557	Agassiz Trawl	Coral	PT1	M1 NPS M4
	Terceira Island	M150-ABH_193§	1535-1557	Agassiz Trawl	Coral	PT1	M1 NPS M4
	Terceira Island	M150-ABH_218§	2898	Agassiz Trawl	Sediment	PT1	M1 NPS M4
	Terceira Island	M150-ABH_271§	1975	Multicorer	Sediment	PT1	M1 NPS M4
	Terceira Island	M150-ABH_284§	2155-2220	Multicorer	Coral	PT1	M1 NPS M4
	Terceira Island	M150-ABH_285§	1500	Agassiz Trawl	Sediment	PT1	M1 NPS M4
	Formigas Bank	M150-ABH_313§	1500	Multicorer	Coral	PT1	M1 NPS M4
	Formigas Bank	M150-ABH_335§	496	Multicorer	Sediment	PT1	M1 NPS M4
	Terceira Island	M150-ABH_383§	151	Hanning grab	Sediment	PT1	M1 NPS M4

* Mission OOM2018_LUSO
Þ mission Deep_Madeira 2019
§ mission M150 BIODIAZ

4.3.2 Actinobacteria isolation

The deep-sea samples were thawed and, whenever applicable, washed with sterile water to remove looser particles (for corals, sponges and the sea-lily sample). The corals, sponges and the sea-lily were cut in small fragments and homogenized with a sterile mortar. One gram of each sample was added to one mL of sterile seawater and subjected to a pre-treatment consisting in incubation in a water bath at 57 °C for 15 min (PT1). For some samples an additional pre-treatment was also applied, which consisted in the incubation of the sample with antibiotics (nystatin, cycloheximide and nalidixic acid at 20 mg mL^{-1}) for 30 min (PT2). Both pre-treatments aimed to select spore-forming bacteria and stimulate the growth of rare and slow growing actinobacteria. The resulting samples were ten-fold diluted until 10^{-4} and an aliquot of 100 µl of each sample (10^{0} – 10^{-4}) was spread in duplicate over the surface of different isolation media, according to the information specified in Table 1. The selective isolation media used were: (i) Nutrient-

poor sediment extract (NPS) (per liter of seawater): 100 mL of marine sediment extract (obtained by washing 900 mL of sediments with 500 mL of seawater) and 17 g of agar; (ii) Raffinose-Histidine (RH) (per liter of 70% seawater and 30% deionized water): 10 g raffinose, 1 g L-histidine, 1 g KH_2PO_4 , 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_20$ and 17 g agar; (iii) M4 agar (per liter of seawater): 2 g chitin and 17 g agar; (iv) M1 agar (per liter of seawater): 10 g soluble starch, 4 g yeast extract, 2 g peptone and 17 g agar. All media were supplemented with cycloheximide, nalidixic acid and nystatin, at 50 mg L^{-1} each, to prevent the growth of fungi and Gram-negative bacteria. The plates were incubated for a period of up to 6 months at two different temperatures, 28 ºC and 5 °C, to enhance the recovery of actinobacteria with different growth requirements and metabolic profiles. Along the incubation period, plates were periodically inspected and all colonies with different morphological characteristics were picked and streaked on new agar plates until obtainment of pure colonies. Each isolate was cryopreserved at -80 ºC in 30% glycerol.

4.3.3 Phylogenetic identification of actinobacterial isolates

For all microorganisms isolated, genomic DNA was extracted from 1 mL of liquid culture (centrifuged for 5 min at 7000 g) using the E.Z.N.A.® Bacterial DNA Kit, following the manufacturer's instructions with a few modification steps as described in Chapter 2. 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), as described in Chapter 2. Purification and sequencing of the amplified DNA was performed by GenCore, I3S (Instituto de Investigação e Inovação em Saúde, Portugal). The obtained 16S rRNA sequences were analysed using the Geneious Prime 2022.1.2 software and the resulting consensus sequences were compared to the GenBank database using the blastn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The consensus sequences were additionally compared with the databases EzTaxon (http://www.ezbiocloud.net/) and Ribosomal Database Project (https://rdp.cme.msu.edu/index.jsp) to confirm the results obtained in the GenBank database.

4.3.4 Preparation of actinobacterial extracts

Actinobacterial crude extracts were prepared by growing each isolate in 100 mL Erlenmeyer flasks containing 30 mL of the respective isolation medium (M1, M4, RH or NPS), but without the supplementation of antibiotics (Table 2). The flasks were incubated at 28 °C, 100 rpm, in the dark for 3-5 days (depending on the growth rate of each strain),

after which 0.5 g of Amberlite® XAD16N resin was added to the cultures and left to incubate for two more days. Cultures were extracted twice with methanol/acetone 1:1 (v/v). The organic layer was dried in a rotary evaporator and the resulting extract was dissolved in pure DMSO (DMSO; ≥ 99.9%) to obtain stock solutions with final concentrations of 10.0 mg mL^{-1} and 1.0 mg mL^{-1}, to be used in the bioactivity assays.

4.3.5 Antimicrobial assays

The antimicrobial activity was assessed using the agar disc diffusion method. Actinobacterial crude extracts were tested against five reference strains: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Salmonella enterica* subsp*. enterica* serovar Typhimurium (ATCC 25241) and *Candida albicans* (ATCC 10231). The reference bacterial strains were suspended in Mueller-Hinton medium (MH) (Liofilchem, Roseto d. Abruzzi, Italy) and the yeast in Sabouraud Dextrose medium (SD) (Liofilchem, Roseto d. Abruzzi, Italy) and the optical density was adjusted to 0.5 McFarland standard (OD_{625}= 0.08−0.13). Blank discs (6 mm in diameter; Oxoid Limited, Hampshire, UK) loaded with 15 µL of each extract at the concentration of 1.0 mg mL^{-1} were placed on agar plates (MH or SD) seeded with the reference strains. Negative control consisted in 15 µL of pure DMSO and positive control consisted in 15 µL of enrofloxacin (1.0 mg mL^{-1}; Sigma-Aldrich, MO, USA) for the bacterial strains and 15 µL of nystatin (1.0 mg mL^{-1}; Sigma-Aldrich, MO, USA) for *C. albicans*. Agar plates were incubated at 37 °C for 24 h, after which the presence of an inhibition halo was analysed and the respective diameter was measured.

4.3.6 Anticancer assays

Anticancer activity was tested in two cancer cell lines, breast ductal carcinoma (T-47D) and liver hepatocellular carcinoma (HepG2), both from Sigma-Aldrich. The extracts were additionally tested on a non-cancer cell line, human brain capillary endothelial cells (hCMEC/D3), (donated by Dr. P. O. Courad, INSERM, France), to evaluate the general cytotoxicity. All the cell lines were cultivated in Dubelco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penincillin (100 IU mL^{-1})/streptomycin (10.0 mg mL^{-1}) and 0.1% (v/v) amphotericin, at 37 °C in a humidified atmosphere containing 5% of CO_2. MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed in 96-well plates seeded with cells at a density of 6.6×10^4 cells mL^{-1}. After 24 h, cells were exposed to actinobacterial extracts at a final concentration of 15 µg mL^{-1}. Solvent and positive controls consisted,

respectively, in 0.5% DMSO and staurosporine (1 µM). After 48 h, MTT (0.2 mg mL^{-1}, Sigma-Aldrich, MO, USA) was added to the plates and cells were incubated for 4 h at 37 °C. After this period, the medium was removed and 100 µL of DMSO were added per well to dissolve formazan crystals, and the plates were after read by spectrophotometry at 570 nm (Synergy HT, Biotek, Bart Frederick Shahr, Germany). The cell viability was measured after 48 h and calculated in all the cell lines. Each extract was tested in triplicate and in two independent assays.

4.3.7 Anti-inflammatory assays

The anti-inflammatory activity was performed using the Mouse macrophage cell line (RAW264.7) maintained in Dulbecco's Modified Eagle's Medium (DMEM) complemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin (100 IU mL^{-1})/streptomycin (10.0 mg mL^{-1}) and 0.1% (v/v) amphotericin, at 37 °C in a humidified atmosphere containing 5% of CO_2. Macrophage cells (density 3.5×10^5 cells mL^{-1}) were seeded in 96-well plates and incubated at 37 °C for 24 h. After this period, the medium was renewed and the cells were exposed to the actinobacterial extracts at a final concentration of 15 µg mL^{-1}, for 24 h. For the anti-inflammatory assays, the cells were treated with LPS (lipopolysaccharide, Sigma-Aldrich, MO, USA) at a final concentration of 200 µg/mL, while for the pro-inflammation assay no LPS was added to the cells. Solvent control consisted of 0.5% DMSO and the positive control of LPS at 200 µg/mL. Nitric oxide (NO) level was analysed by the Griess reaction to evaluate the cells inflammation. Cells culture supernatant (75 µl) was transferred to a new 96-well plate and 75 µl of Griess reagent (1% Sulfanilamide (w/v) and 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (w/v) in 2% phosphoric acid) was added. The plates were incubated for 15 min in the dark and the NO level was evaluated by reading the absorbance at 562 nm. In parallel, MTT assays were performed to determine cell viability under the two conditions, adding MTT to the original 96-well plates at a final concentration of 0.5 mg mL^{-1} and incubating the plates for 45 min at 37 °C. The supernatant was removed and 100 µl of DMSO were added at each well to solubilize the formazan crystals. The absorbance was read at 570 nm (Synergy HT, Biotek, Bart Frederick Shahr, Germany). The actinobacterial crude extracts were tested in triplicate and in two independent assays.

4.3.8 Anti-obesity assays

Zebrafish Nile red assay was performed to analyse the lipid-reducing activity of actinobacterial crude extracts. Zebrafish embryos from one DPF (days after fertilization)

were kept in egg water (60 µg ml^{-1} sea salt dissolved in distilled water) with 200 µM 1-phenyl-2-thiourea (PTU) to inhibit pigmentation. Between three to five DPF, zebrafish larvae were exposed to crude extracts at a final concentration of 10 µg mL^{-1}, with daily renewal of water and extracts, in a 48-well plate with a density of 6 to 8 larvae per well. Solvent control and positive control consisted of 0.1% DMSO and 50 µM resveratrol (REV), respectively. Neutral lipids were stained overnight with Nile red at the final concentration of 10 ng mL^{-1}. Fluorescence was analysed 5 min after the larvae were anesthetized with tricaine (MS-222, 0.03%). The extracts were tested in two independent assays.

4.3.9 Statistical analysis

The results of the cytotoxicity, anti-inflammatory and anti-obesity assays, with a total of six replicates for each extract, were statistically analysed for significant differences in relation to the solvent control. The value of $p < 0.05$ was established as a level of significance in all statistical tests. To verify the normality of the data, the Kolmogorov Smirnov test was used and, while for analysing equal variances, the Barthlett test was applied For parametric data, one-way ANOVA followed by Dunnet's post hoc test was employed. Data not meeting the one-way ANOVA criteria were square root transformed and retested for normality and equal variances. For non-parametric data, the Kruskal-Walli's test was used followed by Dunn's multiple comparison test.

4.3.10 Dereplication of the bioactive extracts

Extracts with activity in the antimicrobial, cytotoxic and/or anti-inflammatory assays were selected for metabolomic analysis, specifically MS-based dereplication and molecular networking analyses. Actinobacterial crude extracts were resuspended in methanol (2 mg mL^{-1}) and analysed by LC-HRESIMS/MS. This analysis was performed using the same approach as described in Chapter 2. The raw data resulting from the dereplication of actinobacterial extracts were converted to mzML format and submitted to the GNPS data analysis workflow employing standard parameters (except precursor ion and fragment ion mass tolerance, which was set to 0.02 Da, to take in consideration high resolution data). To complement all dereplication data, the extracts were submitted to the advanced dereplication analysis tools of GNPS platform. This analysis was carried out with the default parameters (except for the precursor ion and fragment ion mass tolerance, which was established to 0.005 Da, as these are high resolution data) and using the Insilico Peptidic Natural Products Dereplicator workflow and

DEREPLICATOR+ algorithm to *in silico* identification of natural products. For results annotated by the web-based mass spectrometry GNPS platform (using the classical molecular networking, in silico structure annotation and automated chemical classification) a threshold p-value ≤ 10^{-10} for DEREPLICATOR and DEREPLICATOR VarQuest, and a strict value of 15 for DEREPLICATER+ were applied to minimize false matches. All these matches were manually checked to see if they were associated with compounds produced by actinobacteria and/or marine organisms (sponges, corals and sea-lily) and discarded when they did not meet these criteria (Gurevich et al., 2018; Mohimani et al., 2017; Mohimani et al., 2018).

The MolNetEnhancer workflow, performed with the default parameters, was used to combine all the outputs: (i) classical molecular networking; (ii) *in silico* structure annotation (DEREPLICATOR, DEREPLICATOR VarQuest and DEREPLICATOR+); (iii) automated chemical classification (ClassyFire). The molecular network obtained was loaded into Cytoscape v3.6.1 to provide a chemical overview of metabolomic data and the distribution of m/z clusters between the different extracts.

4.4 Results

4.4.1 Actinobacteria isolation and identification

A total of 276 actinobacterial strains were isolated from the analysed deep-sea samples (Table 2). Taxonomic identification revealed that strains are distributed by 20 genera (from the most to the least abundant): *Brevibacterium*, *Microbacterium, Tsukamurella, Streptomyces, Nocardiopsis*, *Micromonospora, Rhodococcus*, *Brachybacterium*, *Dietzia*, *Salinispora*, *Micrococcus, Gordonia*, *Nocardioides*, *Leucobacter*, *Kocuria*, *Dermacoccus*, *Corynebacterium*, *Blastococcus*, *Arthrobacter* and *Actinopolymorpha*. All treatments and media applied allowed the isolation of actinobacterial strains, with the largest fraction belonging to the genera *Brevibacterium* and *Microbacterium*, a result obtained in the samples from both archipelagos (Fig.1). Among the less abundant genera, *Actinopolymorpha*, *Arthrobacter*, *Blastococcus, Dietzia, Leucobacter* and *Nocardioides* were only retrieved from the Madeira archipelago, while *Corynebacterium*, *Dermacoccus, Gordonia*, *Kocuria* and *Salinispora* were obtained only from the Azores archipelago.

Based on the similarity threshold of 98.7% for a new species, as established by Stackebrandt (2006), the taxonomic analysis of the obtained isolates suggests that three

Microbacterium strains (C_014 6, C_014 11 and C_192 3) and one *Brevibacterium* strain (Sed_044 18) might be new species (Table 10).

Table 10 – Taxonomic identification of all actinobacterial isolates recovered from the deep-sea samples collected from Madeira and Azores Archipelagos

Strain	Site	Sample code	Pre-treatment applied	Isolation medium	Closest Taxonomy º	Identity (%)
C_002 1.17	Madeira	OOM2018_002	PT2	M4	*Brevibacterium* sp.	100
C_002 1.2	Madeira	OOM2018_002	PT2	M4	*Nocardiopsis lucentensis*	99.93
C_002 1.9	Madeira	OOM2018_002	PT2	M4	*Nocardiopsis lucentensis*	99.86
C_002 2.6	Madeira	OOM2018_002	PT1	RH	*Brevibacterium* sp.	99.93
C_002 2.7	Madeira	OOM2018_002	PT1	RH	*Streptomyces rubrogriseus*	99.86
C_002 5.1.1	Madeira	OOM2018_002	PT2	M4	*Nocardiopsis lucentensis*	99.93
C_002 5.1.17	Madeira	OOM2018_002	PT2	M4	*Brevibacterium aureum*	99.93
C_002 5.2	Madeira	OOM2018_002	PT2	RH	*Rhodococcus erythropolis*	99.93
C_003 1.1	Madeira	OOM2018_003	PT2	RH	*Brevibacterium* sp.	100
C_003 1.11	Madeira	OOM2018_003	PT2	M4	*Streptomyces* sp.	99.93
C_003 1.12	Madeira	OOM2018_003	PT2	M4	*Tsukamurella* sp.	99.93
C_003 1.16	Madeira	OOM2018_003	PT2	M4	*Nocardiopsis* sp.	99.93
C_003 1.17	Madeira	OOM2018_003	PT2	M4	*Streptomyces* sp.	99.79
C_003 1.18	Madeira	OOM2018_003	PT2	M4	*Streptomyces chumphonensis*	99.93
C_003 1.3	Madeira	OOM2018_003	PT2	RH	*Streptomyces* sp.	99.86
C_003 1.4	Madeira	OOM2018_003	PT2	RH	*Brevibacterium* sp.	99.93
C_003 1.8	Madeira	OOM2018_003	PT2	RH	*Brachybacterium paraconglomeratum*	99.49
C_003 1.9	Madeira	OOM2018_003	PT2	NPS	*Streptomyces xinghaiensis*	99.93
C_003 2.1	Madeira	OOM2018_003	PT1	RH	*Brevibacterium* sp.	99.71
C_003 2.2	Madeira	OOM2018_003	PT1	RH	*Brevibacterium aureum*	99.7
C_003 2.7	Madeira	OOM2018_003	PT1	M4	*Tsukamurella* sp.	99.85
C_003 2.9	Madeira	OOM2018_003	PT1	M4	*Brevibacterium* sp.	99.3
C_003 5.1.4	Madeira	OOM2018_003	PT2	RH	*Brevibacterium* sp.	99.86
C_003 5.1.7.	Madeira	OOM2018_003	PT2	RH	*Streptomyces* sp.	99.56
C_003 5.2.3	Madeira	OOM2018_003	PT1	RH	*Microbacterium aerolatum*	99.64
C_003 5.2.7	Madeira	OOM2018_003	PT1	RH	*Brevibacterium* sp.	100
C_006 1.12	Madeira	OOM2018_006	PT2	RH	*Nocardiopsis lucentensis*	99.85
C_006 1.7	Madeira	OOM2018_006	PT2	RH	*Nocardiopsis lucentensis*	99.92
C_006 2.2	Madeira	OOM2018_006	PT1	RH	*streptomyces* sp.	99.86
C_006 5.1.2	Madeira	OOM2018_006	PT2	RH	*Brevibacterium* sp.	100
C_006 5.1.3	Madeira	OOM2018_006	PT2	RH	*Brevibacterium antarcticum*	98.93
C_006 5.1.4	Madeira	OOM2018_006	PT2	M4	*Blastococcus aggregatus*	99.54
C_006 5.1.5	Madeira	OOM2018_006	PT2	M4	*Rhodococcus erythropolis*	99.93
C_006 5.1.6	Madeira	OOM2018_006	PT2	M4	*Rhodococcus erythropolis*	99.93
C_006 5.2.1	Madeira	OOM2018_006	PT1	NPS	*Rhodococcus erythropolis*	99.93
C_006 5.2.3	Madeira	OOM2018_006	PT1	RH	*Brachybacterium paraconglomeratum*	99.48
C_006 5.2.5	Madeira	OOM2018_006	PT1	RH	*Streptomyces* sp.	99.85

C_006 5.2.7.	Madeira	OOM2018_006	PT1	RH	*Microbacterium aerolatum*	99.5
C_006 5.2.8	Madeira	OOM2018_006	PT1	M4	*Dietzia maris*	100
C_012 10	Madeira	OOM2018_012	PT1	M1	*Microbacterium amylolyticum*	100
C_012 14	Madeira	OOM2018_012	PT1	NPS	*Brevibacterium aureum*	99.93
C_012 16	Madeira	OOM2018_012	PT1	NPS	*Tsukamurella tyrosinosolvens*	100
C_012 2	Madeira	OOM2018_012	PT1	M1	*Brevibacterium siliguriense*	99.28
C_012 6	Madeira	OOM2018_012	PT1	M1	*Microbacterium* sp.	99.71
C_013 1	Madeira	OOM2018_013	PT1	M1	*Tsukamurella* sp.	99.93
C_013 2	Madeira	OOM2018_013	PT1	M4	*Microbacterium aerolatum*	99.93
C_014 1	Madeira	#014	PT1	M1	*Brevibacterium sediminis*	99.86
C_014 10	Madeira	#014	PT1	NPS	*Brevibacterium siliguriense*	99
C_014 11	Madeira	#014	PT1	NPS	*Microbacterium aerolatum**	98.35
C_014 12	Madeira	#014	PT1	NPS	*Tsukamurella tyrosinosolvens*	100
C_014 13	Madeira	#014	PT1	NPS	*Tsukamurella tyrosinosolvens*	99.86
C_014 16	Madeira	#014	PT1	M4	*Rhodococcus erythropolis*	99.71
C_014 6	Madeira	#014	PT1	M4	*Microbacterium ginsengiterrae**	98.71
C_014 7	Madeira	#014	PT1	NPS	*Rhodococcus qingshengii*	99.93
C_014 9	Madeira	#014	PT1	NPS	*Brachybacterium rhamnosum*	99.06
C_017 1	Madeira	#017	PT1	M1	*Microbacterium amylolyticum*	99.71
C_017 2	Madeira	#017	PT1	M1	*Brevibacterium sediminis*	99.64
C_017 3	Madeira	#017	PT1	NPS	*Brevibacterium sediminis*	99.86
C_017 5	Madeira	#017	PT1	NPS	*Microbacterium amylolyticum*	99.93
C_017 6	Madeira	#017	PT1	M1	*Microbacterium aerolatum*	99.78
C_017 7	Madeira	#017	PT1	M4	*Brevibacterium aureum*	100
C_025 1	Madeira	#025	PT1	M1	*Leucobacter komagatae*	99.71
C_025 11	Madeira	#025	PT1	NPS	*Brevibacterium aureum*	99.93
C_025 2	Madeira	#025	PT1	M1	*Micrococcus luteus*	99.43
C_025 3	Madeira	#025	PT1	NPS	*Tsukamurella tyrosinosolvens*	100
C_025 4	Madeira	#025	PT1	M1	*Microbacterium aerolatum*	99.5
C_025 6	Madeira	#025	PT1	NPS	*Brevibacterium aureum*	99.64
C_025 7	Madeira	#025	PT1	NPS	*Microbacterium aerolatum*	99.42
C_025 9A	Madeira	#025	PT1	M4	*Microbacterium aerolatum*	99.86
C_033 11	Madeira	OOM2018_033	PT1	M1	*Actinopolymorpha rutila*	99.71
C_033 13	Madeira	OOM2018_033	PT1	NPS	*Microbacterium aerolatum*	99.54
C_033 14	Madeira	OOM2018_033	PT1	NPS	*Arthrobacter luteolus*	99.71
C_033 18	Madeira	OOM2018_033	PT1	M1	*Rhodococcus erythropolis*	99.93
C_033 4	Madeira	OOM2018_033	PT1	M1	*Brevibacterium aureum*	99.93
C_034 1	Madeira	OOM2018_034	PT1	M4	*Brevibacterium aureum*	99.93

C_034 4	Madeira	OOM2018_034	PT1	NPS	*Tsukamurella strandjordii*	99.26
C_034 5	Madeira	OOM2018_034	PT1	NPS	*Brevibacterium aureum*	99.78
C_034 6	Madeira	OOM2018_034	PT1	NPS	*Brevibacterium aureum*	99.93
C_034 7	Madeira	OOM2018_034	PT1	NPS	*Brevibacterium aureum*	99.78
C_040 6	Madeira	OOM2018_040	PT1	NPS	*Streptomyces* sp.	100
C_051 2	Azores	M150-ABH_051	PT1	M1	*Streptomyces* sp.	100
C_051 4	Azores	M150-ABH_051	PT1	M4	*Nocardiopsis salina*	98.86
C_063 8	Azores	M150-ABH_063	PT1	M1	*Salinispora goodfellowii*	99.78
C_063 1	Azores	M150-ABH_063	PT1	M4	*Microbacterium aerolatum*	99.42
C_063 12	Azores	M150-ABH_063	PT1	M1	*Microbacterium aerolatum*	99.56
C_063 2 (A)	Azores	M150-ABH_063	PT1	M4	*Microbacterium aerolatum*	99.57
C_063 3	Azores	M150-ABH_063	PT1	NPS	*Nocardiopsis prasina*	99.93
C_063 4	Azores	M150-ABH_063	PT1	M1	*Streptomyces sp*	99.93
C_063 5	Azores	M150-ABH_063	PT1	M1	*Microbacterium aerolatum*	99.78
C_063 9	Azores	M150-ABH_063	PT1	M4	*Salinispora goodfellowii*	99.78
C_081 10	Madeira	OOM2018_081	PT1	NPS	*Brevibacterium* sp.	100
C_081 12	Madeira	OOM2018_081	PT1	M1	*Rhodococcus erythropolis*	99.93
C_081 5	Madeira	OOM2018_081	PT1	M1	*Nocardiopsis lucentensis*	99.93
C_081 6 A	Madeira	OOM2018_081	PT1	NPS	*Nocardiopsis lucentensis*	99.86
C_083 10	Madeira	OOM2018_083	PT1	M1	*Brevibacterium* sp.	100
C_083 11	Madeira	OOM2018_083	PT1	M1	*Brachybacterium paraconglomeratum*	99.85
C_083 11 (B)	Madeira	OOM2018_083	PT1	M4	*Rhodococcus erythropolis*	99.93
C_084 5	Madeira	OOM2018_084	PT1	NPS	*Tsukamurella* sp.	100
C_084 6	Madeira	OOM2018_084	PT1	M4	*Micromonospora* sp.	100
C_083 3	Azores	M150-ABH_086	PT1	NPS	*Brevibacterium* sp.	99.86
C_107 6	Azores	M150-ABH_107	PT1	M1	*Streptomyces* sp.	99.93
C_107 7	Azores	M150-ABH_107	PT1	NPS	*Kocuria* sp.	100
C_124 10	Azores	M150-ABH_124	PT1	M1	*Tsukamurella tyrosinosolvens*	99.93
C_124 4	Azores	M150-ABH_124	PT1	NPS	*Brevibacterium aureum*	99.93
C_124 5	Azores	M150-ABH_124	PT1	NPS	*Brevibacterium* sp.	100
C_124 7	Azores	M150-ABH_124	PT1	NPS	*Brachybacterium* sp.	99.42
C_124 8	Azores	M150-ABH_124	PT1	M1	*Micromonospora chokoriensis*	99.56
C_124 9	Azores	M150-ABH_124	PT1	M4	*Tsukamurella tyrosinosolvens*	99.93
C_192 1	Azores	M150-ABH_192	PT1	M1	*Nocardiopsis lucentensis*	99.86
C_192 2	Azores	M150-ABH_192	PT1	M1	*Brevibacterium* sp.	99.93
C_192 3 (B)	Azores	M150-ABH_192	PT1	M1	*Brachybacterium paraconglomeratum*	99.85
C_192 3	Azores	M150-ABH_192	PT1	M1	*Microbacterium aerolatum**	98.48
C_193 3	Azores	M150-ABH_193	PT1	NPS	*Brevibacterium* sp.	99.86
C_218 4	Azores	M150-ABH_218	PT1	M1	*Brevibacterium* sp.	100
C_285 12	Azores	M150-ABH_285	PT1	M1	*Tsukamurella* sp.	100
C_285 13	Azores	M150-ABH_285	PT1	M1	*Microbacterium* sp.	100
C_285 4	Azores	M150-ABH_285	PT1	M1	*Micrococcus luteus*	99.93
C_285 7	Azores	M150-ABH_285	PT1	M1	*Brevibacterium* sp.	100
C_335 1	Azores	M150-ABH_335	PT1	M1	*Brevibacterium aureum*	99.93

C_335 2	Azores	M150-ABH_335	PT1	M4	*Microbacterium* sp.	99.93
C_335 3	Azores	M150-ABH_335	PT1	NPS	*Tsukamurella tyrosinosolvens*	100
L_015 5.1.10	Madeira	OOM2018_015	PT2	NPS	*Rhodococcus erythropolis*	99.93
L_015 5.1.5	Madeira	OOM2018_015	PT2	NPS	*Rhodococcus erythropolis*	99.93
L_015 5.1.6	Madeira	OOM2018_015	PT2	NPS	*Rhodococcus erythropolis*	99.93
L_015 5.1.7	Madeira	OOM2018_015	PT2	NPS	*Rhodococcus erythropolis*	99.93
L_015 5.2.8	Madeira	OOM2018_015	PT1	NPS	*Brevibacterium* sp.	100
S_001 1	Madeira	#001	PT1	M1	*Brevibacterium sediminis*	99.86
S_001 10	Madeira	#001	PT1	M1	*Microbacterium ginsengiterrae*	98.85
S_001 12	Madeira	#001	PT1	NPS	*Tsukamurella tyrosinosolvens*	99.78
S_001 2	Madeira	#001	PT1	M1	*Brevibacterium siliguriense*	98.99
S_001 4	Madeira	#001	PT1	M1	*Microbacterium aerolatum*	100
S_001 5	Madeira	#001	PT1	M1	*Tsukamurella tyrosinosolvens*	99.78
S_004 6	Azores	M150-ABH_004	PT1	M1	*Micromonospora tulbaghiae*	99.93
S_004 11	Azores	M150-ABH_004	PT1	M1	*Microbacterium aerolatum*	99.86
S_004 2	Azores	M150-ABH_004	PT1	M4	*Brevibacterium* sp.	99.35
S_004 4	Azores	M150-ABH_004	PT1	M1	*Microbacterium aerolatum*	100
S_018 1.10	Madeira	OOM2018_018	PT2	M4	*Brevibacterium* sp.	99.64
S_018 1.29	Madeira	OOM2018_018	PT2	RH	*Brevibacterium* sp.	99.93
S_018 1.4	Madeira	OOM2018_018	PT2	M4	*Dietzia cercidiphylli*	99.49
S_018 2.6	Madeira	OOM2018_018	PT1	M4	*Brevibacterium* sp.	99.93
S_018 5.1.1	Madeira	OOM2018_018	PT2	RH	*Streptomyces* sp.	100
S_018 5.1.11	Madeira	OOM2018_018	PT2	RH	*Dietzia* sp.	99.93
S_018 5.1.12	Madeira	OOM2018_018	PT2	M4	*Dietzia* sp.	99.85
S_018 5.1.3	Madeira	OOM2018_018	PT2	M4	*Dietzia* sp.	99.38
S_018 5.2.3	Madeira	OOM2018_018	PT1	M4	*Streptomyces* sp.	99.71
S_018 5.4	Madeira	OOM2018_018	PT2	M4	*Streptomyces* sp.	99.93
S_018 5.8	Madeira	OOM2018_018	PT2	M4	*Brevibacterium antarcticum*	99.86
S_020 10	Madeira	#020	PT1	M1	*Tsukamurella tyrosinosolvens*	99.78
S_020 13	Madeira	#020	PT1	NPS	*Brevibacterium aureum*	99.79
S_020 2	Madeira	#020	PT1	M1	*Tsukamurella tyrosinosolvens*	99.93
S_020 3	Madeira	#020	PT1	M1	*Brevibacterium aureum*	99.93
S_020 4	Madeira	#020	PT1	M1	*Tsukamurella tyrosinosolvens*	99.86
S_020 5	Madeira	#020	PT1	M1	*Brevibacterium sediminis*	99.86
S_020 6	Madeira	#020	PT1	NPS	*Tsukamurella tyrosinosolvens*	99.93
S_020 8	Madeira	#020	PT1	NPS	*Tsukamurella tyrosinosolvens*	99.93
S_020 9	Madeira	#020	PT1	M1	*Tsukamurella strandjordii*	99.14
S_021 1	Madeira	#021	PT1	M4	*Tsukamurella tyrosinosolvens*	99.93
S_021 2	Madeira	#021	PT1	NPS	*Brevibacterium aureum*	99.79
S_021 3	Madeira	#021	PT1	NPS	*Brevibacterium sediminis*	99.35
S_023 3	Madeira	#023	PT1	NPS	*Tsukamurella tyrosinosolvens*	99.86

S_023 4	Madeira	#026	PT1	NPS	*Microbacterium aerolatum*	99.64
S_026 16	Madeira	#026	PT1	NPS	*Microbacterium aerolatum*	99.78
S_026 1	Madeira	#026	PT1	M1	*Brevibacterium sediminis*	99.71
S_026 10	Madeira	#026	PT1	M4	*Brevibacterium aureum*	100
S_026 13	Madeira	#026	PT1	M4	*Brevibacterium aerolatum*	99.71
S_026 15	Madeira	#026	PT1	NPS	*Brevibacterium sediminis*	99.86
S_026 17	Madeira	#026	PT1	NPS	*Microbacterium ginsengiterrae*	98.84
S_026 19	Madeira	#026	PT1	M4	*Brevibacterium sediminis*	99.43
S_026 2	Madeira	#026	PT1	M1	*Microbacterium aerolatum*	99.71
S_026 3	Madeira	#026	PT1	M1	*Microbacterium oxydans*	99.78
S_026 4	Madeira	#026	PT1	M1	*Brevibacterium sediminis*	99.86
S_026 5	Madeira	#026	PT1	M1	*Microbacterium aerolatum*	99.93
S_026 6	Madeira	#026	PT1	M1	*Microbacterium aerolatum*	99.78
S_026 7	Madeira	#026	PT1	M1	*Brevibacterium sediminis*	99.85
S_026 8	Madeira	#026	PT1	NPS	*Brevibacterium sediminis*	99.57
S_026 9	Madeira	#026	PT1	NPS	*Brevibacterium sediminis*	99.93
S_039 10	Madeira	OOM2018_039	PT1	M4	*Brevibacterium aureum*	99.93
S_039 4	Madeira	OOM2018_039	PT1	NPS	*Streptomyces* sp.	100
S_041 11	Azores	M150-ABH_041	PT1	M1	*Microbacterium aerolatum*	99.6
S_041 16	Azores	M150-ABH_041	PT1	NPS	*Nocardiopsis prasina*	99.64
S_041 3	Azores	M150-ABH_041	PT1	NPS	*Brevibacterium siliguriense*	99.2
S_041 8	Azores	M150-ABH_041	PT1	NPS	*Microbacterium aerolatum*	99.57
S_044 20	Madeira	OOM2018_044	PT1	M4	*Streptomyces* sp.	99.93
S_044 25	Madeira	OOM2018_044	PT1	M1	*Streptomyces* sp.	99.93
S_044 3	Madeira	OOM2018_044	PT1	NPS	*Microbacterium amylolyticum*	99.86
S_052 2	Azores	M150-ABH_052	PT1	M4	*Brevibacterium* sp.	99.93
S_052 3 (B)	Azores	M150-ABH_052	PT1	M4	*Brevibacterium* sp.	99.93
S_053 1	Azores	M150-ABH_053	PT1	M4	*Tsukamurella strandjordii*	99.93
S_053 2	Azores	M150-ABH_053	PT1	NPS	*Tsukamurella strandjordii*	99.93
S_053 3	Azores	M150-ABH_053	PT1	M1	*Brevibacterium aureum*	100
S_053 4	Azores	M150-ABH_053	PT1	M1	*Brevibacterium* sp.	99.93
S_071 1.15	Madeira	OOM2018_071	PT2	M4	*Streptomyces xinghaiensis*	99.93
S_071 1.16	Madeira	OOM2018_071	PT2	M4	*Tsukamurella tyrosinosolvens*	99.93
S_071 1.3	Madeira	OOM2018_071	PT2	RH	*Tsukamurella tyrosinosolvens*	99.93
S_071 1.5	Madeira	OOM2018_071	PT2	RH	*Brevibacterium* sp.	99.86
S_071 2.10	Madeira	OOM2018_071	PT1	RH	*Streptomyces intermedius*	99.78
S_071 2.11	Madeira	OOM2018_071	PT1	RH	*Brevibacterium* sp.	99.86
S_071 2.12	Madeira	OOM2018_071	PT1	RH	*Brevibacterium* sp.	100
S_071 2.9	Madeira	OOM2018_071	PT1	RH	*Streptomyces* sp.	99.78
S_071 5.1.30	Madeira	OOM2018_071	PT2	RH	*Nocardioides salarius*	99.12
S_077 1	Azores	M150-ABH_077	PT1	NPS	*Brevibacterium siliguriense*	99.93

S_077 16	Azores	M150-ABH_077	PT1	NPS	*Nocardiopsis lucentensis*	99.93
S_077 3	Azores	M150-ABH_077	PT1	NPS	*Microbacterium aerolatum*	99.93
S_077 4	Azores	M150-ABH_077	PT1	M4	*Microbacterium aerolatum*	99.93
S_077 7	Azores	M150-ABH_077	PT1	M1	*Brevibacterium* sp.	99.93
S_077 9	Azores	M150-ABH_077	PT1	M1	*Tsukamurella tyrosinosolvens*	99.93
S_110 3	Azores	M150-ABH_110	PT1	M1	*Corynebacterium aurimucosum*	100
S_113 4	Azores	M150-ABH_113	PT1	NPS	*Brevibacterium aureum*	99.93
S_113 5	Azores	M150-ABH_113	PT1	M1	*Nocardiopsis lucentensis*	99.86
S_113 7	Azores	M150-ABH_113	PT1	M1	*Nocardiopsis lucentensis*	99.93
Sed_003 2	Azores	M150-ABH_003	PT1	NPS	*Streptomyces* sp.	99.86
Sed_003 5	Azores	M150-ABH_003	PT1	NPS	*Streptomyces globisporus*	99.64
Sed_003 6	Azores	M150-ABH_003	PT1	M1	*Microbacterium aerolatum*	99.86
Sed_004 12	Madeira	#004	PT1	NPS	*Micrococcus luteus*	100
Sed_004 13	Madeira	#004	PT1	NPS	*Brachybacterium paraconglomeratum*	99.35
Sed_004 2	Madeira	#004	PT1	M1	*Microbacterium ginsengiterrae*	98.92
Sed_004 22	Madeira	#004	PT1	M1	*Brevibacterium sediminis*	99.72
Sed_004 27	Madeira	#004	PT1	NPS	*Microbacterium aerolatum*	99.78
Sed_004 28	Madeira	#004	PT1	M4	*Tsukamurella tyrosinosolvens*	100
Sed_018 11	Azores	M150-ABH_018	PT1	NPS	*Micromonospora coriariae*	99.42
Sed_018 14	Azores	M150-ABH_018	PT1	M4	*Brevibacterium aureum*	99.78
Sed_044 10	Azores	M150-ABH_044	PT1	NPS	*Tsukamurella tyrosinosolvens*	99.86
Sed_044 11	Azores	M150-ABH_044	PT1	NPS	*Gordonia sputi*	99.93
Sed_044 16	Azores	M150-ABH_044	PT1	M1	*Tsukamurella tyrosinosolvens*	99.86
Sed_044 18	Azores	M150-ABH_044	PT1	M1	*Brevibacterium antiquum**	98.01
Sed_044 21	Azores	M150-ABH_044	PT1	NPS	*Brachybacterium paraconglomeratum*	99.63
Sed_044 26	Azores	M150-ABH_044	PT1	NPS	*Microbacterium aerolatum*	99.28
Sed_044 27	Azores	M150-ABH_044	PT1	NPS	*Nocardiopsis*	99.93
Sed_044 3	Azores	M150-ABH_044	PT1	M1	*Micromonospora coxensis*	99.93
Sed_044 4	Azores	M150-ABH_044	PT1	M4	*Gordonia sputi*	99.93
Sed_044 5	Azores	M150-ABH_044	PT1	NPS	*Streptomyces badius*	99.85
Sed_044 6	Azores	M150-ABH_044	PT1	NPS	*Brevibacterium siliguriense*	99.21
Sed_044 7	Azores	M150-ABH_044	PT1	NPS	*Gordonia sputi*	100
Sed_058 1	Madeira	OOM2018_058	PT1	M1	*Streptomyces* sp.	100
Sed_058 2	Madeira	OOM2018_058	PT1	M1	*Streptomyces microflavus*	99.93
Sed_058 4	Madeira	OOM2018_058	PT1	NPS	*Tsukamurella tyrosinosolvens*	99.93
Sed_058 5	Madeira	OOM2018_058	PT1	NPS	*Streptomyces* sp.	99.93
Sed_058 8	Madeira	OOM2018_058	PT1	NPS	*Brachybacterium paraconglomeratum*	99.44
Sed_058 9	Madeira	OOM2018_058	PT2	M1	*Rhodococcus cercidiphylli*	99.78
Sed_059 14	Madeira	OOM2018_059	PT1	NPS	*Brevibacterium celere*	99.25
Sed_059 17	Madeira	OOM2018_059	PT1	M4	*Streptomyces* sp.	99.93
Sed_059 3	Madeira	OOM2018_059	PT1	M4	*Streptomyces griseorubens*	100

Sed_069 1	Azores	M150-ABH_069	PT1	NPS	*Nocardiopsis lucentensis*	99.93
Sed_069 12	Azores	M150-ABH_069	PT1	M4	*Nocardiopsis lucentensis*	99.07
Sed_069 4	Azores	M150-ABH_069	PT1	M4	*Microbacterium aerolatum*	99.57
Sed_143 1	Madeira	OOM2018_143	PT1	M1	*Micromonospora schwarzwaldensis*	99.28
Sed_143 3	Madeira	OOM2018_143	PT1	NPS	*Brachybacterium paraconglomeratum*	99.2
Sed_143 7	Madeira	OOM2018_143	PT1	NPS	*Brachybacterium paraconglomeratum*	99.85
Sed_174 1	Azores	M150-ABH_174	PT1	M1	*Salinispora* sp.	99.85
Sed_174 14	Azores	M150-ABH_174	PT1	M1	*Brachybacterium paraconglomeratum*	99.35
Sed_174 3	Azores	M150-ABH_174	PT1	NPS	*Brevibacterium* sp.	100
Sed_174 6	Azores	M150-ABH_174	PT1	NPS	*Micromonospora* sp.	99.78
Sed_271 10	Azores	M150-ABH_271	PT1	NPS	*Microbacterium aerolatum*	99
Sed_271 2	Azores	M150-ABH_271	PT1	NPS	*Brevibacterium* sp.	99.28
Sed_271 3	Azores	M150-ABH_271	PT1	NPS	*Microbacterium amylolyticum*	99.86
Sed_271 6	Azores	M150-ABH_271	PT1	NPS	*Microbacterium aerolatum*	99.5
Sed_271 9	Azores	M150-ABH_271	PT1	M1	*Brachybacterium paraconglomeratum*	99.42
Sed_284 10	Azores	M150-ABH_284	PT1	NPS	*Micromonospora chokoriensis*	99.49
Sed_284 11	Azores	M150-ABH_284	PT1	NPS	*Micromonospora tulbaghiae*	99.86
Sed_284 15	Azores	M150-ABH_284	PT1	NPS	*Brachybacterium* sp.	99.93
Sed_284 2	Azores	M150-ABH_284	PT1	NPS	*Microbacterium* sp.	99.21
Sed_313 11	Azores	M150-ABH_313	PT1	NPS	*Brevibacterium siliguriense*	99.71
Sed_313 15	Azores	M150-ABH_313	PT1	NPS	*Micromonospora tulbaghiae*	99.71
Sed_313 25	Azores	M150-ABH_313	PT1	NPS	*Micromonospora noduli*	99.64
Sed_313 26	Azores	M150-ABH_313	PT1	NPS	*Micromonospora saelicesensis*	100
Sed_313 29	Azores	M150-ABH_313	PT1	NPS	*Microbacterium aerolatum*	99.78
Sed_313 29 (B)	Azores	M150-ABH_313	PT1	NPS	*Micromonospora* sp.	99.78
Sed_313 31	Azores	M150-ABH_313	PT1	NPS	*Micromonospora noduli*	99.86
Sed_313 32	Azores	M150-ABH_313	PT1	M4	*Micromonospora noduli*	99.44
Sed_313 36	Azores	M150-ABH_313	PT1	M4	*Tsukamurella tyrosinosolvens*	99.7
Sed_383 1	Azores	M150-ABH_383	PT1	NPS	*Microbacterium aerolatum*	99.49
Sed_383 10	Azores	M150-ABH_383	PT1	NPS	*Salinispora pacifica*	99.85
Sed_383 11	Azores	M150-ABH_383	PT1	NPS	*Microbacterium aerolatum*	99.49
Sed_383 14	Azores	M150-ABH_383	PT1	NPS	*Micromonospora chokoriensis*	99.42
Sed_383 19	Azores	M150-ABH_383	PT1	NPS	*Dermacoccus profundi*	99.78
Sed_383 3	Azores	M150-ABH_383	PT1	NPS	*Micromonospora tulbaghiae*	99.78

º According to 16S ribosomal RNA sequences (Bacteria and Archaea) from NCBI Blast

* Novel species

The genera *Actinopolymorpha*, *Arthrobacter*, *Blastococcus*, *Kocuria* and *Leucobacter* were isolated only from corals samples, with 121 actinobacterial strains being recovered

in total from these samples. Eighty-four actinobacterial isolates were recovered from the samples of deep-sea sponges, being distributed by the genera *Brevibacterium, Dietzia, Microbacterium, Micromonspora, Nocardiopsis, Streptomyces, Tsukamurella, Corynebacterium* and *Nocardioides,* with the latter two genera being only recovered from a sponge collected in Azores and in Madeira, respectively. Sixty-eight actinobacterial strains were isolated from the sediment samples and these were affiliated with twelve actinobacterial genera: *Brachybacterium*, *Brevibacterium*, *Dermacoccus*, *Gordonia*, *Microbacterium*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus*, *Salinispora*, *Streptomyces* and *Tsukamurella*. For the single sample of sea-lily available, 5 actinobacterial isolates, phylogenetically associated with the genera *Brachybacterium* and *Rhodococcus*, were retrieved.

The selective isolation media NPS and M1 led to the isolation of the highest number of actinobacterial isolates in terms of absolute numbers. However, taking into account the fact that the selective culture media used in our study were not employed for the cultivation of all samples (NPS and M4 media were used for all deep-sea samples (n=66); M1 medium was used for 61 samples and RH medium was used for 5 samples), the medium that allowed retrieving a higher number of isolates per sample was actually RH (Fig. 1).

In terms of the pre-treatments use in this study, PT1 was applied to all deep-sea samples (n=66), which allowed retrieving a high number of actinobacterial isolates (n=233) while PT2 was applied to only 5 samples. Thus, analysing the number of isolates that each pre-treatment allowed to obtain on average per sample, PT2 revealed to be more efficient since it led to the isolation of an average of 10 actinobacterial strains per sample, in contrast to PT1 which allowed the retrieval of an average of 3 actinobacterial strains per sample. Moreover, strains of the genera *Blastococcus* and *Nocardiodes* were only obtained after application of PT2.

The incubation temperature of 28 °C led to the retrieval of a higher number of isolates but incubating at 5 °C allowed the isolation of *Arthrobacter*, *Blastococcus* and *Nocardiodes* species that could not be retrieved at 28 °C (Fig.9).

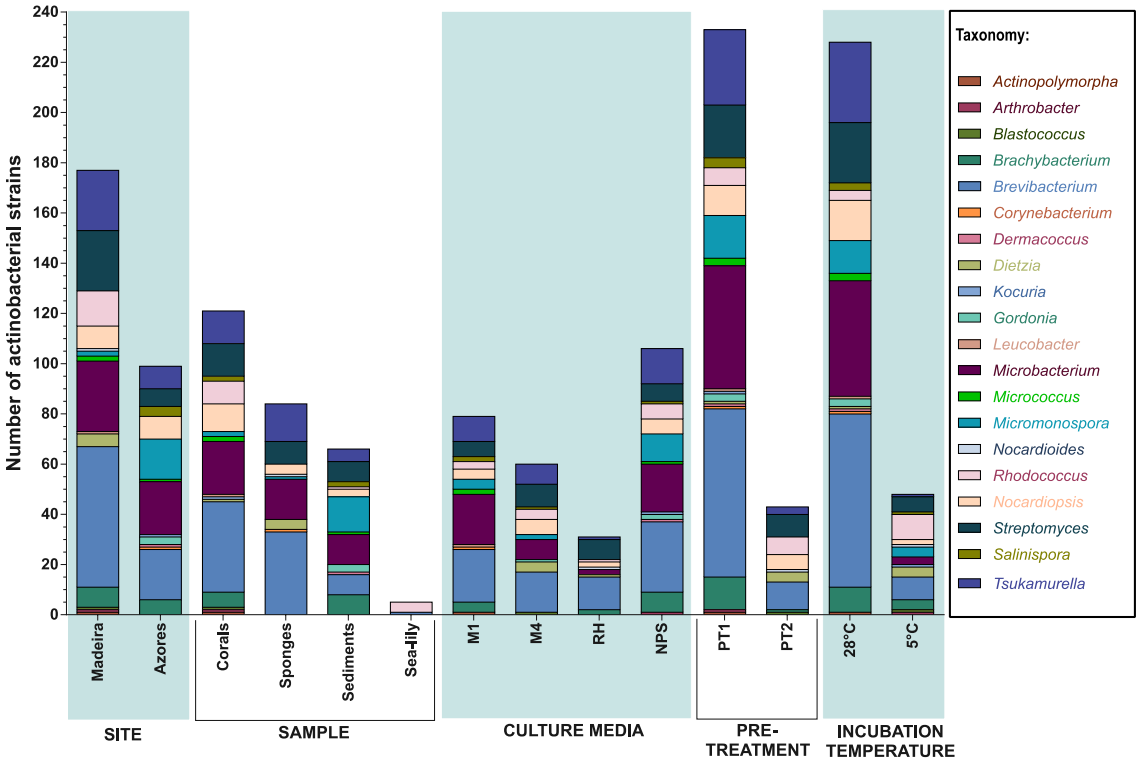

Figure 9 – Distribution of the actinobacterial strains isolated from the deep-sea samples collected in Madeira and Azores archipelagos (NE Atlantic Ocean), according to the abundance: (i) in each archipelago; (ii) per sample type; (iii) per selective culture media employed; (iv) per applied pre-treatments (PT1, Water bath at 57 °C for 15 min; PT2, Incubation with antibiotics (20 mg mL $^{-1}$) for 30 min; (v) per incubation temperatures.

4.4.2 Bioactivities of the actinobacterial isolates

Antimicrobial assays revealed sixteen actinobacterial strains (13 isolated from Madeira and 3 from Azores, belonging to the genera *Brachybacterium*, *Brevibacterium*, *Nocardiopsis* and *Streptomyces*) active against one or more of the tested microorganisms - *Bacillus subtilis*, *Candida albicans* and *Staphylococcus aureus,* producing halos with diameters between 8 and 24 mm (Table 11).

Table 11 – Deep-sea actinobacterial crude extracts with antimicrobial activity indicated by the diameter of inhibition halo (mm) in the agar disk diffusion method

Site	Strain code	Closest taxonomic identification	Disk diffusion method (mm)		
			S. aureus	*B. subtilis*	*C. albicans*
Madeira	C_003 1.3	*Streptomyces* sp.			
Madeira	C_006 2.2	*Streptomyces rubrogriseus*			
Madeira	C_014 9	*Brachybacterium rhamnosum*			
Madeira	C_040 6	*Streptomyces* sp.			
Madeira	S_018 1.29	*Brevibacterium* sp.			
Madeira	S_018 5.1	*Streptomyces* sp.			
Madeira	S_026 1	*Brevibacterium sediminis*			
Madeira	S_039 4	*Streptomyces* sp.			
Madeira	S_044 25	*Streptomyces* sp.			
Madeira	S_071 2.9	*Streptomyces* sp.			
Madeira	Sed_058 1	*Streptomyces* sp.			
Madeira	Sed_058 2	*Streptomyces microflavus*			
Madeira	Sed_058 5	*Streptomyces* sp.			
Azores	C_063 4	*Streptomyces* sp.			
Azores	C_192 1	*Nocardiopsis lucentensis*			
Azores	Sed_003 2	*Streptomyces* sp.			

No halo < 10 mm 10 - 20 mm > 20 mm

Screening of anticancer activity allowed identifying crude extracts of 23 isolates capable of reducing the cellular viability of at least one of the cancer cell lines tested (Fig. 10). Extracts derived from strains assigned to the genera *Brevibacterium* (strains C_034 1, S_026 1 and S_020 1), *Dietzia* (S_018 1.4), *Microbacterium* (C_025 4), *Micrococcus* (C_025 2), *Nocardiopsis* (C_063 3), *Salinispora* (C_063 9) and *Streptomyces* (C_003 1.11, C_051 2, S_044 25, Sed_044 5, Sed_058 2 and Sed_058 5) induced a decrease in the viability of T47-D cells by at least 30%. These same extracts, excluding those from strains C_003 1.11, C_025 4, C_063 3, S_020 1 and Sed_058 2, also showed a reduction in the viability of HepG2 cells in more than 20%. Only against this same cancer cell line, the strains Sed_143 3 (*Brachybacterium* sp.), L_015 5.2.8 (*Brevibacterium* sp.), C_025 1 (*Leucobacter* sp.), Sed_313 29 (*Micromonospora* sp.), Sed_058 1 and C_003 1.17 (both *Streptomyces* sp.) exhibited activity in at least 30%. The extract of *Streptomyces* sp. strain C_063 4 showed the most significant activity against the three cell lines tested, revealing a general cytotoxicity. The strains C_034 1, C_063 9, L_015 5.2.8, S_018 1.4, S_041 16, S_071 16, Sed_044 5, Sed_143 3 and Sed_313 29 showed activity only in the cancer cell lines, but not in the normal cell line hCMEC/D3. In addition, a higher number of actinobacterial strains isolated from the deep-sea samples of Madeira (16 active strains out of a total of 172 strains isolated from Madeira) demonstrated to be active against cancer cell lines when compared to those obtained from Azores (7 active strains out of a total of 104 strains isolated from Azores).

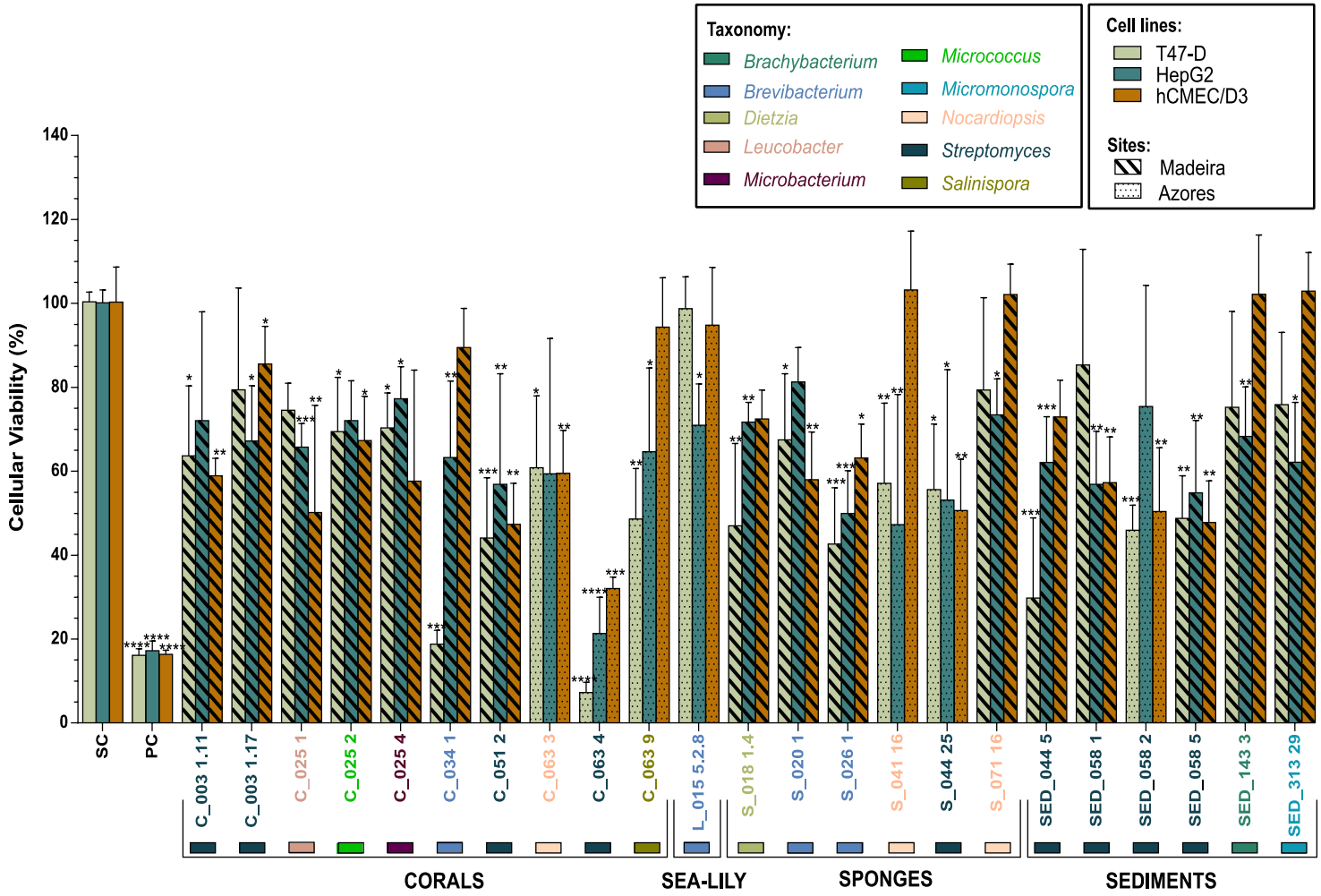

Figure 10 – Deep-sea actinobacterial crude extracts (15 µg mL^{-1}) with anticancer activity in the cancer cell lines, breast ductal carcinoma (T-47D) and liver hepatocellular carcinoma (HepG2), and in the non-cancer cell line hCMEC/D3 (human brain capillary endothelial cells), after 48 h of exposure. Only extracts with statistically significant differences are shown in the graph. SC and PC indicate the solvent and positive controls, respectively. Values are presented as mean ± standard deviation from two independent assays conducted in triplicate and significant differences compared to the solvent control are annotated with asterisks in the graphs (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$.

Anti-inflammatory activity was observed in 28 extracts that significantly reduced NO production by the inflamed cell line RAW264.7 (Fig.11).However, one of these extracts (derived from strain C_063 4) also caused a reduction in the viability of the RAW264.7 macrophage cell line, meaning that the reduction of NO production in the presence of this extract may be a result of the decreased cellular viability rather than an anti-inflammatory effect. All active extracts were capable to reduce the inflammatory response by more than 50%, without showing associated cytotoxicity (Fig. 11). In terms of geographic origin, most actinobacteria with anti-inflammatory activity were isolated from corals collected in the Madeira archipelago.

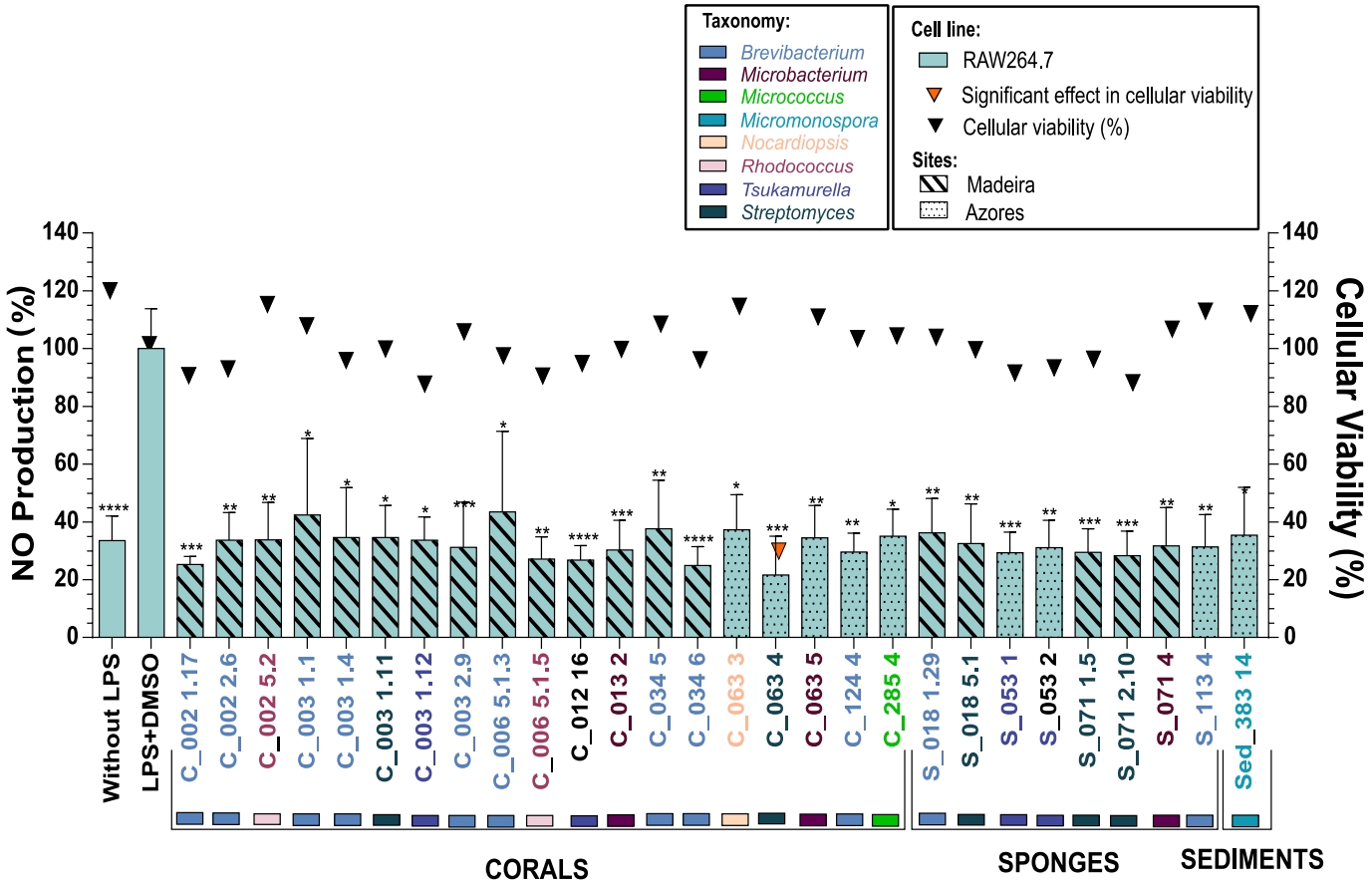

Figure 11 – Deep-sea actinobacterial crude extracts (15 µg mL^{-1})) with anti-inflammatory activity following inflammation induced by LPS exposure (200 µg mL^{-1}).. The inflammatory effect on the RAW 264.4 cell line, as measured by NO production (columns), and the effect on the viability of the same cellular line (triangles) are exhibited after 48 h of exposure. Only extracts with statistically significant differences are shown in the graph. The triangle in orange colour indicates that the extract exhibits anti-inflammatory activity, but also significantly reduces cell line viability. Without LPS and LPS+DMSO refer to positive and solvent with lipopolysaccharide controls, respectively. Values are presented as mean ± standard deviation from two independent assays conducted in triplicate and significant differences compared to the solvent with lipopolysaccharide control are annotated with asterisks in the graph (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$).

Zebrafish Nile red fat metabolism assay tested in 30 crude extracts, revealed that six crude extracts, three from *Brevibacterium* strains (S_071 2.12, S_018 1.29 and S_071 1.5), two from *Streptomyces* strains (C_003 1.3 and C_003 1.18) and one from a *Dietzia* strain (S_018 5.1.12), exhibit a significant neutral lipid-reducing activity after 48 h of exposure (Fig 12).

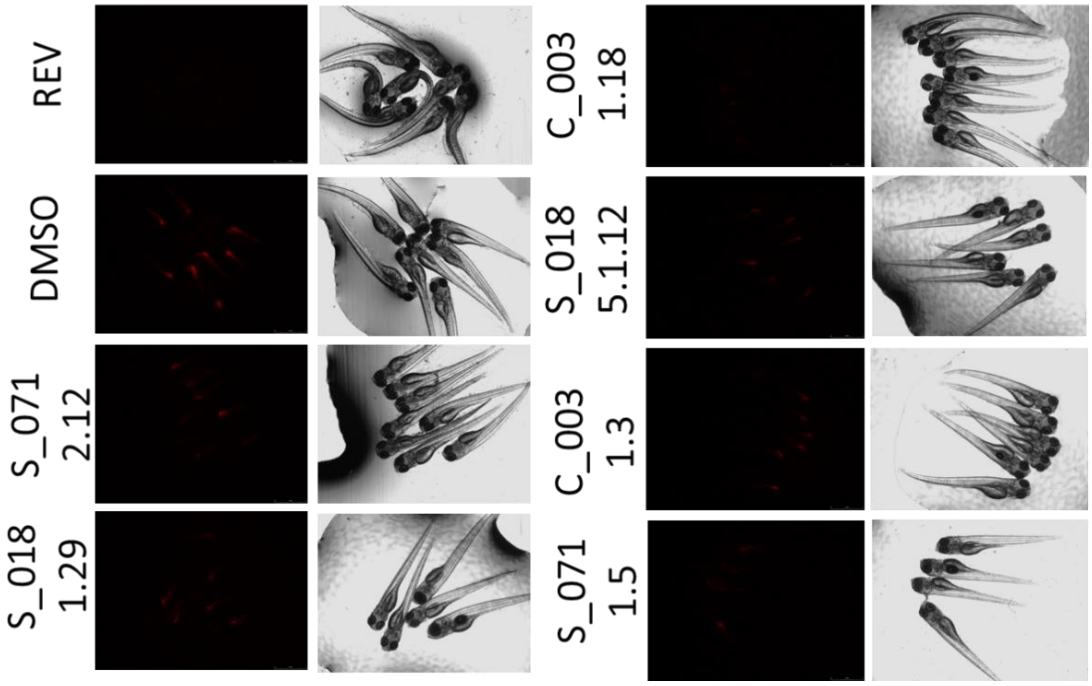

Figure 12 – Deep-sea actinobacterial crude extracts with anti-obesity activity shown by the zebrafish Nile red fat metabolism assay after 48 h of exposure. Solvent control was 0.1% dimethyl sulfoxide (DMSO) and positive control was 50 µM resveratrol (REV). Strong fluorescence signal is present in zebrafish larvae from the solvent control (DMSO) around the yolk sac and stomach/intestine. Crude extracts of the strains shown in the figure decreased the Nile red staining, in contrast to solvent control.

4.4.3 Dereplication analysis

The 56 extracts that showed antimicrobial, anticancer and/or anti-inflammatory activities were subjected to a dereplication analysis in order to understand if these activities could be associated with novel secondary metabolites.

The results showed several clusters associated to different chemical class annotations as indicated (in different colours) by the molecular networking. The most prevalent chemical class annotations were “Carboxylic acid and derivatives”, “Peptidomimetics” and “Polypeptides”, all of them containing different actinobacterial peptide-related compounds (Fig. 13). The chemical class “Carboxylic acid and derivatives” comprised the compounds Antibiotic PN00053, Bisucaberin, Bonactin, Champacyclin, Deferrioxamine E and G, Glycinocin D, Hymenamide F, Langkolide, Phepropetin A and D, Surugamide A-D, Roflamycoin, Telomycin, Virginiamycin, Wainunuamide and

Zelkomycin. The “Peptidomimetics” class included the cyclic depsipeptides Antibiotic A0341A, Antibiotic A54145, Enduracidin B, Skyllamycin A-B, Grividomycin-III, Sch486058, Shurimycin A, Triculamin and Valinomycin, and the cyclic peptide, Peptidolipin F. Lastly the “Polypeptides” chemical class encompassed the polyketides Axinastatin 4, Callyaerin H and Venepeptide.

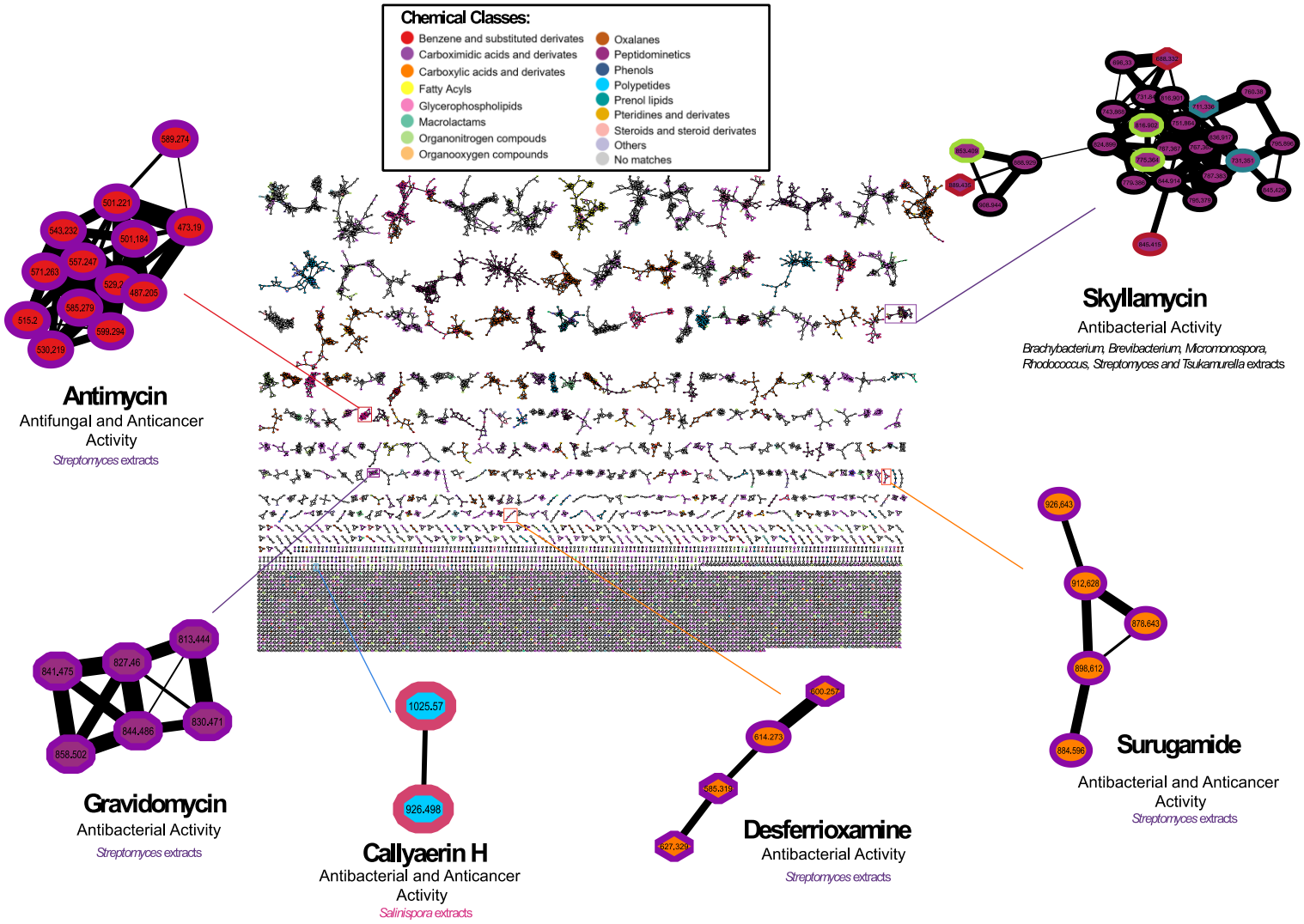

Figure 13 – Molecular networking of the bioactive actinobacterial crude extracts, using MS/MS data with a positive ionization mode (ESI+). Nodes represent parent ions and edge width differs according to the cosine score which measure relatedness between MS/MS spectra. Nodes colours represent the chemical classes to which each compound corresponds, according to the legend. Some compounds associated with Actinobacteria are highlighted as examples of the most prevalent of chemical class displayed in the molecular networking.

4.5 Discussion

In this study, we employed culture-dependent methods to investigate the taxonomic diversity and bioactive potential of actinobacteria associated with various deep-sea samples, which included sediments, sponges, corals and a sea-lily, collected in Madeira and Azores archipelagos. A total of 276 actinobacterial isolates, distributed by 20 genera, were recovered from the deep-sea samples, with the highest fraction of these isolates belonging to the genera *Brevibacterium* and *Microbacterium*. The dominance of these genera in the deep-sea, especially in deep-sea sediments, has been reported before in other studies. Meena et al. (2019) isolated 123 actinobacterial strains from six deep-sea sediment samples collected around the active volcanic island of Barrern, in the Andaman Sea, and taxonomic identification revealed that the genera *Streptomyces, Dietzia* and *Brevibacterium* were the most dominant ones. Prieto-Davó et al. (2016) reported the isolation of more than 400 actinobacterial strains from 662 deep-sea sediments of Madeira Archipelago, affiliated to the genera *Streptomyces*, *Micromonospora*, *Salinispora*, *Brevibacterium*, *Gordonia* and *Microbacterium*. In addition, we recently reported a study on the isolation of 49 actinobacterial strains from deep-sea sediments of the Artic, Madeira and Azores regions, that were mostly associated to the genera *Brachybacterium*, *Microbacterium* and *Brevibacterium* (Ribeiro et al., 2023). The results obtained in these works are in line with those obtained in the present study, in which we also isolated actinobacteria associated with all described genera from our deep-sea sediment samples, also obtaining agreement regarding the most dominant genera.

Sponges are known to establish symbiotic relationships with numerous groups of microorganisms, with actinobacteria being one of the most common bacteria living in symbiosis with these organisms (Dat et al., 2018). In this study, we isolated several actinobacterial genera from the analysed deep-sea sponges, such as *Brevibacterium*, *Dietzia*, *Microbacterium*, *Micromonospora*, *Nocardiopsis*, *Streptomyces*, *Tsukamurella*, *Corynebacterium* and *Nocardioides*. Other studies also revealed deep-sea sponges as good hosts of actinobacteria. Xu et al. (2018) described the isolation of the genera *Streptomyces*, *Actinomycetospora*, *Agrococcus*, *Leifsonia*, *Nocardiopsis*, *Promicromonospora*, *Rhodococcus*, *Salinispora* and *Tsukamurella* from several deep-sea sponges collected in different sites of the Pacific Ocean. Xin et al. (2011) identified 24 actinobacterial strains associated with Antarctic deep-sea sponges, which were affiliated to the genera *Brachybacterium*, *Brevibacterium*, *Dietzia*, *Nocardiopsis*, *Pseudonocardia* and *Streptomyces*. A study carried out to analyse the microbial diversity associated with four deep-sea sponges collected in the Atlantic Ocean, identified several

actinobacterial genera, including *Micrococcus, Kocuria, Micromonospora, Modestobacter, Citricoccus* and *Chryseoglobus* (Williams et al., 2020).

Very few studies have assessed the cultivable actinobacterial community associated with deep-sea corals, and in this respect our study is an important contribution to enlarge this knowledge. Here we show that a large number of actinobacterial genera can be found associated with deep-sea corals, having identified 16 genera in the 29 samples of corals analysed. Other studies have also shown the association of actinobacteria with deep-sea corals. For example, a study carried out with the deep-sea coral *Lophelia pertusa* collected off Cabo de Santa Maria di Leuca, in Southern Italy, revealed an abundant diverse microbial community, which included actinobacteria (Yakimov et al., 2006). Sarmiento-Vizcaíno et al. (2017) studied the diversity of actinobacteria associated with North Cantabrian Sea invertebrates, that included deep-sea corals and sponges, and identified 18 actinobacterial strains, mainly from corals, assigned to the genera *Streptomyces*, *Micromonospora*, *Pseudonocardia* and *Myceligenerans*.

Regarding the sea-lily sample analysed in the present study, to our knowledge no studies on the isolation of actinobacteria from this marine animal are available, although actinobacteria have been identified in the intestinal microbiota of other echinoderms (like in the sea cucumber species *Holothuria glaberrima* and *Apostichopus japonicus*) (Pagán-Jiménez et al., 2019; Zhao et al., 2019).

The different culture media used in our study affected the diversity of the isolated actinobacteria. Several studies indicate that the use of oligotrophic media can be beneficial for the isolation of actinobacteria compared to media with more complex carbon sources (Hames-Kocabas et al., 2012). Nonetheless, the use of more complex media, such as M1, RH, and chitin-based media, has been also successful in the isolation of marine actinobacteria (Liu et al., 2019; Williams et al., 2020). In our study, the medium that revealed more efficient in the isolation of actinobacteria was the RH medium. All the selective culture media led to the isolation of a significant diversity of deep-sea actinobacteria, including rare actinobacterial genera, indicating that these media are suitable for the isolation of deep-sea actinobacteria.

Most of the actinobacterial strains obtained were isolated at 28 °C. This is in agreement with other studies that reported the optimum growth temperature for the successful cultivation of deep-sea actinobacteria generally between 25 and 30 °C (Kamjam et al., 2017; Pesic et al., 2013). It is curious that deep-sea actinobacteria seems to still prefer this range of temperatures over lower temperatures, such as the 5 °C tested in our study to mimic the temperature that might be observed at the deep-sea. Nevertheless, incubation at this latter temperature allowed the recovery of unique genera, not recovered at 28 °C, as was the case of *Arthrobacter*, *Blastococcus* and *Nocardioides*.

In our study, all actinobacterial crude extracts were submitted to a preliminary screening of their antimicrobial, anticancer and anti-inflammatory activities. Antimicrobial assays showed 16 actinobacterial strains active against one or more of the tested microorganisms - *Bacillus subtilis*, *Candida albicans* and *Staphylococcus aureus.* In the dereplication analysis, we detected molecules associated to the antibiotics classes cyclic depsipeptides, cyclic peptides, non-ribosomal peptides and lipopeptides in 12 of the actinobacterial extracts exhibiting antimicrobial activity (C_006 2.2, C_014 9, C_040 6, C_063 4, S_018 5.1, S_026 1, S_039 4, S_044 25, SED_003 2, SED_058 1, SED_058 2 and SED_058 5). These molecules were reported to be produced by diverse *Streptomyces* strains, except for Peptidolipin F, that was isolated from a marine *Nocardia* sp. All of the identified antibiotics exhibit activity against Gram-positive bacteria and/or have associated antifungal activity, which may explain the activities exhibited by the mentioned 12 actinobacterial strains. For the remaining 4 extracts, derived from *Nocardiopsis* strain C_192 1, *Streptomyces* strain S_071 2.9, *Streptomyces* strain C_003 1.3 and *Brevibacterium* strain S_018 1.29, no hits of compounds produced by actinobacteria that could justify the observed antimicrobial activity were obtained, and therefore these strains may be a promising source of new antibiotics. Deep-sea actinobacteria have already proven to be capable of producing new antimicrobial molecules, such as Tunicamycin E, an antibiotic produced by *Streptomyces xinghaiensis* SCSIO S15077, isolated from a deep-sea sediment collected in the South China sea and exhibiting moderate antifungal activity against *C. albicans* (Zhang et al., 2020); and Desotamide B (cyclic petptide) and Lopophorin H (spirotetronate poliketide), two natural compounds produced by deep-sea derived *Streptomyces* strains collected in the South China sea, with activity against *S. aureus* and *B. subtilis*, respectively (Pan et al., 2013; Song et al., 2014).

The analysis of anticancer activity showed 23 actinobacterial extracts active against at least one of the tested cancer cell lines. Some of these extracts were able to inhibit only the cancer cell lines tested and not the non-cancer cell line, indicating a selective anticancer activity. In the dereplication analysis, hits to compounds that may explain the anticancer activity were found in 16 extracts. These compounds included siderophores [Bisucaberin (Kameyama et al., 1987) and Desferrioxamine E (Salis et al., 2014)], macrocyclic [Ikarugamycin (Popescu et al., 2011) and Langkolide (Helaly et al., 2012)] and non-ribossomal peptides [Surugamide A-B, D and G (Takada et al., 2013)] all reported to be produced by *Streptomyces* strains, and the cyclic peptides Wainunuamide (Tabudravu et al., 2001), Axinastatin 5 (Mechnich et al., 1996) and Callyaerin H (Ibrahim et al., 2010), isolated from the marine sponges *Stylotella aurantium*, *Axinella* sp. and *Callyspongia truncate*, respectively. However, for the remaining 8 extracts that exhibited

anticancer activity, no hits associated with compounds that could justify this activity were found, suggesting that these extracts may contain new molecules with anticancer activity. In this regard, several new compounds with anticancer activity have been isolated from deep-sea actinobacteria, like Ammosamides A-B, two chlorinated alkaloids produced by *Streptomyces* sp. (Gaudêncio et al., 2008); Dermacozines A-G, a new phezanine family produced by *Dermacoccus abyssi*, with moderate activity against the leukemia cell line K562 (Abdelfattah et al., 2016); Microbacterins A-B, two new peptaibols isolated from *Microbacterium sediminis*, presenting significant inhibitory effect against several human cancer cells, including the human hepatoma cell line and the human gastric cancer cell line (Liu et al., 2015); and Pseudonocardians A-C, three new diazaanthraquinone derivatives with anticancer and antimicrobial activity, recovered from a *Pseudonocardia* strain (Li et al., 2011).

There have been limited studies investigating the capacity of deep-sea actinobacteria to produce anti-inflammatory compounds. In our study, 28 actinobacterial extracts exhibited anti-inflammatory activity. However, when the dereplication analysis was performed in these extracts, hits capable of explaining this activity were obtained for none of them, indicating that these can be highly promising in terms of the discovery of new anti-inflammatory compounds. This is particularly relevant in the light of the fact that only a few anti-inflammatory compounds have been hitherto discovered to be produced by marine actinobacteria, as is the case of Actinoquinolines A-B, two quinoline alkaloids isolated from *Streptomyces* sp. derived from deep-sea sediments (Hassan et al., 2016); Diazepinomicin, a farnesylated dibenzodiazepinone alkaloid produced by a *Micromonospora* sp. with strong anti-inflammatory activity (Charan et al., 2004); and Strepsesquitriol, a new sesquiterpene isolated from a deep-sea *Streptomyces* sp. with moderate inhibitory activity against lipopolysaccharide induced TNFα production in RAW264.7 macrophages (Yang et al., 2013).

The zebrafish fat metabolism assay has proven useful in uncovering bioactive compounds with anti-obesity properties, originating from marine fungi and plant polyphenols. This technique offers the advantage of simulating the complexity of a whole small animal model, enabling the identification of extracts and compounds that have significant physiological effects (Noinart et al., 2017; Urbatzka et al., 2018). In the present study, 6 actinobacterial extracts were identified to have anti-obesity activity. Santos et al. (2019) also reported two actinobacterial strains, isolated from a marine sponge collected on the continental shelf at Berlengas, Portugal, that significantly reduced the level of fluorescence in Zebrafish Nile red assay. Nonetheless, this bioactivity is scantly explored and to date there are no reports of natural products with this activity produced by actinobacteria. The fact that the dereplication analysis of our extracts exhibiting anti-

obesity activity did not show any hits to molecules capable of explaining this activity, indicates the presence of potential new anti-obesity compounds.

4.6 Conclusion

This work investigated the diversity of deep-sea actinobacteria in the unexplored archipelagos of Madeira and Azores and their biosynthetic potential. Our findings suggest that the deep-sea is an important reservoir of actinobacteria, including novel phylogenies, and that these are a source of new bioactive compounds. One of the most notable results found in our work was the significant anti-inflammatory and anti-obesity activities of several of our actinobacterial extracts, two bioactivities that are almost entirely unreported for actinobacteria. The metabolomic profile of bioactive extracts revealed some metabolites associated with already known NP but, most importantly, it also revealed the possible presence of new molecules, since no hits to NP accounting for the observed bioactivities were obtained. These results demonstrate that prospecting actinobacteria in unexplored environments, such as the deep-sea of Madeira and Azores, is a promising approach for discovering new molecules with pharmaceutical applications.

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CHAPTER 5

Deep-sea coral-associated *Streptomyces*: A potential hotspot for new molecules

Ribeiro I, Freitas S, Urbatzka R, Leão PN, Carvalho MF. "Deep-sea coral-associated *Streptomyces*: A potential hotspot for new molecules". *In prep*

5.1. Abstract

Streptomyces are widely distributed in the marine environment, although only a few studies on their associations with deep-sea corals have been reported. The investigation of the metabolic profile of the deep-sea coral-associated *Streptomyces chumphonensis* strain C_003 1.18, isolated from Madeira Archipelago in Chapter 4, is described in this chapter. This strain was selected due to its relevant anti-obesity activity and also due to the fact that no compound that could justify this activity was identified in the dereplication analysis. Large-scale culturing of this strain was performed, but the resultant extract became toxic to zebrafish larvae. In view of this result, the analysis of the metabolic profile of strain C_003 1.18 changed to a mass-guided isolation, targeting other promising masses presented in the extract. With mass-guided isolation, four compounds were isolated using high performance liquid chromatography (HPLC), including the well-known Piericidin A1 and three novel compounds. Our study suggests that deep-sea coral-associated *Streptomyces* represent an underexplored promising source for drug discovery.

5.2. Introduction

Scientists are driven by the constant need of discovering valuable molecules, leading them to explore atypical habitats in search of novel natural products that can meet the increasing pharmaceutical demand. To address this challenge, they have adopted various strategies, including studying extreme biomes such as the deep-sea, as it is believed that harsh abiotic conditions found there can lead to the development of diverse metabolic and genetic characteristics that may translate in a complex chemistry (Ramesh et al., 2009). Consequently, expeditions to the deep-sea are now being switched from geological and ecological studies to bioprospecting for natural bioactive compounds (Leary et al., 2009).

The phylum Actinomycetota, covers a diverse and complex group of bacteria with a wealth of natural antibiotic mechanisms (Anandan et al., 2016; Barka et al., 2016). They are a prolific source of metabolites of pharmaceutical and ecological interest. *Streptomyces*, in particular, have contributed notably to the progress of global health by producing a large number of antibiotics to treat several diseases (Donald et al., 2022; Subramani et al., 2019). Members of this phylum are successful colonizers of several extreme environments and studies indicate that Actinomycetota constitutes one of the most abundant phylum in deep-sea environments (Chen et al., 2016).

Corals are the most remarkable members of the coral reefs that support a wide range of marine life and provide important ecological services (Buddemeier, 2004). These unique structures provide habitats for diverse prokaryotic communities, including Bacteria and Archaea (Rohwer et al., 2002). The coral-associated actinobacteria play an important role in maintaining coral health by regulating diverse functions, including the supply and recycling of nutrients, and the production of antimicrobial compounds to protect against pathogens (Siro et al., 2022).

Overall, actinobacteria from deep-sea corals are an important source of research from ecological and pharmaceutical standpoints. Further studies on the microbiome of these organisms may uncover new insights into their biological activities, providing opportunities to find novel molecules. In this study, we isolated a *Streptomyces* strain associated with the deep-sea coral collected in the Madeira archipelago, (NE Atlantic) and explored its bioactive potential. We report the metabolic profile of this strain and the identification of some of the secondary metabolites produced with antibacterial activity. Finally, we report the isolation of three new compounds, which highlights the importance of studying deep-sea coral-associated *Streptomyces* for discovering novel natural products.

5.3. Materials and methods

5.3.1. Strain isolation

Strain C_003 1.18 was isolated from a deep-sea coral (OOM2018_003), collected at 1980 m depth in the Madeira archipelago with a ROV during the mission OOM2018_LUSO (Table 9, chapter 4). The isolation process is described Chapter 4. Briefly, one gram of coral was cut in small fragments, homogenized with a sterile mortar and subjected to a pre-treatment that consisted in the incubation of the sample with antibiotics (nystatin, cycloheximide and nalidixic acid at 20 mg mL^{-1}) for 30 min (PT2). The resulting sample was ten-fold diluted until 10^{-4} and an aliquot of 100 µl of each dilution (10^{0} - 10^{4}) was spread over the surface of M4 agar. The media were supplemented with cycloheximide, nalidixic acid and nystatin, at 50 mg L^{-1} each, to prevent the growth of fungi and Gram-negative bacteria. The plates were incubated for a period of up to 6 months at 28 ºC. Strain C_003 1.18 was purified and cryopreserved at -80 ºC in 30% glycerol.
The phylogenetic identification of this strain is described in Chapter 4.

5.3.2. Small-scale culturing and organic extraction

Small-scale culturing and organic extraction were described in Chapter 4. Briefly, strain C_003 1.18 was cultured in 30 mL of M4 liquid medium, at 28 °C, 100 rpm, in the dark for 3 days. After this initial growth period, 0.5 g of Amberlite® XAD16N resin was added to the culture medium and left to incubate for two more days. Cells and resin were then harvested, immediately frozen, and freeze-dried. Cells and resin were extracted twice with acetone/methanol 1:1 (v/v), dried and the resulting extract was dissolved in pure DMSO (DMSO; ≥ 99.9%) to obtain stock solutions (with final concentrations of 10.0 mg mL^{-1} and 1.0 mg mL^{-1}) to be used in the bioactivity assays.

5.3.3. Large-scale culturing and organic extraction

Large-scale culturing of strain C_003 1.18 was carried out in 5 L Erlenmeyer flasks containing 2.5 L of M4 liquid medium, for 3 weeks under the same growth conditions used previously. After growing for 2 weeks, 16.7 g/L of Amberlite® XAD16N resin was added to the cultures and left to incubate for one additional week. In total, 18 L of this culture were grown. Cells and resin were harvested by centrifugation (1792 g, for 10

min), frozen and freeze-dried. Freeze-dried biomass from large-scale culturing (22.3 g, dry weight (d.w.).) was extracted using a mixture of acetone/methanol 1:1 (v/v) by stirring for 20 minutes at room temperature. This procedure was repeated five times under heated conditions (40 °C), or sonication, until the solvent content was no longer coloured. A total extract mass of 9.2 g was obtained.

5.3.4. Fractionation of the large-scale extract

The crude extracts were fractionated by normal-phase Vacuum Liquid Chromatography (VLC) using silica gel 60 (0.015–0.040 mm, Merck). The extract was dry-loaded onto the column using 9 g of silica (according to extract mass). A mixture of ethyl acetate/n-hexane (EtOAc/n-hex) 1:9 (v/v) was used to start the separation, and a stepwise gradient to 100% EtOAc and then to 100% methanol (MeOH) was used. A total of eleven fractions were obtained (A-K).

Fractions D-H (112.05 mg) containing the mass features of interest (*m/z* 479.3949) were pooled, (eluting with EtOAc/MeOH 9:1 (v/v) to 1:1 (v/v)) and further fractionated using reverse-phase C18 flash chromatography with a SiliaSep PREMIUM Flash Cartridge C18 (25g, 25 µm, 90 Å, SiliClycle), with a stepwise gradient from 20 % methanol aqueous (MeOH(aq)) to 100% MeOH then to 100% isopropanol (IPA) . A total of eight fractions were obtained (D-H_1-8). Fraction D-H_6 (23.26 mg), eluting with 60 % MeOH(aq), was selected for reversed-phase semipreparative High-Performance Liquid Chromatography (HPLC) separation using a Synergi Fusion-RP C18 column (80 Å, 150 × 4.6 mm, 5 µm, Phenomemex). An isocratic elution program was performed, using 95% MeOH(aq) over 25 min, and a final cleaning step with 100% MeOH before returning to the initial conditions. A total of 3 fractions were obtained (D-H_6_A-C). Fraction D-H_6_C (21.97 mg) was selected to continue the fractionation steps used the same system, but with isocratic program using 90 % MeOH(aq), over 50 min. This procedure yielded nine fractions (D-H_6_C_1-9). HPLC fraction D-H_6_C_3 (4.2 mg) containing the mass of interest was further separated by analytical reversed-phase HPLC with a Surf-C18 column (100 Å, 250 × 4.6 mm, 5 µm, iChem). An isocratic elution program with 87 % MeOH(aq) was used, over 35 min. A total of seven fractions were obtained (D-H_6_C_3_A-G), including the fraction D-H_6_C_3_C (0.63 mg) and the fraction D-H_6_C_3_D (1.23 mg) which contained the compound corresponding to 372.3479 *m/z*. Fraction D-H_6_C_3_C contained the mass of interest (*m/z* 479.3949), but only partially purified and with low amount, so the adjacent fraction (D_H_5) was fractionated using the same conditions, to obtain additional compound. The final combined fractions (2.28 mg), containing this mass of interest, were obtained.

During the fractionation process of fraction D-H_5, subfraction D-H_5_B was obtained. This contained the partially purified *m/z* 416.2797, and was thus further separated by analytical reversed-phase HPLC with an ACE Excel CN-ES (100 Å, 75 × 4.6 mm, 2 µm, ACE). The separation was performed using an isocratic elution program with 50 % acetonitrile aqueous (ACN(aq)), over 30 min. A total of three fractions were obtained, including the purified fraction D-H_5_B_2 corresponding to the compound with *m/z* 416.2797 (1.41 mg).

Fraction D-H_8 (20.90 mg) containing the compound corresponding to *m/z* 663.4544, was fractionated by reversed-phase semipreparative HPCL, using a Synergi Fusion-RP C18 column (80 Å, 150 × 4.6 mm, 5 µm, Phenomemex). The isocratic elution program consisted in 90% / 10% IPA, over 20 min. Eleven fractions were obtained (D-H_8_A-K), including the fraction D-H_8_F (1.05 mg) with the mass of interest.

Fraction J (2551.4 mg) containing the mass of interest *m/z* 766.89345 was further separated by reverse-phase C18 VLC using a C18 silica (40-60 µm, 100 Å, Phenomenex), with a stepwise gradient from 5 % MeOH(aq) to 100 % MeOH and then 100 % IPA and 100 % dichloromethane (DCM). Eleven fractions were obtained (J_1-9). Fractions J_5 and J_6 (eluting with 40 % to 50 % MeOH/(aq)) were pooled and fractionated again by reverse-phase using reverse-phase C18 flash chromatography with a SiliaSep PREMIUM Flash Cartridge C18 (10g, 25 µm, 90 Å, SiliClycle), with a stepwise gradient from 30 % MeOH(aq) to 100 % MeOH then to 100 % IPA. In total, twelve fractions were obtained (J_5_A-L). Flash chromatography fraction J_5_H (3.34 mg) contained the mass of interest and was fractionated by analytical reversed-phase HPLC with a Surf-C18 column (100 Å, 250 × 4.6 mm, 5 µm, iChem). An isocratic elution program with 65 % ACN(aq), over 25 min. In total six fractions were obtained (J_5_H_1-6), including fraction J_5_H_1 which contains the mass of interest (1.2 mg).

Reverse-phase C18 flash chromatographies were performed with a Pure FlashPrep C-850 Chromatography System (BUCHI) and separations were monitored at 210, 254, 300 and 500 nm wavelength. For the HPLC separations were used a JASCO PU-4180 HPLC pump and an MD-4010 photodiode array detector, and separations were monitored at 210, 254, 320 nm and full wavelength (200-600 nm). All solvents used were American Chemical Society (ACS) grade, with the exception of HPLC which used HPLC grade solvents.

5.3.5. Bioactive assays

The bioactive assays for the small-scale crude extract, including the antimicrobial, anticancer, anti-inflammatory and anti-obesity activity are described in Chapter 4. The antimicrobial and anti-obesity activity of the fractions was analysed as described in Chapter 4.

Briefly, the antimicrobial activity was assessed using the agar disc diffusion method. Strain crude extract was tested against five reference strains: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 25241) and *Candida albicans* (ATCC 10231). The presence of an inhibition halo was analysed, and the respective diameter was measured, after 24 h of incubation.

Anticancer activity was tested against two cancer cell lines, breast ductal carcinoma (T-47D) and liver hepatocellular carcinoma (HepG2), both from Sigma-Aldrich, and human brain capillary endothelial cells (hCMEC/D3), (donated by Dr. P. O. Courad, INSERM, France), to evaluate the general toxicity. The cell viability using MTT assay was measured and calculated in all the cell lines, after 48 h of exposure.

The anti-inflammatory activity was analysed using the Mouse macrophage cell line (RAW264.7) treated with LPS (lipopolysaccharide, Sigma-Aldrich, MO, USA), while for the pro-inflammation assay no LPS was added to the cells. Nitric oxide (NO) level was analysed by the Griess reaction to evaluate the cells inflammation, after 24h of exposure. In parallel, MTT assays were performed to determine cell viability.

Zebrafish Nile red assay was performed to analyse the lipid-reducing activity of strain crude extracts and fractions. Zebrafish larvae were exposed to crude extracts at a final concentration of 10 µg mL-1, with daily renewal of water and extracts, in a 48-well plate with a density of 6 to 8 larvae per well. Neutral lipids were stained overnight with Nile red at the final concentration of 10 ng mL^{-1} and the fluorescence was measured 5 min after the larvae were anesthetized with tricaine (MS-222, 0.03%).

Statistical analysis of the results of anticancer and anti-inflammatory activity are described in Chapter 4. For the results of anti-obesity activity respective to the fractions, statistical analysis was performed according to the procedures described in Chapter 4.

5.3.6. Dereplication analysis

The crude extract and all fractions obtained in each fractionation step were subjected to MS-based dereplication. Crude extracts were resuspended in methanol (2 mg mL^{-1}) and the fractions in MeOH or IPA, depending on the best elution solvent (0.5 mg mL^{-1}) and

analysed by liquid chromatography-high resolution electrospray ionization tandem mass spectrometry (LC-HRESIMS/MS). This analysis was performed using the same approach as described in Chapter 2. The raw data resulting from the dereplication of extracts and fractions were converted to mzML format and submitted to the GNPS data analysis workflow using three in silico tools (Insilico Peptidic Natural Product Dereplicator, Dereplicator VarQuest and Dereplicator+) with default parameters, excluding ion mass precursor tolerance and fragment ion mass tolerance (set to 0.005 Da). For results annotated by the web-based mass spectrometry GNPS platform, a threshold p-value ≤ 10^{-10} for DEREPLICATOR and DEREPLICATOR VarQuest, and a strict value of 15 for DEREPLICATER+ were applied to reduce false matches. All these matches were manually checked to see if they were associated with compounds produced by actinobacteria and/or marine corals and removed when they did not meet these criteria (Gurevich et al., 2018; Mohimani et al., 2017; Mohimani et al., 2018). The fractions masses from *m/z* values were submitted to two databases of natural products, the DNP (version 27.1, CRC Press, Abingdon, UK) and the NPA database (van Santen et al., 2022) to confirm if they corresponded to compounds previously reported from actinobacteria and/or marine corals.

5.3.7. NMR spectrometry

Two pure fractions obtained from the fractionation of the crude extract of *S. chumphonensis* strain C_003 1.18 were analysed by 1D Nuclear Magnetic Resonance (NMR) spectrometry. This analysis was carried out at the Materials Center of the University of Porto (CEMUP) in a Bruker Avance III, 400 MHz, controlled by TopSpin 3.2, to examine the structure of molecules of interest. Fraction D-H_5_B_2 was analysed in MeOH deuterated (MeOH-d4, Sigma) and fraction D-H_8_F in chloroform deuterated (chloroform-d, Sigma). NMR data were analyzed in MNova 12.0.4.

5.4. Results

5.4.1. Mass-guided isolation

Crude extract of *Streptomyces chumphonensis* strain C_003 1.18 exhibited a significant neutral lipid-reducing activity after 48 h of exposure (described in Chapter 4).
According to this promising result, the strain was cultivated on a large-scale to obtain sufficient amounts of the natural product responsible for the biological activity result. The

strain was grown in large scale (18 L), which yielded 22.3 g of dry biomass. Repeated organic extraction with acetone/methanol 1:1 (v/v) afforded an organic extract (9.2 g). The large-scale culturing extract was retested to confirm the anti-obesity activity but the results were not what one would expect, as the extract became toxic to zebrafish larvae after 48 hours of exposure (Fig 14).

In an attempt to try to separate the compounds that confer toxicity from those that could have anti-obesity activity, a VLC was performed in the organic extract to obtain 13 fractions of increasing polarity. All 13 fractions were retested, and the results demonstrate that the fractions D-H showed toxicity to zebrafish larvae and the others (Fractions A-C and I-K) had no anti-obesity activity (Fig 14).

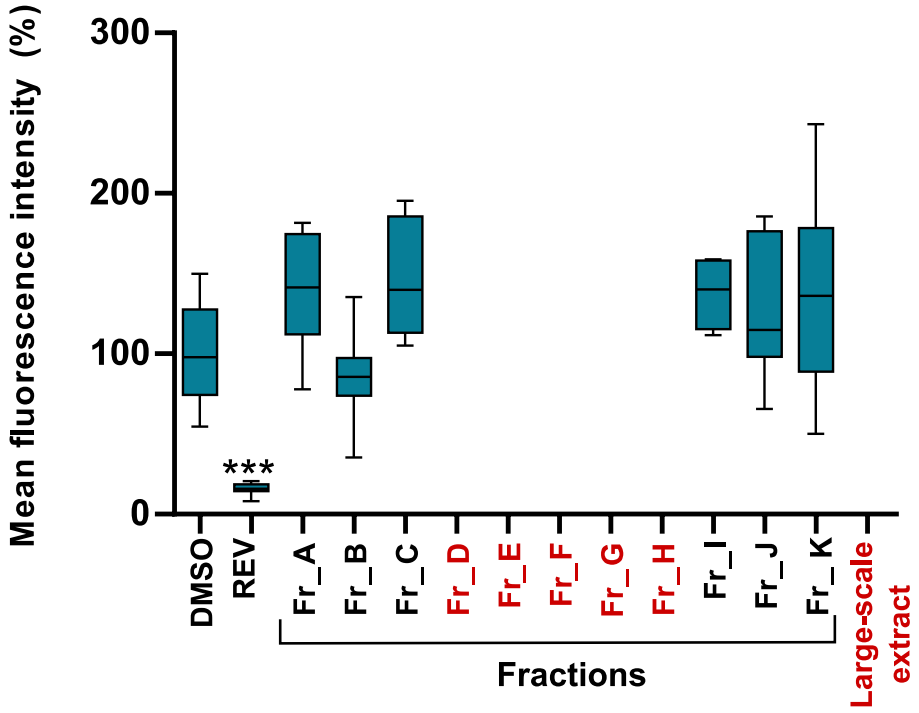

Figure 14 – Results obtained in the zebrafish Nile red fat metabolism assay after 48 h of exposure. In red are represented the fractions and the large-scale extract that cause toxicity to zebrafish larvae. Solvent control was 0.1% dimethyl sulfoxide (DMSO) and positive control was 50 µM resveratrol (REV). Values are shown as interval displaying the data distribution through their quartiles with 5-95% confidence from two independent assays conducted in triplicate and line within the box-whisker represents the median of the fluorescent intensity (%). Significant differences compared to the solvent control are annotated with asterisks in the graphs (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$).

Fractions were also tested against antimicrobial activity, and the results showed that the same fractions with toxicity against zebrafish larvae also exhibited activity against *B. subtilis* and *S. aureus* with inhibition halos of 9 - 19 mm (Table 12).

Table 12 – Fractions with antimicrobial activity indicated by the diameter of inhibition halo (mm) in the agar disk diffusion method

Fractions	Disk diffusion method (mm)	
	S. aureus	***B. subtills***
Fr_D	12	15
Fr_E	15	19
Fr_F	14	18
Fr_G	9	10
Fr_H	11	15

Fractions were dereplicated using the in silico GNPS tools DEREPLICATOR, DEREPLICATOR VarQuest and DEREPLICATOR as well as manual searched on the DNP and NP Atlas databases and the results showed hits to the antibiotic compounds Albocyclin, Antibiotic NK170204B, Foromacid A, Piericidins and Piericidinol, At the same time, LC-HRESIMS/MS analysis revealed the presence of four masses features in fractions D-H and one mass in fraction J, enabling an MS-guided isolation approach. The fractions D-H combined (112.05 mg) and fraction J (2551.40 mg), were used to follow the targeted masses features over a series of chromatographic procedures by LCHRESIMS/MS analysis. Reverse-phase C18 flash chromatography, and semipreparative and analytical HPLC were required to obtain the partially pure compounds: A (*m/z* 479.3940, 2.28 mg), B (*m/z* 416. 2797, 1.65 mg), C (*m/z* 372.3479, 1.23 mg), D (*m/z* 663.4544, 1.05 mg) and E (*m/z* 766.8934, 1.2 mg). The chromatographic peaks corresponding to the m/z values from the new compounds isolated are presented in Fig 15.

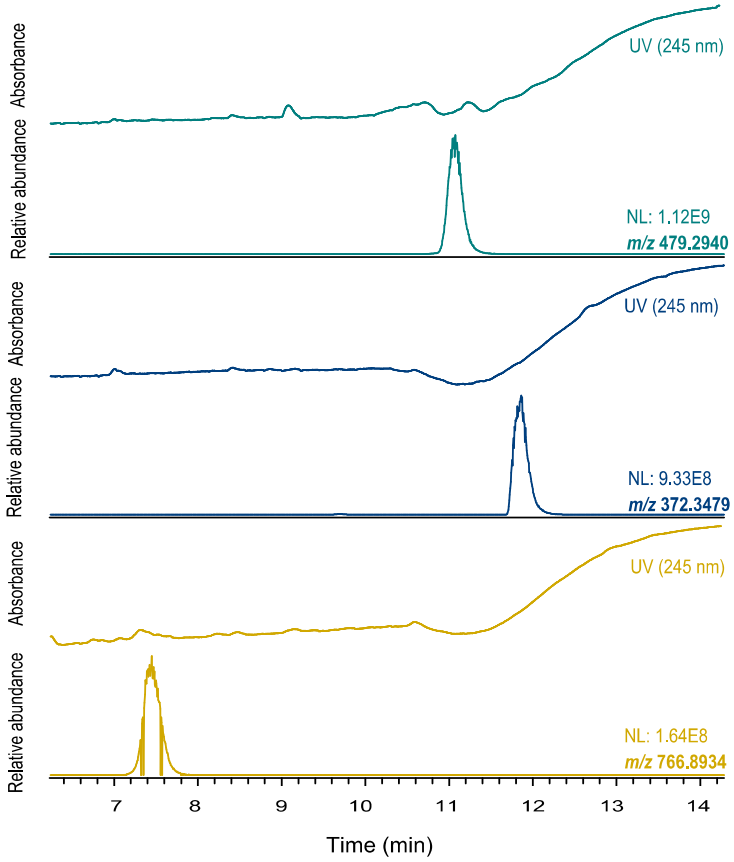

Figure 15 – Extracted ion chromatograms (EIC) from the three novel compounds and the respective UV chromatogram The NL (normalization level) value corresponds to the base peak intensity of the m/z value when compared with the mass composition of the actinobacterial fraction.

All the *m/z* values were dereplicated using DNP and NP Atlas databases and the results show no hits for the *m/z* 479.3940, 372.3479 and 766.8934, confirming the novelty of the compounds. For the *m/z* 416.3940 one hit associated to the antibioitic Piercidin A1 was found and for the *m/z* 663.4544 was obtained one hit related to the compound Tris(2,4-di-tert-butylphenyl)phosphate. Inspection of the ^{1}H NMR data (400 MHz, MeOH-d4 or chloroform-d) from these two compounds, confirmed the molecular structures of the know isolated compounds, Piercidin A1 and Tris(2,4-di-tert-butylphenyl)phosphate, by comparison with reported ^{1}H NMR data for these compounds (Fig. S4 and S5).

5.5. Discussion

Corals are complex hosts, harbouring a diverse range of microorganisms associated with functions related to host health and defence. The concept of the coral holobiont is

comparable to the idea of complex symbiosis, which explains the intricate relationship between the coral animal and the microorganisms it associates with (Bourne et al., 2009). We report here that Streptomyces *chumphonensis* strain C_003 1.18, from deep-sea coral collected in Madeira archipelago, produce bioactive secondary metabolites and novelty natural products with both ecological and pharmacological interest.

Streptomyces is a highly prolific genus, known for its ability to produce secondary metabolites with diverse biotechnological applications (Donald et al., 2022). This strain was selected for large-scale culturing for its potential anti-obesity activity. The zebrafish fat metabolism assay has been employed to discover bioactive compounds originating from marine fungi and plant polyphenols with anti-obesity activity. This method provides the benefit of replicating the intricacy of a complete small animal model, allowing the identification of extracts and compounds with physiologically significant activities. (Noinart et al., 2017; Urbatzka et al., 2018). However, anti-obesity activity is a little explored bioactivity and that to date there are no reports of natural products produced by actinobacteria with anti-obesity activity. Our study shows that the small-scale culturing extract exhibits an activity that appears not to be reproducible under large-scale cultivation, once the large-scale crude extract became toxic to zebrafish larvae.

The dereplication analysis allowed to identify antibiotics associated with strain C_003 1.18 that may explain the anti-microbial activity obtained, including Albocyclin, macrolide-type antibiotic with activity against methicillin resistant Staphylococcus aureus (MRSA) (Chatare et al., 2017); Antibiotic NK 170204B, closed related to Piericidins compounds with antibacterial and antifungal activity (Buckingham et al., 2010); Foromacid A, a broad-spectrum antibiotic (Corbaz et al., 1956); and Piericidins an important class of biologically active natural products known to exhibit antimicrobial, antifungal and insecticidal activity (Schnermann et al., 2005). All these compounds were originally isolated by *Streptomyces* sp. strains from terrestrial environments, however the presence of Piericidins has already been reported in the marine-derived *Streptomyces chumphonensis* KK1-2T and S*treptomyces* sp. SCSIO 40063 (Peng et al., 2022; Phongsopitanuna et al., 2021).

Our study is the only one that reports, as far as we know, the isolation of Piericidin A1 from a deep-sea coral-associated actinobacteria, although it has been isolated from actinobacteria of deep-sea sediments. This discovery is in line with some hypotheses that suggest that coral-associated bacteria acts as a protective barrier against invasive pathogens, potentially through competition for space and occupation of coral niches or by producing antimicrobial compounds that directly impede the growth of invasive microbes in corals (Mahmoud et al., 2016; McDevitt-Irwin et al., 2017).

The other compound isolated in this study, Tris(2,4-di-tert-butylphenyl) phosphate, has been already reported from the co-cultures of the fungi *Bionectria* sp. with *Bacillus subtilis* or with *Streptomyces lividans* (Kamdem et al., 2018). Tris(2,4-di-tert-butylphenyl) phosphate is an organophosphate compound used as a stabilizer in polymers and we believe that its presence in the extract of strain C_003 1.18 is probably an artifact derived from the degradation of polymers (Pelzl et al., 2018). This artifact may have originated during organic extraction, as some steps involved the use of plastic material.

For *m/z* 479.3940, 372.3479 and 766.8934 no hits were associated in the dereplication analysis, confirming the novelty of the compounds. This finding mainly highlights the idea that *Streptomyces* associated with deep-sea coral, represent a promising resource for the discovery of novel natural products. Two studies reported the isolation of *Streptomyces* species producing known bioactive compounds and many other unidentified compounds from deep-sea coral reefs collected in the Central Cantabrian Sea (Braña et al., 2015; Sarmiento-Vizcaíno et al., 2017). From these studies, the authors were able to isolate the novel natural product, designated as Lobophorin K, from the marine-derived *Streptomyces* sp. M-207, previously isolated from the cold-water coral *Lophelia pertusa,* collected at 1800 m of depth (Braña et al., 2017).

5.6. Conclusion

In this study, we have explored a deep-sea coral-associated Streptomyces for its novel natural products. This resulted in identification of known actinobacterial antibiotics and the isolation of three novel compounds, whose identities remain to be elucidated in future studies. Our study emphasizes that *Streptomyces* associated with deep-sea corals, represent a promising resource for the discovery of novel natural products and make us aware that this species may be essential for the preservation of these unique and delicate marine ecosystems.

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CHAPTER 6

General Discussion and Conclusions

6.1 General discussion

The continuous search for new pharmaceutical compounds is essential to guarantee human survival. As drug leads continue to dwindle, the search for new chemical scaffolds has moved to unexplored/underexplored environments, such as the deep-sea. This environment displays a diverse and untapped biodiversity thriving under extreme conditions, translating into a huge potential for the discovery of new natural products. Actinobacteria, with their inherent metabolite-producing abilities, are a focus of interest for natural products research. These bacteria occupy various ecological niches, including deep-sea habitats.

In this thesis, the diversity and biotechnological potential of actinobacteria dwelling in deep-sea environments of the Arctic and Atlantic oceans (specifically in the Azores and Madeira archipelagos) was studied, together with their capacity to produce new compounds with antimicrobial, anticancer, anti-inflammatory or anti-obesity activities. It includes studies that comprehensively assess the diversity of actinobacteria and of their biosynthetic potential in underexplored deep-sea regions, which are scarcely found in the literature.

From the deep-sea samples analysed in the present thesis, a great diversity and abundance of actinobacteria was obtained, more than 300 actinobacteria distributed by 21 genera, thus corroborating the perception that the deep-sea is home to a high diversity of these bacteria. The fact that the isolation process included selective culture media with different carbon and nitrogen sources, oligotrophic media, application of selective pre-treatments and also a long period of incubation together with different incubation temperatures, certainly contributed to this result. These procedures allowed the isolation of genera, such as *Arthrobacter, Actinopolymorpha*, *Blastococcus* and *Nocardiodes*, that were only obtained under certain isolation conditions, like the use of a particular medium or incubation at a certain temperature. In addition, the isolation strategies employed also allowed retrieving genera that were not reported so far to occur in deep-sea environments, as is the case of *Actinopolymorpha*, *Actinotalea Blastococcus*, *Leucobacter*, *Dietzia* and *Rhodococcus*. This result calls the attention to the importance of employing diverse culture strategies for a greater success in isolating a wide range of actinobacteria. Furthermore, the high diversity of deep-sea actinobacteria isolated in the scope of this thesis is also a very important contribution to increase the knowledge in this field.

During the process of isolation and identification of actinobacteria from the analysed deep-sea samples, five strains were identified as potential new phylogenies. A deeper

taxonomic characterization process was carried out for one of these strains, allowing certifying that this strain is indeed a new *Streptomyces* species. The remaining strains associated with the genus *Brevibacterium* (n=1) and *Microbacterium* (n=3) are also under characterization. This finding not only supports the perception that the deep-sea is a source of new actinobacterial phylogenies, but also contributes to enlarge the number of actinobacterial species discovered from deep-sea environments.

The results obtained in this thesis also demonstrate that deep-sea actinobacteria are a valuable source of bioactive compounds. Notably, significant anti-obesity and anti-inflammatory activities were found in the tested actinobacterial extracts, which is particularly remarkable given that these bioactivities remain largely unreported for these bacteria, opening doors for the discovery of new drugs for the treatment of inflammatory diseases and obesity disorders. In addition to the prolific and well-reported genus *Streptomyces*, other genera known to be less conventional in producing molecules of interest also showed interesting bioactivities, such as *Brachybacterium*, *Brevibacterium*, *Dietzia*, *Leucobacter*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, *Rhodococcus*, *Salinispora* and *Tsukamurella*.

Metabolic analysis revealed that the bioactive deep-sea actinobacterial extracts contained some metabolites associated with natural products already isolated from other actinobacteria or from marine organisms. These results indicate that although these actinobacteria were isolated from deep-sea environments, they can also produce compounds that were first isolated from actinobacteria from other environments, indicating that some of these are cross-genera. One of the most notable results found in this work was that no hits to natural products accounting for the observed bioactivities were obtained in some actinobacterial extracts and, therefore, the corresponding strains constitute a promising source of new bioactive compounds (antibiotics, anticancer, anti-inflammatory and anti-obesity drugs). These results demonstrate that prospecting deep-sea actinobacteria is a promising approach for discovering new molecules with pharmaceutical applications, though in the present thesis the novelty of the unmatched molecules could not be characterized.

The metabolic profile of *Streptomyces chumphonensis* strain C_003 1.18, isolated from a deep-sea coral collected in Madeira archipelago (described in Chapter 4), revealed the presence of known antibiotics, but also of three potentially new molecules. This strain was selected for large-scale cultivation due to its potential anti-obesity activity, which is a scantly explored bioactivity and, so far, there are no reports of natural products produced by actinobacteria with this activity. However, when growing the strain in a larger scale for chemical analysis, it was found that the extract from small-scale cultivation

exhibited activity that appeared not to be reproducible in large-scale cultivation, since the extract became toxic to zebrafish larvae. Mass-guided isolation was then performed to isolate other interesting masses that were present in the extract and that did not show hits to known compounds in the dereplication analysis. During mass-guided isolation, the compound Piericidin A1, known for its antimicrobial properties, was isolated. Isolation of this compound has never been reported for deep-sea coral-associated actinobacteria, although it has been isolated from actinobacteria of deep-sea sediments. This discovery is in line with the hypothesis that suggests that coral-associated actinobacteria might act as a protective barrier against invasive pathogens, potentially through competition for space and occupation of coral niches or by producing antimicrobial compounds that directly hamper the growth of invasive microbes in corals. Three novel compounds were isolated (*m/z* 479.3940, 372.3479 and 766.8934) from *S. chumphonensis* strain C_003 1.18, and dereplication analysis showed no hits to these compounds, confirming their novelty. This finding suggests that *Streptomyces* associated with deep-sea corals represent a promising resource for the discovery of novel natural products with both ecological and pharmacological interest.

Together, the results presented in this thesis highlight the importance of bioprospecting underexplored environments, such as the deep-sea, not only for finding new phylogenies but also for the discovery of new natural products. Further research in this field has the potential to yield significant discoveries and advance the frontiers of deep-sea bioprospection.

As future work, it will be important to conclude the process of taxonomic characterization of the new strains and elucidate the chemical structure of the three new masses identified in the extract of *S. chumphonensis* strain C_003 1.18 and screen if they hold bioactivity. It will be also important to chemically characterise the extracts of all strains that exhibited anti-inflammatory and anti-obesity activities, in order to isolate new molecules with these bioactivities. Furthermore, it will also be very interesting to investigate the capacity of all actinobacterial isolates obtained in this work to grow under pressure conditions and analyse if these conditions could lead to the production of new bioactive molecules.

6.2 Conclusion

This thesis investigated the biodiversity and biosynthetic potential of actinobacteria inhabiting deep-sea environments of the Arctic and Atlantic oceans (including Azores and Madeira archipelagos), with the ultimate goal of identifying novel bioactive metabolites that could have significant biotechnological applications. The deep-sea samples

analysed in the scope of this thesis showed a high diversity of actinobacteria, including new phylogenies, with some of them showing a promising bioactive potential. It is particularly notorious the anti-inflammatory and anti-obesity activities that were found in some of the actinobacterial extracts tested, since these bioactivities remain largely unexplored for actinobacteria. In addition, three new molecules were isolated from a *Streptomyces* strain, strengthening the prolific potential of this genus.

Overall, the results obtained in this thesis contribute to increase the scarce knowledge available on actinobacteria associated with deep-sea environments and on their bioactive potential. Furthermore, metabolic analysis of some of the extracts obtained in this thesis pave the way for the discovery of new natural products with pharmaceutical relevance.

Appendix

Appendix I

Chapter 2

Table S1 – Taxonomic identification of the actinobacterial isolates retrieved from analysed the deep-sea sediments and corresponding GenBank accession number

Campaign	Geographical locations	Isolate	Closest identification*	Similarity (%)†	Sequence length (bp)	GenBank accession number
MarMine Arctic Mid-Ocean Ridge (AMOR) 2016	Artic Ocean	78.9	*Micrococcus yunnanensis*	99.64	1375	OQ363622
		78.10	*Micrococcus yunnanensis*	99.78	1378	OQ363620
		78.3	*Brachybacterium paraconglomeratum*	99.76	1269	OQ363621
		79_1.6	*Brachybacterium paraconglomeratum*	99.35	1378	OQ363628
		79_1.24	*Brevibacterium antiquum*	**98.61**	1323	OQ363625
		79_1.25	*Brevibacterium antiquum*	**98.42**	1391	OQ363626
		79_1.12	*Brevibacterium antiquum*	**98.61**	1373	OQ363623
		79_1.12 (A)	*Brachybacterium paraconglomeratum*	99.77	1327	OQ363624
		79_1.4	*Brachybacterium paraconglomeratum*	99.62	1301	OQ363627
		80_1.6	*Brevibacterium antiquum*	**98.49**	1392	OQ363630
		80_1.4	*Brachybacterium paraconglomeratum*	99.73	1273	OQ363629
		80_1.7	*Brachybacterium paraconglomeratum*	99.70	1326	OQ363631
		81_2.5	*Brevibacterium antiquum*	**98.22**	1400	OQ363633
		81_1.2	*Streptomyces* sp.	99.69	1290	OQ363632
		82_2.6	*Brevibacterium antiquum*	**98.35**	1392	OQ363637
		82_2.13	*Streptomyces* sp.	99.71	1379	OQ363636
		82_1.3	*Brachybacterium paraconglomeratum*	99.83	1310	OQ363634
		82_2.10	*Brachybacterium paraconglomeratum*	99.79	1288	OQ363635
		82_2.8	*Brachybacterium paraconglomeratum*	99.79	1331	OQ363638
		136.12	*Actinotalea* sp.	**97.85**	1395	OQ363614
		136.30	*Brevibacterium antiquum*	**98.54**	1367	OQ363619
		136.19	*Actinotalea ferrariae*	**98.40**	1370	OQ363617
		136.2	*Dietzia psychralcaliphila*	100	1362	OQ363618
		136.13	*Actinotalea subterranea*	**98.32**	1371	OQ363615
		136.15	*Brevibacterium antiquum*	**98.21**	1389	OQ363616
IH mission SEDMA R 1/2017	Madeira region	E147_1.1	*Brevibacterium salitolerans*	98.99	1394	OQ363651
		E147_1.8	*Brachybacterium paraconglomeratum*	99.69	1303	OQ363652

		MA3_2.11	*Brevibacterium sediminis*	99.93	1376	OQ36365
		MA3_2.14	*Streptomyces resistomycificus*	100	1391	OQ363656
		MA3 2.13	*Streptomyces* sp.	**97.37**	1405	OQ363655
		MA3_1.18	*Brachybacterium paraconglomeratum*	99.79	1300	OQ363653
		MA3_2.6	*Leucobacter komagatae*	100	1272	OQ363657
EMEPC/PEPC/LUSO/2016	Azores region	DS1_6	*Microbacterium testaceum*	99.33	1352	OQ363641
		DS1_10.2	*Microbacterium testaceum*	99.33	1352	OQ363639
		DS1_10.4	*Microbacterium testaceum*	99.34	1365	OQ363640
		DS3_6.1	*Microbacterium testaceum*	99.34	1368	OQ363645
		DS3_17.1	*Microbacterium testaceum*	99.25	1206	OQ363642
		DS3_17.2	*Microbacterium testaceum*	99.30	1285	OQ363643
		DS3_19.2	*Microbacterium testaceum*	99.34	1369	OQ363644
		DS4_2.1	*Microbacterium testaceum*	99.34	1365	OQ363647
		DS4_2.3	*Microbacterium testaceum*	99.35	1375	OQ363648
		DS4_3	*Rhodococcus* sp.	100	1167	OQ363650
		DS4_18	*Rhodococcus* sp.	100	1190	OQ363646
		DS4_20	*Rhodococcus yunnanensis*	99.83	1171	OQ363649

*According to 16S ribosomal RNA (Bacteria and Archaea type strains) database from NCBI BLAST.
†Values in bold indicate potential new species.

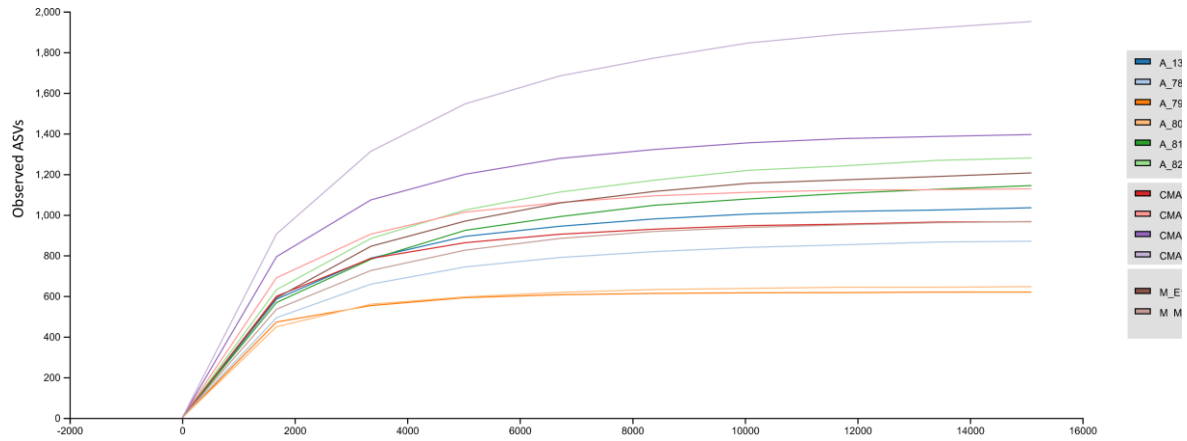

Figure S1 – Rarefaction plot based on the number of ASVs for all samples

Table S2 - Unique Metabolite List for all bioactive actinobacterial extracts that correspond to annotated molecules produced by actinobacteria strains. This list features DEREPLICATOR, DEREPLICATOR VarQuest and DEREPLICATOR+ annotations that are sorted by score, mass, compound name, adduct and FDR %. For analysis of the results, a threshold p-value ≤ 10-10 for DEREPLICATOR and DEREPLICATOR VarQuest, and a very strict score value of 15 on DEREPLICATOR+ were selected to reduce false matches. NA – Not applicable.

DEREPLICATOR							
Strain ID	**Compound Name**	**Score**	**p-Value**	**Peptide Mass**	**Spectrum Mass**	**Adduct**	**Peptide FDR %**
82_2.13	Surugamide B	16	4.80E-48	897.61	898.61	M+H	0
MA3_2.14	Surugamide A	16	7.30E-48	911.62	912.63	M+H	0
MA3_2.14	Surugamide D	12	1.50E-35	897.61	898.61	M+H	0
DEREPLICATOR VARQUEST							
Strain ID	**Compound Name**	**Score**	**p-Value**	**Peptide Mass**	**Spectrum Mass**	**Adduct**	**Peptide FDR %**
DS3_6.1	Amphomycin	10	3.30E-16	1289.65	703.395	M+2H	0
136_2	Antibiotic A0341A_3-Hydroxy	6	7.90E-14	1140.52	592.785	M+2H	0
136_2	Antibiotic A54145_3-Demethoxy	7	3.20E-15	1627.77	781.896	M+2H	0
79_1.6	Antibiotic A54145_3-Demethoxy	12	8.70E-19	1627.77	872.457	M+2H	0
79.4	Antibiotic A54145_3-Demethoxy,_3'''-deoxy	8	1.80E-18	1611.78	761.383	M+2H	0
79_1.6	Antibiotic A54145_3-Demethoxy,_3'''-deoxy	13	5.90E-21	1625.79	873.431	M+2H	0
79_1.6	Antibiotic A54145_3'''-Deoxy,_3-O-de-Me	12	6.50E-19	1627.77	845.42	M+2H	0
80_1.6	Antibiotic A54145A	14	1.20E-22	1643.77	844.421	M+2H	20
80_1.6	Antibiotic A54145D	13	5.50E-21	1657.78	828.925	M+2H	0
136_2	Antibiotic A54145F	6	5.10E-12	1629.75	801.911	M+2H	0
80_1.6	Antibiotic A54145F	11	1.50E-15	1629.75	872.457	M+2H	0
MA3_2.14	Champacyclin	11	8.00E-33	897.605	884.596	M+H	0
79_1.6	Daptomycin	10	2.70E-15	1619.71	847.912	M+2H	0
80_1.6	Enduracidin-C	10	2.60E-10	2380.97	797.084	M+3H	0
79.4	Glycinocin B	6	5.90E-14	1260.66	639.329	M+2H	0
DS1_10.4	Phepropeptin A	10	1.30E-26	682.442	764.469	M+H	0

DS1_10.4	Phepropeptin D	10	9.00E-27	730.442	778.485	M+H	0
80_1.6	Skyllamycin A	12	1.60E-18	1480.69	692.836	M+2H	0
80_1.6	Skyllamycin B	12	1.90E-19	1468.66	751.867	M+2H	0
82_2.13	Surugamide A	21	9.60E-58	911.621	982.634	M+H	0
MA3_2.14	Surugamide C	15	6.60E-44	897.605	878.643	M+H	0
MA3_2.14	Surugamide D	10	1.40E-26	897.605	458.332	M+2H	0
79_1.6	Telomycin	10	4.00E-17	1271.55	600.782	M+2H	0
136_2	Zelkovamycin	7	1.40E-16	779.306	426.719	M+2H	0
DEREPLICATOR+							
Strain ID	**Metabolite**	**Score**	**p-Value**	**Metabolite Mass**	**Spectrum MZ**	**Adduct**	**Metabolite FDR %**
MA3 2.13	Antibiotic A54556D	16	NA	720.385	721.388	M+H	0
DS3_6.1	Antibiotic H668	18	NA	688.44	689.45	M+H	0
MA3 2.13	Antibiotic YL 02107Q-A	17	NA	850.493	851.505	M+H	0
82_2.13	Antimycin A	15	NA	478.195	479.203	M+H	0
MA3 2.13	Concanamycin B	17	NA	808.497	809.506	M+H	0
82_2.13	Deferoxamine	15	NA	658.463	659.47	M+H	0
MA3_2.14	Deferoxamine	16	NA	636.385	637.391	M+H	0
136.30	Dermostatin A	27	NA	720.445	721.456	M+H	0
136.30	Dermostatin B	33	NA	734.461	735.473	M+H	0
MA3 2.13	Desferrioxamine G	16	NA	618.359	619.367	M+H	0
DS1_10.4	Erythromycin	20	NA	717.466	718.47	M+H	0
DS1_10.4	Flavofungin II	15	NA	664.419	665.424	M+H	0
DS3_6.1	Flavofungin II	18	NA	664.419	665.425	M+H	0
DS4.3	Landomycin A	15	NA	1068.46	535.236	M+2H	0
136.30	Langkolide	16	NA	1446.75	724.381	M+2H	0
79_1.6	Malacidin A	19	NA	1248.62	625.315	M+2H	0
79.4	Monensin M1	15	NA	672.445	673.45	M+H	0

79_1.12(A)	Monensin M1	18	NA	672.445	673.45	M+H	0
78.3	Monensin M2	17	NA	688.44	689.449	M+H	0
79_1.12	Monensin M2	18	NA	688.44	689.451	M+H	0
MA3 2.13	Mycolactone F	16	NA	714.507	715.514	M+H	0
79_1.6	Nocardamine	16	NA	584.353	585.361	M+H	0
82_2.13	Nocardamine	21	NA	584.353	585.361	M+H	0
DS4.20	Piericidin	15	NA	385.262	386.269	M+H	0
DS4.20	Piericidin a1	15	NA	415.272	416.279	M+H	0
82_2.13	Proferrioxamine A1	17	NA	546.338	547.346	M+H	0
MA3 2.13	VacidinA	15	NA	1112.57	557.294	M+2H	0

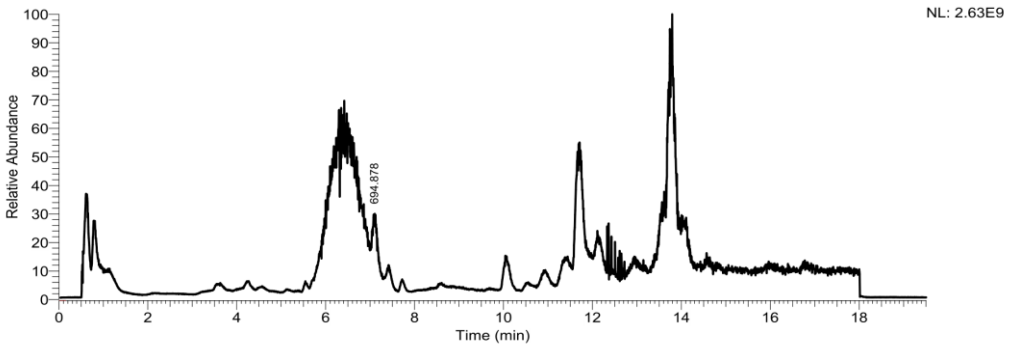

Figure S2 – Total ion chromatogram (TIC) of the actinobacterial crude extract from *Microbacterium* sp. DS3_6.1, recovered from the Artic region. The *m/z* value 694.878 of the major compound (Fig.6) is represented. The NL (normalization level) value corresponds to the base peak intensity.

Appendix II

Chapter 3

DPG = Diphosphatidylglycerol

PE = Phosphatidylethanolamine

GPL = Glycophospholipid

PL = Phospholipid

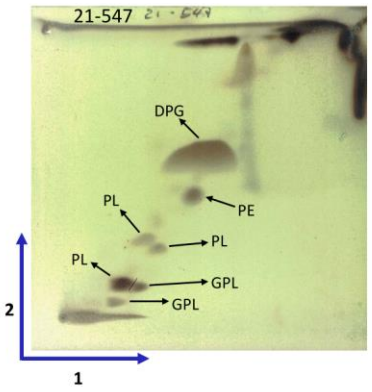

Figure S3 – Polar lipids profile of strain MA3_2.13^T obtained from an exponential culture grown in GYM 50SW agar medium.

Table S3 – Cellular fatty acids composition of strain MA3_2.13^T

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2	GC-MS
1.692	4.24E+8	0.027	----	7.005	SOLVENT PEAK	----	< min rt		
2.312	133	0.018	----	8.176		----	< min rt		
2.646	686	0.025	----	8.810		----	< min rt		
3.185	430	0.028	----	9.828		----			
4.534	569	0.030	----	11.556		----			
5.269	930	0.033	----	12.262		----			
7.014	1238	0.039	0.987	13.619	14:0 ISO	0.57	ECL deviates 0.001	Reference 0.001	OK
7.547	432	0.033	0.976	13.998	14:0	0.20	ECL deviates -0.002	Reference -0.001	OK
8.528	2119	0.038	0.962	14.622	15:0 ISO	0.95	ECL deviates 0.001	Reference 0.002	OK
8.669	6169	0.039	0.960	14.712	15:0 ANTEISO	2.76	ECL deviates 0.001	Reference 0.002	OK
8.826	412	0.038	----	14.812		----			
9.119	3824	0.039	0.955	14.999	15:0	1.70	ECL deviates -0.001	Reference -0.001	OK
9.873	25042	0.055	0.948	15.445	16:1 ISO G	11.08	ECL deviates 0.003		Identified as double peak of 16:1 ISO CIS 9 and 16:1 ISO CIS 4
10.180	109876	0.041	0.945	15.627	16:0 ISO	48.47	ECL deviates 0.001	Reference 0.002	OK
10.500	7933	0.045	0.943	15.817	16:1 CIS 9	3.49	ECL deviates 0.000		OK
10.805	5812	0.041	0.941	15.997	16:0	2.55	ECL deviates -0.003	Reference -0.003	OK
10.891	796	0.039	0.940	16.047	unknown 16.048	0.35	ECL deviates -0.001		
11.539	3527	0.044	0.937	16.418	16:0 9? METHYL	1.54	ECL deviates 0.002		Identified as 17:1 ISO CIS 9
11.735	13753	0.049	0.936	16.530	17:1 ANTEISO C	6.01	ECL deviates 0.005		Identified as double peak of 17:1 ANTEISO CIS 9 and 17:1 ANTEISO CIS 4
11.908	1355	0.038	0.935	16.629	17:0 ISO	0.59	ECL deviates 0.000	Reference 0.000	OK
12.071	29416	0.043	0.934	16.723	17:0 ANTEISO	12.83	ECL deviates 0.001	Reference 0.000	OK
12.195	7054	0.042	0.934	16.793	17:1 CIS 9	3.07	ECL deviates 0.001		OK
12.362	1744	0.049	0.933	16.889	17:0 CYCLO	0.76	ECL deviates 0.001	Reference 0.000	Identified as 17:0 CYCLO CIS 9
12.553	1586	0.044	0.932	16.999	17:0	0.69	ECL deviates -0.001	Reference -0.002	OK
13.278	1655	0.044	0.930	17.408	17:0 10METHYL	0.72	ECL deviates -0.002		Identified as 18:1 ISO CIS 9
13.386	936	0.048	0.930	17.469	18:1 ISO H	0.41	ECL deviates 0.009		Identified as 18:1 ISO CIS 11
13.612	1141	0.069	0.929	17.597	UNKNOWN	0.49	ECL deviates 0.002		Not confirmed
13.920	1384	0.044	0.929	17.770	18:1 CIS 9	0.60	ECL deviates 0.001		OK
14.015	390	0.037	0.929	17.824	Sum In Feature 7	0.17	ECL deviates -0.001	18:1 TRANS	Identified as 18:1 CIS 11
----	390	---	----	----	Summed Feature 7	0.17	18:1 CIS 11/t 9/t 6	18:1 TRANS	
----	-----	---	----	----		----	18:1 TRANS		

ECL Deviation: 0.003 Reference ECL Shift: 0.002 Number Reference Peaks: 11
Total Response: 229525 Total Named: 227184
Percent Named: 98.98% Total Amount: 214269

Appendix

Chapter 5

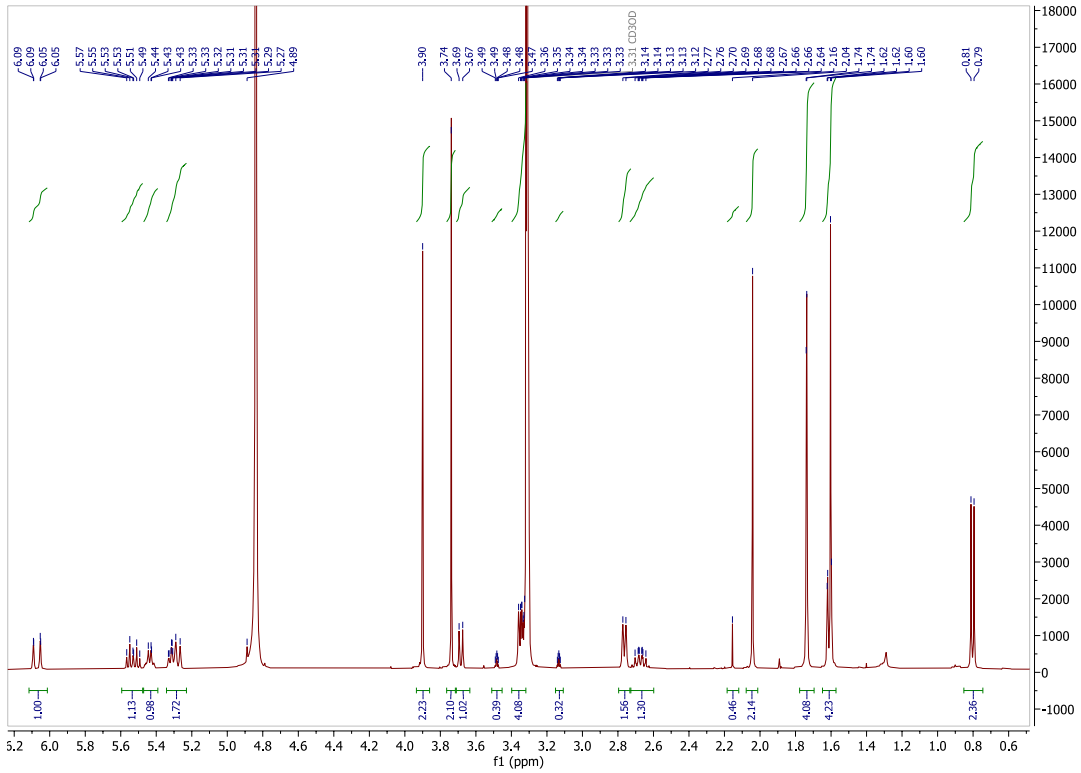

Figure S3 – ^{1}H NMR spectrum (400 MHz, MeOH-d4) of Piericidin A1 isolated from *S. chumphonensis* strain C_003 1.18

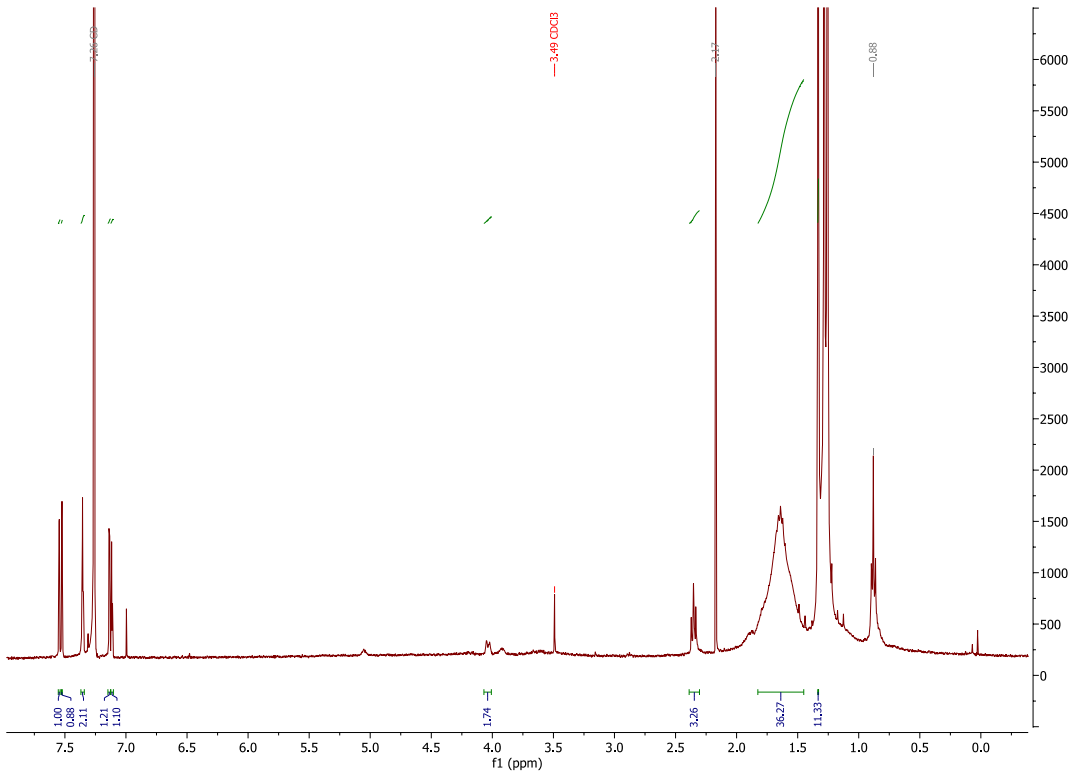

Figure S4 – ^{1}H NMR spectrum (400 MHz, chloroform-d) of Tris(2,4-di-tert-butylphenyl)phosphate isolated from *S. chumphonensis* strain C_003 1.18