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Dissertation for the master's degree
in Biotechnology

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

A resistência aos antibióticos é uma ameaça grave à saúde global, uma vez que o tratamento de um número crescente de infeções bacterianas se tem tornado um problema sério. Adicionalmente, o cancro é responsável pela morte de milhões de pessoas todos os anos e a resistência às terapias existentes é preocupante.

As Actinobactérias são produtoras prolíficas de compostos bioativos com aplicações farmacêuticas. A bioprospecção de ambientes não explorados ou sub-explorados, como é o caso do mar profundo, poderá ser a chave para a descoberta de novas moléculas bioativas. Este trabalho teve como foco o estudo da biodiversidade de Actinobactérias cultiváveis associadas a amostras de mar profundo de Portugal, e a investigação do potencial antimicrobiano e citotóxico das estirpes isoladas. Nove amostras de mar profundo, incluindo esponjas, corais e sedimentos, foram recolhidas no Arquipélago da Madeira a profundidades entre os 463 m e 865 m, utilizando o submersível Lula1000. De modo a promover a seleção de Actinobactérias, foi utilizado um pré-tratamento com calor e usados três meios de cultura seletivos suplementados com antibióticos. Foram isoladas 68 estirpes de Actinobactérias a partir das amostras analisadas, afiliadas com os géneros *Brevibacterium*, *Tsukamurella*, *Microbacterium*, *Micrococcus*, *Leucobacter*, *Rhodococcus*, *Brachybacterium* e *Streptomyces*. Dois destes isolados actinobacterianos poderão representar novas espécies de *Microbacterium*, uma vez que a similaridade dos respetivos genes 16S rRNA se encontra abaixo do valor de referência de 98.7%, utilizado para distinguir entre espécies. Os extratos brutos das estirpes de Actinobactérias isoladas foram avaliados quanto às suas atividades antimicrobiana e anticancerígena, utilizando o método de difusão em disco e o ensaio MTT, respetivamente. Duas estirpes de Actinobactérias, associadas aos géneros *Brevibacterium* e *Brachybacterium*, tiveram atividade contra uma ou mais estirpes de referência testadas, nomeadamente *Candida albicans*, *Bacillus subtilis* e *Staphylococcus aureus*, exibindo valores de MIC de 1000 μg mL^{-1}. Os ensaios de citotoxicidade revelaram 23 estirpes capazes de reduzir a viabilidade celular de pelo menos uma das linhas celulares testadas (T47-D, HepG2 e hCMEC/D3). Este trabalho contribuiu para aumentar o conhecimento sobre a diversidade de Actinobactérias associadas a amostras de mar profundo em Portugal e o seu potencial bioativo. No futuro, será realizada a desreplicação dos extratos bioativos para procurar novos compostos bioativos.

Palavras-chave: Actinobactérias, anticancro, antimicrobiano, mar profundo

ABSTRACT

Antibiotic resistance is a big threat to global health, as the treatment of a growing number of bacterial infections is becoming a serious problem. In addition, cancer is responsible for the death of millions of people every year and resistance to available therapies is of big concern.

It is well established that Actinobacteria are prolific producers of bioactive compounds with pharmaceutical applications. Bioprospecting unexplored or underexplored environments, like the deep-sea, may be a key for the discovery of new bioactive molecules. This work aimed to study the biodiversity of the cultivable Actinobacteria associated with deep-sea samples from Portugal, and investigate the antimicrobial and cytotoxic potential of the isolated strains. Nine deep-sea samples, that included sponges, corals and sediments, were collected at the Madeira archipelago at depths between 463 m and 865 m, using the submersible Lula1000. A heat pre-treatment and three selective culture media supplemented with different antibiotics were used to promote the selection of Actinobacteria. Sixty-eight actinobacterial strains were isolated from the analyzed samples, being affiliated with the genera *Brevibacterium*, *Tsukamurella*, *Microbacterium*, *Micrococcus*, *Leucobacter*, *Rhodococcus*, *Brachybacterium* and *Streptomyces*. Two of these actinobacterial isolates may represent new *Microbacterium* species as their 16S rRNA gene similarity was below the cut-off value of 98.7%, used to discriminate between species. The crude extracts of the isolated actinobacterial strains were screened for antimicrobial and cytotoxic activities, using the disk diffusion method and MTT assay, respectively. Two actinobacterial strains associated with the genera *Brevibacterium* and *Brachybacterium* were active against one or more of the reference strains tested, namely *Candida albicans*, *Bacillus subtilis* and *Staphylococcus aureus,* exhibiting MIC values in the range of 1000 μg mL^{-1}. Cytotoxic assays revealed 23 strains capable of reducing the cellular viability of at least one of the cell lines tested (T47-D, HepG2 and hCMEC/D3). This work contributed to increase the knowledge about the diversity of Actinobacteria associated with deep-sea samples of Portuguese environments and of their bioactive potential. In the future, dereplication of the bioactive extracts will be performed to look for the presence of new bioactive compounds.

Key words: Actinobacteria, anticancer, antimicrobial, deep-sea

INDEX

LIST OF ABBREVIATIONS/ACRONYMS

DMEM – Dubelco's Modified Eagle Medium

DMSO – Dimethyl Sulfoxide

HBV – Chronic Hepatitis B Virus

HCC – Hepatocellular Carcinoma

HCV – Chronic Hepatitis C Virus

IDSA – Infectious Diseases Society of America

MDR – Multi-drug resistant

MH – Mueller-Hinton Agar

MIC – Minimal Inhibitory Concentration

NC – Negative Growth Control

NCV – Chronic Hepatitis C

NP – Natural Products

NRPS – Non-ribosomal Polyketide Synthetases

PC – Positive Growth Control

PKS – Polyketide Synthetases

SD – Sabouraud Dextrose Agar

WHO – World Health Organization

FIGURE INDEX

TABLE INDEX

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Antibiotic-resistant bacterial infections and cancer

For the last few years, scientific and medical communities have been very concerned about antibiotic resistance[1]. Multidrug-resistant bacteria (MDR) are responsible for a high mortality in Europe and in United States of America, due to the lack of effective compounds to treat patients infected with these microorganisms[1]. The main causes for the spread of antibiotic resistance include the massive and inappropriate use of antibiotics along with the emergence of antibiotic resistance genes (**Figure 1**)[1,2].

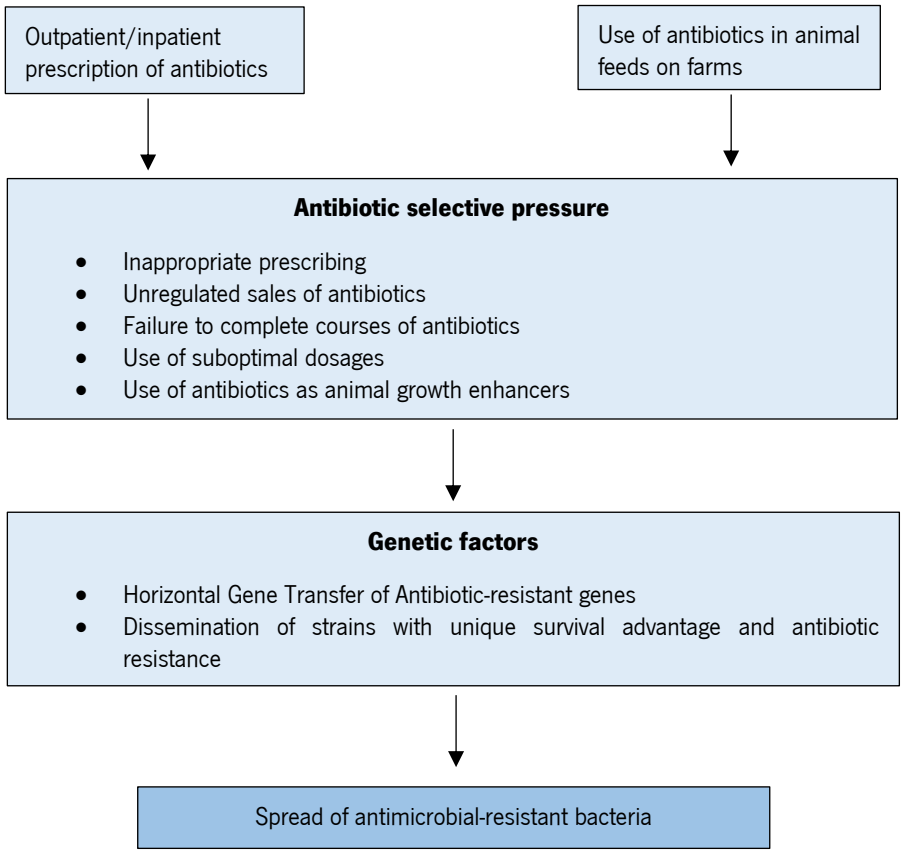

Figure 1. Sources of Antibiotic Resistance.

Bacteria have a genetic plasticity that allows them to respond to physiological threats, as is the case of the presence of antibiotics[3]. Some bacterial cells develop mutations in genes that interfere with the activity of drugs, consequently allowing their survival[3]. Moreover, bacteria can exchange genetic material, through transformation, conjugation and transduction processes that contribute to acquire antibiotics resistance[3,4,5].

Bacteria can also produce enzymes that catalyze chemical changes to antimicrobial molecules, leading to their inactivation[3]. Gram-negative bacteria have porins on the outer membrane, which are transmembrane channels that enable the diffusion of metabolites to the periplasmic space, including antibiotics[3]. Bacteria have mechanisms that interfere with the function, type and level of porins expressed, resulting in a reduction of the uptake of antimicrobial molecules and preventing them to reach their target[3]. Both Gram-positive and Gram-negative organisms also have many classes of efflux pumps that allow the extrusion of antibiotics[3].

The Infectious Diseases Society of America (IDSA) described a group of microorganisms that is able to "escape" antibiotics action and that is responsible for many nosocomial infections, "the ESKAPE pathogens"[6]. This group includes *Staphylococcus aureus*, which is one of the most common antibiotic-resistant Gram-positive bacteria, and the Gram-negative bacteria *Escherichia coli*, among other pathogens[6].

It is estimated that by 2050, antibiotic-resistant microorganisms will cause around 300 million premature deaths[3]. Therefore, the failure of conventional antimicrobials action against some infectious agents and the prevalence of pathogens with increased antibiotic resistance raise the need to search for new compounds able to treat infections caused by these microorganisms[7].

Along with antibiotic-resistant infections, the prevalence of cancer in human population is another serious health problem of our times. According to the World Health Organization (WHO), cancer is the second leading cause of death worldwide[8]. In 2018, approximately 9.6 million people died due to this ilness[8]. Cancer results from an interaction between genetic factors and physical, chemical or biological carcinogens[8]. The main cancer risk factors include age, alcohol abuse, unhealthy diet, sleep disturbance and physical inactivity[8–14]. About 70% of deaths occur in low- and middle-income countries, mainly because of late-stage diagnosis and inaccessible treatment[8].

Breast cancer is the most common cancer affecting women, being responsible for 1.700.000 new cases every year[15]. In women with less than 45 years old, breast cancer is the main cause of cancer-related deaths and constitutes an especially relevant burden in developing countries[15]. Even though the scientific community has made big efforts in the breast cancer field, resistance to therapies and substantial improvement in survival rates still represent the main challenges[15].

On the other hand, hepatocellular carcinoma (HCC) is the fifth most frequent cancer worldwide, and the second leading cause of cancer-related mortality in men[16,17]. In the last years, an increasing

incidence of this cancer and mortality have been observed in USA and in many European countries[16,17]. Chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV), and/or alcohol abuse are major risk factors for the development of HCC[16,17].

The toxicity associated with some cancer treatments along with their side effects, makes the discovery of new anticancer drugs a priority.[18]

1.2. Natural products and their applications

The discovery of penicillin by Alexander Fleming sparked the interest of the scientific community for natural products (NP) research, prompting the discovery of new natural compounds[19].

NP can be produced by plants, fungi, bacteria and animals and have a wide range of applications in human and veterinary medicine, as well as in agriculture[20]. These compounds hold important bioactive properties such as antidiabetic, antitumor, antibacterial, antifungal, antiobesity, antitubercular, immunomodulator, anthelmintic, herbicidal, insecticidal, surfactant and food preservative[20].

Microbial NP, in particular, have been widely explored for medicinal and pharmaceutical purposes[21]. They usually result from the secondary metabolism of microorganisms and generally provide advantages to the producing species[20]. So far, approximately 22500 bioactive microbial metabolites have been discovered, and a great percentage are derived from Actinobacteria[22].

1.3. Actinobacteria as a source of bioactive natural products

Actinobacteria are a group of Gram-positive bacteria that can form long and branched filaments, similar to the fungal mycelia[22,23]. In fact, when this phylum was firstly discovered, their members were thought to be a transitional type between fungi and bacteria[22,23]. It is now well established that Actinobacteria have evolved 2.7 billion years ago, representing one of the most primitive lineages among prokaryotes[22]. Phylogenetic studies showed that Actinobacteria, Cyanobacteria and Deinococcus shared a common ancestor[24]. It is also suggested that the first Actinobacteria were obligate anaerobes, did not form spores or filaments, and their morphology was simple rod/coccus[24]. Even though recent lineages have the ability to grow filaments and form spores, a few groups lost this characteristic[24].

Actinobacteria genome can be linear or circular and has a high G+C content (65%-75%)[23]. They usually have only one chromosome, but they may also have large plasmids[24]. Linear chromosomes can

be found in the genera *Gordonibacter*, *Kineococcus*, *Rhodococcus* and *Streptomyces*[24]. According to Sharma *et al.* (2018), the genome size of actinobacterial strains varies between 2.5 Mbp and 9.7 Mbp, while Verma *et al.* (2018) suggest that it can reach 12.7 Mbp[22,23].

Actinobacteria is considered one of the largest taxonomic units within the domain Bacteria[25]. This phylum is divided into 6 classes (Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria and Thermoleophilia) and 63 families[25]. The class Actinobacteria includes most of the microorganisms that produce bioactive compounds and contains 16 orders and 43 families[25].

Actinobacteria morphology can vary from coccoid to rod coccoid, with various species presenting fragmenting hyphal forms or branched hyphae[22,25]. They can form either aerial or vegetative (or substrate) mycelia and their hyphae are about 1.0-1.5 μM or less in diameter[23]. Moreover, this group produces diverse pigments which color depends on the strain, the culture medium used, the growth conditions and the age of the culture[23,25]. These pigments can be found in the vegetative or aerial mycelium or diffused in the culture medium[23], and even though they are not crucial for the growth and development of the microorganisms, they may improve their survival and competitiveness, as they can have important bioactivities that protect them against pathogens[25,26].

Actinobacteria are also known for the characteristic earthy-smell when it rains, due to the production of the volatile metabolite geosmin[23]. These prokaryotes are mostly aerobic, motile or nonmotile, and may produce spores on the substrate and/or the aerial mycelium[22,25]. The structure and appearance of spores are very important in the taxonomy of Actinobacteria[25]. They can consist in single cells, in chains of different lengths or in vesicles, and flagella may also be present[25]. Actinobacteria exhibit a complex life cycle and can reproduce asexually by fragmentation of mycelia or through formation of spores or conidia (**Figure 2**)[22].

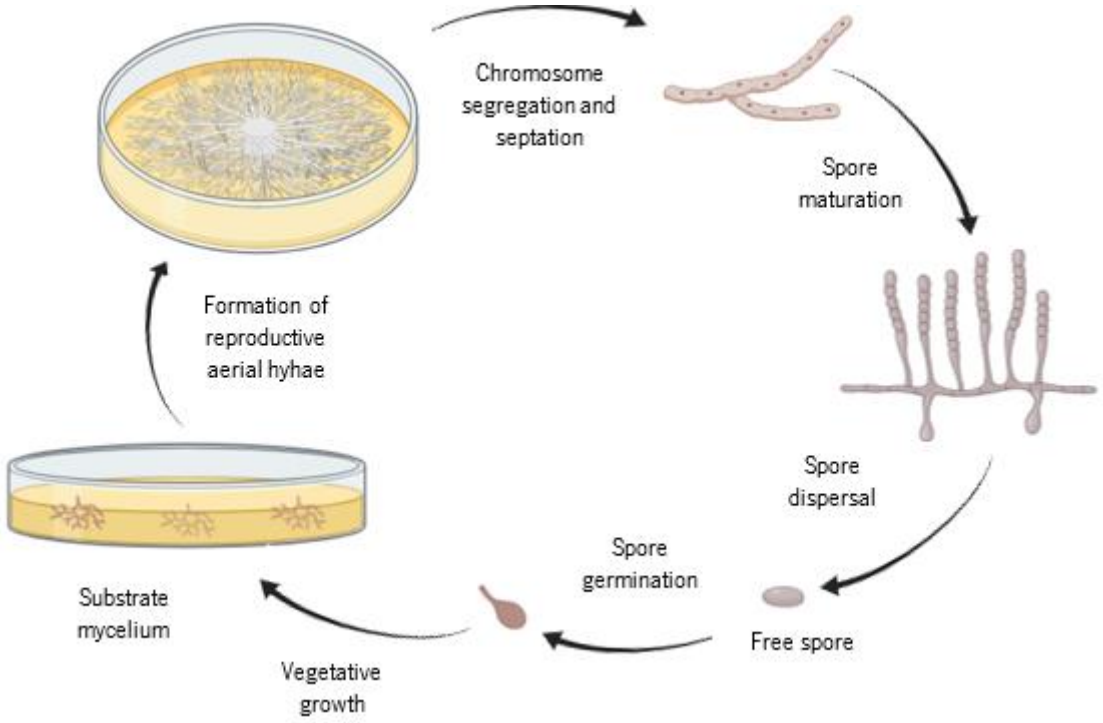

Figure 2. Life cycle of sporulating Actinobacteria.

These microorganisms are ubiquitous in the environment[22]. They are present in a wide range of habitats, from which soil is the most common[22]. Actinobacteria play an important ecological role, by contributing to humus formation, recycling of biomaterials and breaking down complex polymers of humans, animals and fungi[22].

Approximately 45% (ca. 10000) of the bioactive microbial metabolites described were isolated from Actinobacteria[22]. These microorganisms are one of the most biotechnologically important prokaryotes, producing compounds with a wide range of applications[27]. In fact, Actinobacteria are a major source of bioactive natural compounds, including clinically relevant antibiotics[22]. It was the discovery of the antibiotics actinomycin, streptothricin and streptomycin, produced by soil Actinobacteria, in the 1940s, that led to an increasing interest in this group of bacteria for the search of new bioactive compounds[20].

The main classes of antibiotics produced by Actinobacteria include ß-lactams, aminoglycosides, chloramphenicol, tetracyclines, glycopeptide antibiotics, macrolide-lincosamide-streptogramin B, quinolones, and sulphonamides[23]. For instance, the antibiotics vancomycin, teicoplanin and daptomycin, all produced by actinobacterial strains, are widely used to fight Gram-positive infections as well as methicillin-resistant *S. aureus*[20]. These compounds come mostly from the secondary metabolism of Actinobacteria, when microbial growth ceases[28].

About 60% of the antibiotics clinically used were obtained from *Streptomyces* species[29]. For instance, neomycin is a well-known antibiotic naturally produced by *Streptomyces fradiae*[22]. The antibiotic kanamycin was isolated from *S. kanamyceticus* and is often used to treat multi-drug resistant tuberculosis[30]. Ribostamycin, which is considered one of the most critically important antimicrobials by WHO, is produced by *S. ribosidificus*[30]. Other actinobacterial NP are used against serious fungal and bacterial infections in humans, such as amphotericin B and daptomycin, respectively[20]. Aside from the actinobacterial compounds that have already reached the drug market, some other promising ones are under clinical trials[31], such as lancovutide (ClinicalTrials.gov).

Several secondary metabolites isolated from Actinobacteria have also shown promising anticancer activities[27]. However, only a few studies have evaluated the cytotoxic potential of the natural products obtained from this outstanding group of bacteria[31].

Approved anticancer drugs produced by Actinobacteria include mitomycin C (used for treatment of anal, bladder, breast, cervical, colorectal, head and neck cancers), bleomycin (melanoma, lymphoma, sarcoma, testicular and ovarian cancer) and actinomycin D (Wilm's tumor, ovarian cancer, Ewing's sarcoma, osteosarcoma, soft tissue sarcoma, childhood rhabdomyosarcoma, etc)[20].

It is thought that non-ribosomal polyketide synthetases (NRPS) and polyketide synthases (PKS) are the main enzymatic complexes mediating the production of the majority of the bioactive metabolites isolated from Actinobacteria[31]. Moreover, genomic studies revealed that Actinobacteria with moderate and large genomes, in particular, hold many natural-product-biosynthetic gene clusters[32].

1.4. Actinobacteria from marine environments

Due to the intensive exploitation of Actinobacteria from terrestrial sources, scientists started to look for the presence of new bioactive compounds in Actinobacteria inhabiting less explored environments, as is the case of marine environments[33].

Oceans comprise diverse ecosystems and it is estimated that up to 9% of marine biodiversity is represented by Actinobacteria[22]. Marine Actinobacteria take part in the mineralization of organic matter, immobilization of mineral nutrients, fixation of nitrogen, improvement of physical parameters and protection of the environment (through the production of various metabolites)[22]. The first marine Actinobacteria to be discovered and characterized was *Rhodococcus marinonascene*[22]. However, other genera, such as *Dietzia*, *Streptomyces*, *Micromonospora*, *Nocardia*, *Salinispora*, *Serinicoccus*,

Marinophilus, *Larmerjespora*, *Salinibacterium*, *Aeromicrobium*, *Verrucosispora*, *Marinactinospora*, *Marinospora* and others, are also present in marine ecosystems[33]. Some of those genera are considered indigenous, with *Salinispora*, in particular, being the first obligate marine actinobacterial genus to be discovered[28].

It is estimated that less than 1% of all Actinobacteria have been identified[29]. Marine rare Actinobacteria, which include those strains that are recovered with less frequency, are particularly difficult to culture because of their specific growth requirements[34]. By unveiling the microbial community present in a given sample, cultivation-independent studies have allowed the improvement of culture strategies to recover yet non-cultivated Actinobacteria, including optimization of sample pre-treatment and supplementation of isolation media[29]. From 2007 to 2017, 177 new species of marine rare Actinobacteria were described, including 3 novel families and 29 new genera[29]. Compounds produced by these microorganisms exhibited important antimicrobial, antiparasitic, anticancer and antimalarial activities[29,31].

Underexplored or unexplored habitats, like deep-sea environments, are home for new species of Actinobacteria (including rare species) with potential to produce chemically diverse and unique metabolites[29,34]. **Table 1** lists some of the new actinobacterial species recovered from marine sediments, sponges and corals. In some cases, new actinobacterial genera were proposed.

Table 1. New species of Actinobacteria isolated from marine sediments, sponges and corals, in the last five years

Species	Source	Sample identification	Origin	Reference
Glycomyces sediminimaris	Marine sediment	Not applicable	Persian Gulf, Bushehr Province, Iran	[35]
Rubrobacter indicoceani	Deep-sea sediment	Not applicable	Indian Ocean	[36]
Marinitenerispora sediminis	Marine sediment	Not applicable	Tioman Island, Malaysia	[37]
Streptomyces reniochelinae	Marine sponge	*Reniochalina stalagmitis*	Sansha, Hainan Province, China	[38]
Streptomyces diacarni	Marine sponge	*Diacarnus megaspinorhabdosa*	Sansha, Hainan Province, China	[38]
Cellulosimicrobium arenosum	Marine sediment	Not applicable	Not available	[39]
Rhodococcus electrodiphilus	Marine coral	Not available	Gujarat, India	[40]
Corynebacterium alimapuense	Marine sediment	Not applicable	Valparaíso bay, Chile	[41]
Micromonospora craniellae	Marine sponge	*Craniella* sp.	South China Sea	[42]
Geodermatophilus marinus	Marine sponge	*Leucetta chagosensis*	South China Sea	[43]

Actinoplanes sediminis	Marine sediment	Not applicable	Megas Gialos, Syros, Greece	44
Streptomyces verrucosisporus	Marine sediment	Not applicable	Chumphon Province, Thailand	45
Micromonospora orduensis	Marine sediment	Not applicable	Black Sea coast, Ordu, Turkey	46
Nocardioides flavus	Marine sediment	Not applicable	Western Pacific Ocean	47
Psedonocardia profundimaris	Deep-sea sediment	Not applicable	Western Pacific Ocean	48
Streptomyces otsuchiensis	Marine sediment	Not applicable	Otsuchi Bay, Iwate Prefecture, Japan	49
Actinomadura craniellae	Marine sponge	*Craniella* sp.	South China Sea	50
Actinoalloteichus fjordicus	Marine sponge	*Antho dichotoma*	Trondheim fjord, Norway	51
Micromonospora globispora	Marine sediment	Not applicable	Black Sea coast, Ordu, Turkey	52
Micromonospora fluostatini	Marine sediment	Not applicable	Panwa Cape, Phuket Province, Thailand	53
Williamsia spongiae	Marine sponge	*Amphimedon viridis*	Guaecá beach, São Paulo, Brazil	54
Salinispora cortesiana	Marine sediment	Not applicable	Sea of Cortez, Mexico	55
Salinispora fenicalii	Marine sediment	Not applicable	Fiji	55
Salinispora goodfellowii	Marine sediment	Not applicable	Madeira Island, Portugal	55
Salinispora mooreana	Marine sediment	Not applicable	Fiji	55
Salinispora oceanensis	Marine sediment	Not applicable	Fiji	55
Salinispora vitiensis	Marine sediment	Not applicable	Fiji	55
Nocardia xestospongiae	Marine sponge	*Xestospongia* sp.	Andaman Sea, Phuket Province, Thailand	56
Kocuria subflava	Marine sediment	Not applicable	Indian Ocean	57
Streptomyces atlanticus	Marine sponge	*Aplysina fulva*	St. Peter and St. Paul Archipelago	58
Saccharopolyspora spongiae	Marine sponge	*Scopalina ruetzleri*	St. Peter and St. Paul Archipelago	59
Spongiactinospora rosea	Marine sponge	*Craniella* sp.	South China Sea	60
Williamsia aurantiacus	Marine sponge	*Glodia corticostylifera*	Guaecá beach, São Paulo, Brazil	61
Streptomyces ovatisporus	Marine sediment	Not applicable	Black Sea Coast, Samsun, Turkey	62
Actinomarinicola tropica	Marine sediment	Not applicable	South China Sea	63
Glutamicibacter mishrai	Marine coral	*Favia veroni*	Andaman Sea, India	64
Micromonospora pelagivivens	Deep-sea sediment	Not applicable	Kagoshima, Japan	65
Amycolatopsis albispora	Deep-sea sediment	Not applicable	Indian Ocean	66
Nesterenkonia salmonea	Dep-sea sediment	Not applicable	Southern Atlantic Ocean	67
Nesterenkonia sphaerica	Deep-sea sediment	Not applicable	Southern Atlantic Ocean	67
Rubrobacter tropicus	Deep-sea sediment	Not applicable	South China Sea	68
Rubrobacter marinus	Deep-sea sediment	Not applicable	South China Sea	68

Saccharopolyspora coralli	Marine coral	*Porites* sp.	Qiongdong Sea, Hainan Province, China	[69]

Table 2 lists new bioactive compounds produced by marine Actinobacteria discovered in the last five years, showing that *Streptomyces*, along with other actinobacterial genera, are prolific producers of these compounds[70].

Table 2. New bioactive compounds obtained from Actinobacteria isolated from marine sediments, corals and sponges, in the last five years

Compound	Species	Sampling site	Type of sample	Bioactivities	Reference
Anthraquinones N-acetyl-N-demethylmayamycin and Streptoanthraquinone A	*Streptomyces* sp.	East China Sea	Sediment	Antibacterial Cytotoxic	[71]
Polycyclic tetramic acid macrolactam Isoikarugamycin	*Streptomyces zhaozhouensis*	Utonde, Equatorial Guinea	Sediment	Antifungal Antibacterial	[72]
Nivelactam B	*Streptomyces varsoviensis*	East China Sea	Sediment	Antifungal Cytotoxic	[73]
Medermycin analogue	*Streptomyces* sp.	Zhejiang province, China	Sediment	Cytotoxic	[74]
Spiroindimicins E and F Lagunapyrones D and E	*Streptomyces* sp.	Trondheim Fjord, Norway	Sediment	Cytotoxic	[75]
Dokdolipids A-C	*Actinoalloteichus hymeniacidonis*	Dokdo Island, Reublic of Korea	Sediment	Cytotoxic	[76]
Aureolic acids	*Streptomyces* sp.	Not available	Sediment	Antimicrobial Cytotoxic	[77]
Ananstreps C	*Streptomyces anandii*	Guangdong province, China	Sediment	Cytotoxic	[78]
Terrosamycin B	*Streptomyces* sp.	Prince Edward Island	Sediment	Antimicrobial Cytotoxic	[79]
3-Hydroxyquinaldic acid derivatives	*Streptomyces cyaneofuscatus*	Cantabrian Sea	Coral	Cytotoxic	[80]
Bagremycins F and G	*Streptomyces* sp.	Jintang Island of Zhoushan, China	Marine mud	Antibacterial	[81]
Nocardiotide A	*Nocardiopsis* sp	Red Sea	Sponge	Cytotoxic	[82]
Quinomycin G	*Streptomyces* sp.	Hainan Province of China	Sponge	Antibacterial Antitumor	[83]
6-Lavandulyl-7-methoxy-5,2',4'-trihydroxylflavanone and 5'-lavandulyl-4'-methoxy-2,4,2',6'-tetrahydroxylchalcone	*Streptomyces* sp.	Son Tra island, Vietnam	Sponge	Antimicrobial	[84]

Strepchazolin A	*Streptomyces chartreusis*	Hainan Island, China	Sediment	Antibacterial	85
Tetrocarcin Q	*Micromonospora carbonacea*	Ling shui Bay, Hainan Province of China	Sponge	Antibacterial	86
Phocoenamicins B and C	*Micromonospora* sp.	Canary Islands	Sediment	Antibacterial	87
(2-(hydroxymethyl)-3-(2-(Hydroxymethyl)-3-methylaziridin-1-yl) (2-hydroxyphenyl) methanone	*Verrucosispora* sp.	East China Sea	Sponge	Antimicrobial	88
Madurastatin D1 and D2	*Actinomadura* sp.	Not available	Sponge	Antimicrobial	89
Alageninthiocin	*Streptomyces* sp.	Kanyakumari, India	Sediment	Antimicrobial Cytotoxic	90
Naphthalenepropanoic acid analog	*Micromonospora* sp.	East China Sea, Zhejiang province, China	Sediment	Antimicrobial Cytotoxic	91
Petrocidin A	*Streptomyces* sp.	Milos, Greece	Sponge	Cytotoxic	92
Indolocarbazoles	*Streptomyces* sp.	Zhejiang Province, China	Sediment	Cytotoxic	93
Cyclizidine-type alkaloids	*Streptomyces* sp.	Hainan Island, China	Sediment	Cytotoxic	94
Anthracimycin B	*Streptomyces cyaneofuscatus*	Cantabrian Sea	Coral	Antibacterial	95
N-acetylborrelidin B and borrelidin	*Streptomyces mutabilis*	Red Sea	Sediment	Antimicrobial Cytotoxic	96
(2E, 6E)-3,7,11-Trimethyldodeca-2,6-dienedioic acid	*Streptomyces* sp.	Vancouver, Canada	Sediment	Antibacterial	97
Microsporanates A–F and Tetrocarcin P	*Micromonospora harpali*	South China Sea	Sediment	Antibacterial	98
Mathermycin	*Marinactinospora thermotolerans*	South China Sea	Sediment	Antibacterial	99
Fradiamine A	*Streptomyces fradiae*	Sagami Bay, Japan	Sediment	Antibacterial	100
2-[(2R-Hydroxypropanoyl)amino] benzamide	*Nocardiopsis* sp.	Vietnam's East Sea	Sediment	Antimicrobial	101
Lavandulylated flavonoids	*Streptomyces* sp.	Vietnam's East Sea	Sponge	Antimicrobial	102
Neoantimycins A and B	*Streptomyces antibioticus*	Guangdong province, China	Sediment	Cytotoxic	103
Niphimycins C–E	*Streptomyces* sp.	Heishijiao Bay, Dalian, China	Sediment	Antimicrobial; Cytotoxic	104
Streptomyceamide C	*Streptomyces antibioticus*	Xinhui, Guangdong, China	Sediment	Cytotoxic	105
Pteridic acids E–G	*Streptomyces fradiae*	South China Sea	Coral	Antibacterial	106

Glycerol 1-hydroxy-2,5-dimethyl benzoate	*Verrucosispora* sp.	South China Sea	Sediment	Antibacterial	[107]
Quinoline alkaloid and 1,4-dioxane derivative	*Micromonospora* sp.	Cát bà peninsula, Vietnam	Sediment	Antibacterial	[108]
Neo-actinomycins A	*Streptomyces* sp.	Heishijiao Bay, Dalian, China	Sediment	Antimicrobial Cytotoxic	[109]
Paulomycin G	*Micromonospora matsumotoense*	Cantabrian Sea	Sediment	Cytotoxic	[110]
Lobophorin K	*Streptomyces* sp.	Cantabrian Sea	Coral	Cytotoxic	[111]

Among marine Actinobacteria, the rare genera are promising targets for drug discovery as they are underexplored taxonomic groups, which reduces the chance of re-discovery of known compounds and may lead to the discovery of novel scaffolds for the production of new drugs[29]. A classic example is Salinosporamide A ("marizomib", "NPI-0052"), originally isolated from the marine Actinobacteria *Salinispora tropica*. This metabolite successfully completed Phase I clinical trials in patients with lymphoma, glioma, myeloma, melanoma, lung and pancreatic cancers (ClinicalTrials.gov). Phase II clinical trials were recently completed in patients with relapsed/refractory multiple myeloma (ClinicalTrials.gov). A phase III trial is currently being run in patients with newly diagnosed glioblastoma (ClinicalTrials.gov).

1.5. Actinobacteria from deep-sea environment

From sea surface to >10000 meters depth, the marine environment provides different habitats to bacteria[112]. Coastal area represents about 7 to 8% of the total sea surface.[33] Deep sea, which is much more vast and scientifically less explored, is divided into three regions: the bathyal zone (200-2000 m), the abyssal zone (2000-6000 m) and the hadal zone (below 6000 m)[113]. It is generally characterized by an increase of pressure with depth, low temperature that reduces chemical reaction rates, exponential decrease of light with depth, and variable salinity and oxygen concentrations[33,113]. Those harsh conditions are a driver for the evolution of differentiated biochemical and physiological mechanisms in the deep sea-living inhabitants in order to enhance their survival under such conditions, which may translate in the production of new bioactive metabolites[33,113].

Several new actinobacterial species were isolated from deep-sea samples between 2006 and 2016, mostly coming from depths higher than 2000 m[113]. Novel species described during this period are affiliated with the genera *Microbacterium* (*M. marinum*, *M. indicum*, *M. sediminis* and *M. profundi*),

Brevibacterium (B. oceani), *Dermacoccus* (*D. abyssi*), *Pseudonocardia* (*P. antitumoralis*), *Sciscionella* (*S. marina*), *Streptomyces* (*S. indicus*, *S. oceani* and *S. nanhaiensis*), among others[113]. Some authors compare deep-sea muds to tropical rain forests regarding species richness and diversity[29].

The presence of Actinobacteria in marine sediments has been widely described in the literature[112,114]. In fact, several new compounds produced by Actinobacteria living in marine sediments were discovered[34]. Recently, more attention has been given to deep-sea sediments[34]. For instance, *Pseudonocardia* sp., isolated from a deep-sea sediment, was found to produce pseudonocardians which showed antimicrobial activity against *S. aureus*, *Enterococcus faecalis* and *Bacillus thuringensis*, as well as cytotoxicity against three tumor cell lines (SF-268, MCF-7 and NCI-H460)[34]. *Marinactinospora thermotolerans* was obtained from a deep-sea sediment collected from South China Sea, and produces marthiapeptide A that has cytotoxic activity against several cancer cell lines, and antibacterial activity against *B. subtilis, B. thuringiensis, S. aureus and Micrococcus luteus*[34].

Furthermore, Actinobacteria can be found living in symbiosis with free-swimming and sessile marine vertebrates and invertebrates[112,114]. Coral reefs support high biodiversity in the tropical and sub-tropical marine environments[70]. It is estimated that Actinobacteria represent about 10 to 50% of the total coral bacteria, where they usually inhabit in mucus, tissue and in the coral calcium carbonate skeleton[70,115]. Actinobacteria produce compounds with antibacterial activity, that protect the corals from pathogens, keeping them healthy[70]. This latter feature offers an opportunity to discover new antimicrobials that can also be used against human pathogens[111]. Actinobacteria have been also associated with deep-sea corals. A meta-analysis of 16S rRNA amplicon for the investigation of the microbiome of deep-sea stony and soft corals revealed that Actinobacteria are commonly present in all coral hosts[116]. Lawler *et al.* (2016) found Actinobacteria associated with the deep-sea coral *Alcyonium grandiflorum* collected at Norfolk[117]. Sarmiento-Vizcaíno *et al.* (2017) demonstrated that deep-sea corals are inhabited by a high diversity of Actinobacteria with the potential to produce bioactive comounds[118]. In that study, the authors recovered actinobacterial isolates belonging to the genera *Streptomyces* and *Micromonospora*, and some extracts of these isolates exhibited activity against drug-resistant human pathogens, including Gram-positive and Gram-negative bacteria, and fungi[118]. Furthermore, several bioactive compounds have been recovered from Actinobacteria isolated from deep-sea corals. For example, lobophorin K was obtained from *Streptomyces* sp. isolated from the deep-sea coral *Lophelia pertusa*[111]. The compound displayed moderate activity against *S. aureus* and cytotoxic activity against MCF-7, MiaPaca-2 and THLE-2 cell lines[111].

Aside from corals and sediments, marine sponges also represent an important source of marine Actinobacteria. Up to 40% of the total sponges biomass is represented by microorganisms[70]. Sponges are commonly inhabited by Actinobacteria that produce bioactive secondary metabolites which act as a chemical defense[112]. On their turn, sponges provide favorable environmental conditions to microorganisms[70]. Actinobacteria associated with sponges are usually specific and are considered an important phylum among the sponge-associated microorganisms[70]. It is suggested that ca. 58% of marine actinobacterial natural compounds are derived from sponge-associated Actinobacteria (**Figure 3**)[70].

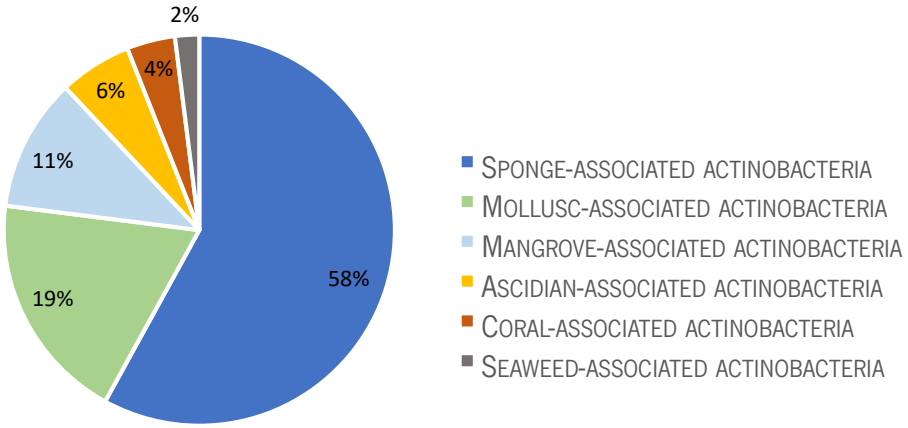

Figure 3. Percentage of actinobacterial natural products obtained from different marine organisms.

There is a growing number of studies showing that deep-sea sponges, in particular, hold a high diversity of Actinobacteria with the potential to produce bioactive metabolites[119,120]. Recently, Xu *et al.* (2018a) isolated 50 strains of marine Actinobacteria from deep-sea sponges and found that more than half of the strains exhibited antibacterial and antifungal activity[119]. Kennedy *et al.* (2014) found several actinobacterial taxa in 3 deep-sea sponges[120]. Deep-sea sponge-associated Actinobacteria have also been reported to exhibit various bioactivities[121,122]. For instance, two *Streptomyces* strains, isolated from deep-sea sponges collected at North Atlantic Ocean, exhibited antimicrobial activity against clinically relevant yeast species[121]. Xu *et al.* (2018b) obtained three new nocardiopsistins from a *Nocardiopsis* strain isolated from a deep-sea sponge[122]. The compounds showed promising antibacterial activity against methicillin-resistant *S. aureus*[122].

1.6. Aim and outline of this thesis

Actinobacteria are ubiquitous microorganisms with high ability to produce important bioactive compounds that may help tackling antimicrobial resistance as well as cancer diseases. The extreme conditions that Actinobacteria find in deep-sea environments may lead to the production of novel

metabolites, but this biosynthetic potential is scantly explored in deep-sea Actinobacteria, particularly in those inhabiting Portuguese marine regions.

As such, the aim of this thesis was to investigate the diversity of culturable Actinobacteria associated with several deep-sea samples, including sponges, corals and sediments, collected at Madeira Archipelago and assess their bioactive potential.

The present thesis is organized in five sections. The first section starts with an introduction where several important subjects related to the developed work are addressed, like the problematic of multidrug-resistant microorganisms and cancer diseases and the need of finding new compounds to tackle these illnesses; the importance of natural products and their applications in different fields; a general perspective of Actinobacteria, including their distribution and environmental importance; and the presence, biodiversity and importance of Actinobacteria in marine environments, with a special focus in deep-sea environments. In section 2, information about the sampling site and the samples obtained is included, as well as the materials and methods applied in the study for the isolation of Actinobacteria, their identification, the preparation of crude extracts and the bioactivity assays. The third section includes the results obtained and their discussion, focusing on the phylogenetic identification of the Actinobacteria isolated from the deep-sea samples, and the antimicrobial and cytotoxic potential of the extracts obtained from them. Finally, the fourth and fifth sections consist on main conclusions and future work and references, respectively.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Sampling site and sample collection

Nine deep-sea samples, including sponges (5), corals (3) and sediments (1), were collected at the South Coast of Madeira archipelago using a manned submersible (Lula1000). Sampling was performed in September 2019 at depths ranging between 463 and 865 meters (**Figure 4** and **Table 3**). Samples were collected to sterile tubes, frozen at -20°C, and transported and conserved at this temperature until its processing in the laboratory.

Figure 4. Localization on the map of the samples collected at the South Coast of Madeira Archipelago.

Table 3. Information on the sampling sites and the collected samples

Sampling site	Latitude	Longitude	Sample	Taxonomic identification	Locality	Depth (m)	Temperature (ºC)
S1	32°38.596'N	17°04.399' W	Sponge (#020)	*Desmosponge* sp.	Ribeira Brava Canyon	463	13.23
	32°38.596'N	17°04.399' W	Sponge (#021)	*Desmosponge* sp.	Ribeira Brava Canyon	463	13.23
	32°38.596'N	17°04.399' W	Sponge (#023)	*Desmosponge* sp.	Ribeira Brava Canyon	463	13.23
S2	32°38.031'N	17°04.937' W	Coral (#014)	*Narella versluysi*	Ribeira Brava Canyon	804	10.43
	32°38.031'N	17°04.937' W	Coral (#017)	*Lophelia pertusa*	Ribeira Brava Canyon	804	10.43
S3	32°38.229'N	17°05.698' W	Sediment (#004)	Not applicable	Ribeira Brava Canyon	747	10.85
	32°38.229'N	17°05.698' W	Sponge (#001)	*Desmosponge* sp.	Ribeira Brava Canyon	747	10.85
S4	32°36.985'N	16°51.451' W	Coral (#025)	*Corallium* sp.	Garajau/Lazareto	593	11.89
S5	32°36.670'N	16°51.337' W	Sponge (#026)	*Desmosponge* sp.	Garajau/Lazareto	865	10.30

2.2. Isolation of Actinobacteria

Corals and sponges samples were rinsed with sterile sea water to remove sand and loosely attached particles and microorganisms. Samples were then macerated in a sterile mortar to release the endophytic microorganisms and incubated in a water bath at 60 °C for 15 min to select spore-forming bacteria. Three ten-fold dilutions were prepared using sterile sea water and 100µL of each dilution was plated on three selective isolation media, M1, M4 and NPS (**Table 4**), supplemented with the selective antibiotics cycloheximide (50 mg L^{-1}), nystatin (50 mg L^{-1}) and nalidixic acid (50 mg L^{-1}) in order to prevent the growth of fungi and Gram-negative bacteria.

Table 4. Composition of the media used for the isolation of Actinobacteria from the nine deep-sea samples

Medium	Composition (per litre of seawater)	References
M1	Starch, 10 g; yeast extract, 4 g; peptone, 2 g; agar, 15 g.	[123]
M4	Chitin, 2 g; agar, 18 g.	[123]
NPS	Agar, 15 g; 100 mL of marine sediment extract obtained by washing 900 mL of sediments with 500 mL of seawater.	[123]

The plates were incubated at 28 °C and 5 °C for a period up to six months. Plates were periodically visually inspected, and whenever morphologically different colonies were observed they were peaked and streaked on new agar plates until obtainment of pure colonies.

Pure isolates were grown in 5 mL of liquid selective medium, according to the medium where they were isolated, at 28 °C with shaking at 100 rpm, for cryopreservation and phylogenetic identification purposes. Each pure isolate was cryopreserved at -80°C in 30% (v/v) glycerol.

2.3. Phylogenetic identification of the isolates

Biomass for DNA extraction was obtained from the cultures aforementioned, by centrifuging 1 mL of cultures for 5 minutes at 13500 rpm and storing the pellet at -20 °C.

The E.Z.N.A.® Bacterial DNA kit (Omega Bio-Tek, GA, United States) was used for extraction of DNA, following the instructions of the manufacturer, with some modification steps: (i) in the lysozyme addition step, the samples were incubated at 37 °C for 30 min, instead of 10 min; (ii) in the optional step, two zirconia beads (2.3 mm in diameter) were added together with the glass beads to the samples; (iii) the incubation time with proteinase K was increased to 2 h and a concentrated stock (10 mg mL^{-1}) was used instead of the solution from the kit; (iv) the centrifugation speed described in the protocol was changed in all stages from 10,000 g to 13,000 g; (vi) in the final DNA elution step, 25 µL of elution buffer was added to the HiBind® DNA Mini column (step performed twice), instead of 50-100 µL. 16S rRNA

gene was amplified by Polymerase Chain Reaction (PCR) using the universal primers 1492R (5'-GGTTACCTTGTTACGACTT-3') and 27F (5'-GAGTTTGATCCTGGCTCAG-3').[124] The PCR mixture (final volume of 10 µL) consisted of 5 µL of Qiagen Multiplex PCR Master Mix, 1 µL of each primer and 3 µL of DNA template. For the negative control, 3 µL of DNA-free water were used instead of the DNA template. The PCR reaction started with an initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 48 °C for 90 seconds and extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min.

PCR products were separated in a 1.5x agarose gel containing 0.5 µL of SYBR Safe (Thermo Fisher Scientific, MA, United States). The gel was run at 150 V for 30 min and visualized in a transilluminator using IMAGELAB *software*.

Purification and sequencing of the amplified fragments was carried out by GenCore, i3S (Instituto de Investigação e Inovação em Saúde, Portugal). The sequences were analyzed using *Geneious* software package (version 11.1.4). The most similar sequences in *GenBank* were found using BLASTN (Basic Local Alignment Search Tool). In order to establish the taxonomic affiliation of the isolates, the obtained consensus sequences were compared to those present in the 16S ribossomal RNA (Bacteria and Archaea) database from NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the Identify tool from EzTaxon (https://www.ezbiocloud.net/) and the Sequence Match tool from the Ribosomal Database Project (http://rdp.cme.msu.edu/). For putatively new species, phylogenetic trees were constructed. The respective sequences of these isolates were aligned with the eight closest neighbor sequences found in the GenBank for each isolate. This alignment was used to construct a phylogenetic tree with the help of the Molecular Evolutionary Genetics Analysis software (MEGA X), using the Maximum Likelihood method with 1000 bootstraps.

2.4. Preparation of crude extracts for bioactivity assays

For obtaining crude extracts from the actinobacterial strains, each isolate was grown in 5 mL of liquid selective medium (without the addition of cycloheximide, nalidixic acid and nystatin), for 2-3 days at 28 °C and 100 rpm. At this point, two selective media were used: M1 (for isolates obtained in this same medium) and Marine Broth (for isolates obtained from M4 and NPS media). After this period, cultures were transferred to 100 mL Erlenmeyer flasks containing 30 mL of selective liquid medium (with no antibiotics added) and incubated in the dark at 28 °C with shaking at 100 rpm. Approximately 3-5 days later (according to the growth rate of each microorganism), 0.5 g of Amberlite XAD16N resin (Sigma-Aldrich, MO, United States) was added to the medium in order to adsorb the metabolites produced by

the actinobacterial isolate, and the cultures were incubated for three additional days. After this incubation period, the cultures were centrifuged at 4000 rpm for 10 minutes and the resulting pellet, consisting of biomass and resin, was washed two times with deionized water and lyophilized in order to remove all water. The lyophilized pellet was extracted with a mixture of acetone/methanol (1:1 ratio (v/v)) and the organic layer was dried in a rotary evaporator. The resulting crude extract was then used to prepare stock solutions in dimethyl sulfoxide (≥ 99.9%, DMSO, Sigma-Aldrich, USA), at the concentrations of 10 mg mL^{-1}.

2.5. Antimicrobial activity screening

Antimicrobial activity was screened using the disk diffusion method, against five reference microbial species: *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC 29213) and *Salmonella thyphimurium* (ATCC 24241).

Bacterial strains were grown in Mueller-Hinton Agar (MH), while *C. albicans* was grown in Sabouraud Dextrose Agar (SD). The OD (625 nm) of the reference strains in the corresponding liquid medium was set according to 0.5 McFarland Standard (0.08-0.130). The cultures grown in the liquid medium were used to inoculate agar plates (with the same composition of the liquid medium) by evenly streaking on the surface of the plates a swab dipped in the grown liquid cultures. Blank paper disks with 6 mm of diameter were placed on top of the agar and were impregnated with 15 µL of each crude extract at a concentration of 1 mg mL^{-1}. Negative control disks were impregnated with DMSO. Positive controls consisted in 15 µL of enrofloxacin (1 mg mL^{-1}; Sigma-Aldrich; MO; United States) for bacterial strains, and nystatin (1 mg mL^{-1}; Sigma-Aldrich; MO; United States) for *C. albicans*. Plates were incubated at 37 °C and the results were observed after 18 hours for the presence of inhibition halos, whose diameter was measured. Each extract was tested in triplicate.

The extracts showing antimicrobial activity were further tested to determine their minimal inhibitory concentration (MIC) or, in other words, the lowest concentration that prevented growth of the reference strain. The broth dilution susceptibility test was used, in which different concentrations of the extracts were inoculated with a standard density of the reference strain. Liquid cultures of the standard reference strains were obtained as for the disk diffusion assay. Twelve solutions (S1-S12) were prepared in Eppendorfs corresponding to different concentrations of the extracts (0.487-1000 µg ml^{-1}). The first solution (S1) contained 450 µL of Mueller-Hinton broth for bacterial strains, or Sabouraud-Dextrose broth

for *C. albicans*, and 50µL of the stock solution (10 mg mL^{-1}). Each one of the other solutions (S2-S12) contained 250 µL of the corresponding broth and 250 µL of the previous solution (**Figure 5**).

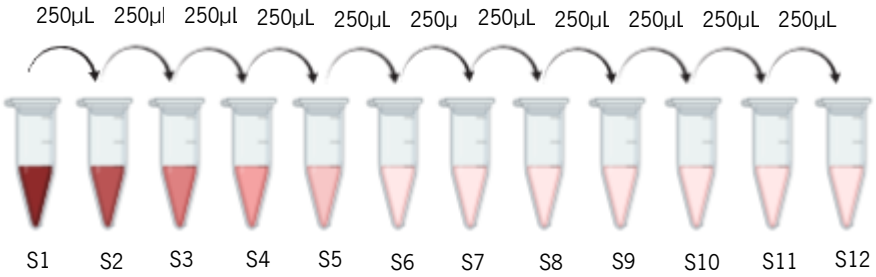

Figure 5. Scheme for the preparation of the extracts dilutions for the MIC assay.

The MIC assay was performed in 96 well plates (**Figure 6**). In each well, 50 µL of microbial inoculum (diluted 1:100) were incubated with 50 µL of each extract dilution. Triplicates were made for each extract dilution. Positive growth control (PC) consisted in 50 µL of microbial inoculum and 50 µL of medium broth, and negative growth control (NC) consisted in 100 µL of medium broth.

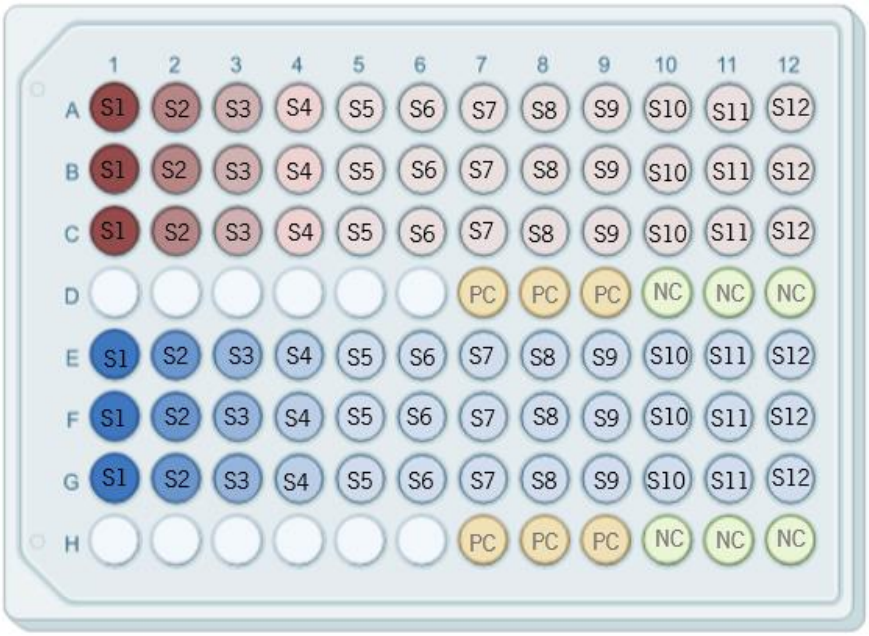

Figure 6. Ninety-six-well plate prepared for MIC determination, showing the incubation of the diluted extracts with the reference microbial strains.

After 18 hours of incubation at 37 °C, the OD (625 nm) was read. The lowest concentration of the extract resulting in no microbial growth corresponded to the MIC.

2.6. Cytotoxic activity screening

In order to evaluate the cytotoxic potential of the actinobacterial extracts, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used and the following cancer cell lines were tested: T47-D (breast ductal carcinoma) and HepG2 (liver cancer), both from Sigma-Aldrich (St. Louis, Missouri, USA). The cell line hCMEC/D3 (human brain capillary endothelial cells) was used to test for general toxicity. Cells were grown in Dubelco's Modified Eagle Medium (DMEM) from Gibco (Thermo Fischer Scientific, Waltham, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 1% (v/v) penicillin/streptomycin (Biochrom) at 100 IU mL^{-1} and 10 mg mL^{-1}, respectively, and 0.1% (v/v) amphotericin (GE Healthcare, Little Chafont, United Kingdom). The cells were incubated at 37 °C in a humidified atmosphere containing 5% of CO_2.

The cells were seeded in 96-well plates at a density of 6.6×10^4 cells mL^{-1}. After 24 h, cells were exposed to the extracts at a final concentration of 15 μg mL^{-1}. Negative and positive controls consisted in 20% DMSO (Sigma-Aldrich, USA) and 0.5% staurosporine, respectively. Cell viability was evaluated at 24 and 48 h, after the addition of MTT (final concentration of 0.2 mg mL^{-1}) and incubation for 4 h at 37 °C. The medium was removed and 100 μL of DMSO was added per well. The absorbance was read at 550 nm (Synergy HT, Biotek, USA). Cell viability was expressed as a percentage relative to the negative control. The assays were performed in triplicate at two independent times.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. Replicates were tested for significant differences in comparison to the solvent control, DMSO. The level of significance was set to $p<0.05$ for all tests. Data was checked for its normal distribution using Kolmogorov Smirnov test. If data did not follow a normal distribution pattern, it was square root transformed. One-Way ANOVA or non-parametric Kruskal-Wallis test were applied, as well as Dunn's multiple comparison test.

CHAPTER 3

RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

3.1. Culturable Actinobacteria associated with the analyzed deep-sea samples

Nine deep-sea samples were collected at the South Coast of Madeira archipelago at depths ranging from 463 to 865 meters. Samples were pre-treated by incubation at 60 °C for 15 min and spread on three selective media (M1, M4 and NPS). The plates were incubated for a period up to six months, during which growth of colonies with different morphologies was obtained. The observed colonies were, in general, small, with regular or irregular shape, bright or opaque with, in some cases, a translucent halo, and, occasionally, with spores.

The dominant colors of the colonies were cream, white, yellow and orange, but red and dark brown/black colonies were also observed (**Figure 7**).

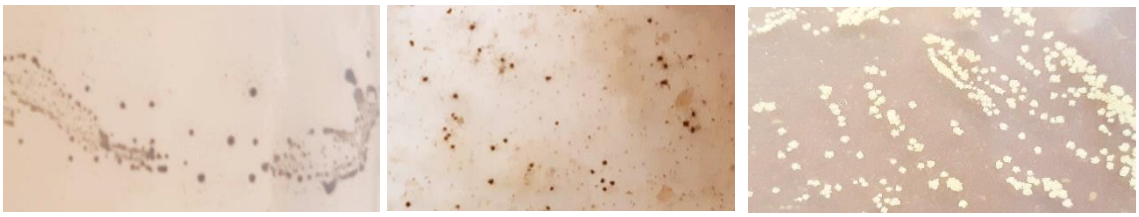

Figure 7. Examples of bacterial colonies obtained from deep-sea samples collected at the Madeira archipelago.

It is well established that Actinobacteria morphology is diverse and that they produce a variety of pigments, from yellow, orange and red, to brown or black.[25] Spores production is also common in this group of bacteria[25].

In total, 68 actinobacterial isolates were recovered from the 9 deep-sea samples analyzed, as revealed by 16S rRNA gene analysis. These isolates were distributed by the genera *Brevibacterium* (25), *Microbacterium* (20), *Tsukamurella* (14), *Leucobacter* (2), *Micrococcus* (2), *Rhodococcus* (2), *Brachybacterium* (2) and *Streptomyces* (1) (**Figure 8** and **Table 5**). The most abundant isolates were affiliated to the genera *Brevibacterium*, *Microbacterium* and *Tsukamurella*, and were recovered from the three types of samples analyzed (sponges, corals and sediments) (**Figure 8**).

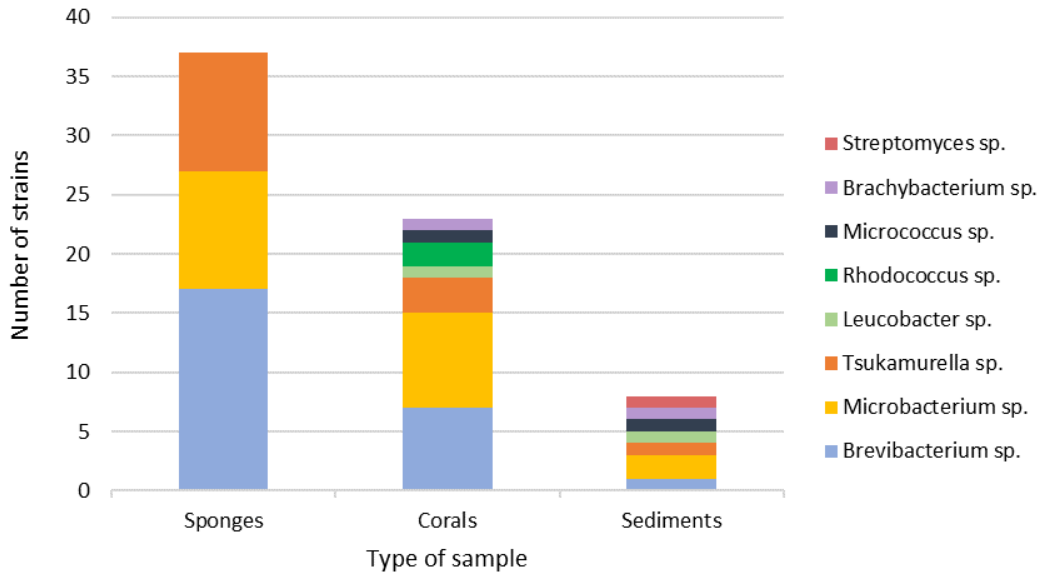

Figure 8. Actinobacterial genera identified in the deep-sea samples (comprising corals, sponges and sediments) collected at the Madeira archipelago.

A total of 37 actinobacterial isolates were recovered from the samples of deep-sea sponges, being distributed by the genera *Microbacterium*, *Brevibacterium* and *Tsukamurella* (**Figures 8**, **9a**, **9b**, **9c**, **9d** and **9e**). The latter two genera were also identified in other studies, in deep-sea sponges collected at United States, Gulf of Mexico, Caribbean Sea and Antartica[119,125]. In contrast to our results, Williams *et al.* (2020) identified several actinobacterial genera (*Micrococcus*, *Kocuria*, *Micromonospora*, *Modestobacter*, *Citrococcus* and *Chryseoglobus*) in 4 deep-sea sponges collected at different sites across the equatorial Atlantic, but none of which was recovered from sponges in the present study [126].

Regarding coral samples, 23 isolates distributed by 7 actinobacterial genera (*Brevibacterium*, *Microbacterium*, *Tsukamurella*, *Leucobacter*, *Rhodococcus*, *Micrococcus* and *Brachybacterium*) were recovered (**Figures 8**, **9f**, **9g** and **9h**). Six isolates were obtained from sample #017, which consisted in the stony coral *Lophelia pertusa*, whereas 9 and 8 actinobacterial strains were isolated from samples #014 and #025, consisting in the soft corals *Narella versluysi* and *Corallium* sp, respectively. Very few studies focus on Actinobacteria associated with deep-sea corals. Sarmiento-Vizcaíno *et al.* (2017) studied the diversity of Actinobacteria living in association with six deep-sea corals of the orders Scleractinia, Gorgonacea and Alcyonaea and of the species *Lophelia pertusa,* collected in the Cantabrian Sea[118]. The authors did not identify any of the actinobacterial genera recovered in the present study, having rather identified the genera *Streptomyces* and *Micromonospora*, indicating that it may be a specific association of Actinobacteria to different coral species[118]. Actinobacteria was also found to be an important fraction of

deep-sea corals of the species *Lophelia pertusa*, inhabiting the Central Mediterranean Sea, though the community was not characterized at the genus level[127].

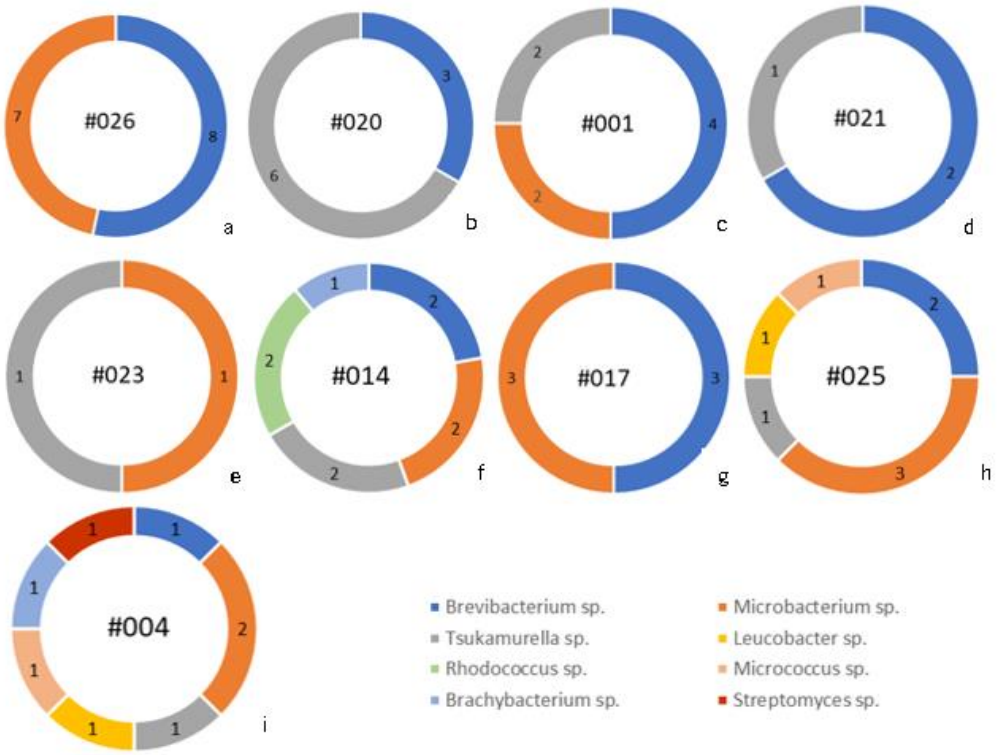

Figure 9. Distribution of actinobacterial genera for each analyzed deep-sea sample. a, b, c, d and e are relative to sponges; f, g and h are relative to corals, and i is relative to the sediment sample.

In the only sediment sample analyzed, 8 actinobacterial isolates afilliated with *Brevibacterium*, *Micrococcus*, *Microbacterium*, *Tsukamurella*, *Leucobacter*, *Brachybacterium* and *Streptomyces* genera were identified, with the he latter genus being recovered only from this sample (**Figures 8** and **9i**). Strains of many of these genera, specifically *Brachybacterium*, *Brevibacterium*, *Microbacterium* and *Micrococcus*, were also isolated by Zhang *et al.* (2014) from Arctic deep-sea sediments[128]. *Tsukamurella*, *Microbacterium* and *Streptomyces* strains were isolated from subseafloor sediments collected at Nankain and Okinawa Troughs[129]. Chen *et al.* (2016) studied the actinobacterial diversity of deep-sea sediments collected along the Southwest Indian Ridge, using 16S rRNA gene pyrosequencing and culture-based methods, and isolated the same genera identified in the present study, which revealed to be abundant according to both culture-dependent and independent methods[130]. Ettoumi *et al.* (2016) were able to cultivate *Micrococcus*, *Brevibacterium*, *Brachybacterium*, among other actinobacterial genera from deep-sea sediments collected from Tyrrhenian Sea[131]. In a study developed by Silva *et al.* (2013), the authors isolated actinobacterial strains belonging to the genera *Brevibacterium* and *Micrococcus* from deep-sea samples collected from the South Atlantic Ocean[132]. Chen *et al.* (2016) reported a new *Brevibacterium*

species, *Brevibacterium sediminis*, from deep-sea sediments collected at Carlsberg and Southwest Indian Ridges[133]. Verma *et al.* (2017) recovered *Streptomyces* strains from deep-sea sediments collected at the Bay of Bengal and at volcanic Barren Island in the Andaman Sea[134]. The results obtained in the present study, together with the referenced studies, suggest that the genera *Brevibacterium*, *Micrococcus*, *Microbacterium*, *Tsukamurella*, *Leucobacter*, *Brachybacterium* and *Streptomyces* are common in deep-sea sediments. According to the literature, *Micromonospora*, *Rhodococcus* and *Streptomyces* species are among the most dominant actinobacterial genera in the marine habitat, including in the deep-sea[33]. Interestingly, in our study, we were not able to isolate *Micromonospora* strains and *Rhodococcus* and *Streptomyces* were among the least dominant actinobacterial isolates, with only two and one isolates being identified, respectively.

From the three selective media used in the present study for the isolation of Actinobacteria, the media NPS and M1 were the ones that led to the isolation of a higher number of actinobacterial strains, 31 and 28 isolates, respectively (**Figure 10**). Eight actinobacterial genera were recovered from NPS medium, while M1 allowed the isolation of 5 genera (**Figure 10**). Medium M4 led to the isolation of the lowest number of actinobacterial strains (9 isolates), affiliated with 4 genera. *Brevibacterium*, *Microbacterium* and *Tsukamurella* strains were recovered from the three selective media, while *Brachybacterium* and *Streptomyces* isolates were obtained only in NPS medium.

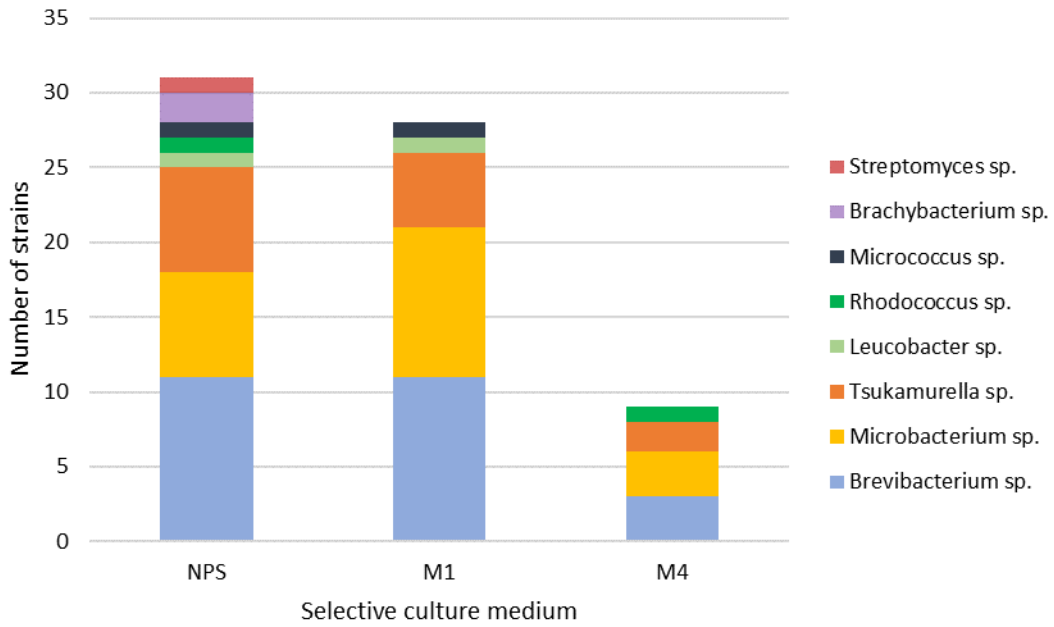

Figure 10. Distribution of actinobacterial genera according to the selective media used in this study.

Isolation of deep-sea Actinobacteria is influenced by several factors, including pre-treatment, medium composition, dilution factor, seawater requirement and incubation time[113]. Pre-treatment with

heat not only inhibits growth of fungi and fast growing bacteria, but also enhances the isolation of actinobacterial microorganisms[113]. However, heat pre-treatment may also result in the reduction of the number and diversity of Actinobacteria as some strains can be sensitive to heat[113]. For example, Kamjam *et al.* (2017) reported that less Actinobacteria were isolated from Norwegian fjord sediments when using a heat pre-treatment at 55 °C than when using one at 50 °C[113].

The results obtained in the present study indicate that medium composition influences the abundance and diversity of actinobacterial isolates. Studies show that the use of nutrient poor media may be more efficient for the isolation of Actinobacteria than complex organic and rich media[135]. Nutrient-poor media, such as NPS, have been successfully used for the isolation of marine Actinobacteria, including obligate marine Actinobacteria, due to their ability to mimic the conditions found in marine environments[123]. Sediment extracts and natural sea water, which are the components of NPS medium, are usually used alone or as a supplement to mimic environmental conditions[123]. On the other hand, M1 medium has already been used for the selective isolation of marine Actinobacteria, including rare species[34,123,136]. In addition, Williams *et al.* (2020) report the successful use of a variety of selective culture media, including M1, to cultivate actinobacterial strains from deep-sea sponges[137]. Our results show the importance of testing different isolation media and culture conditions in order to allow the isolation of Actinobacteria with different nutritional requirements and metabolism.

In the present study, the deep-sea sediment analyzed (#004) and two coral samples (#014 and #025) yielded a higher actinobacterial diversity. Interestingly, in previous studies, deep-sea sponges have shown a higher actinobacterial diversity than that found in our study. For instance, 12 strains from the genera *Pseudonocardia*, *Rhodococcus*, *Streptomyces*, *Salinispora* and *Mycobacterium* were isolated from the deep-sea sponge *Discodermia* sp. and 18 strains related to the genera *Rhodococcus*, *Streptomyces*, *Promicromonospora*, *Agrococcus*, *Pseudonocardia* and *Actinomycetospora* were recovered from the deep-sea sponge *Forcepia* sp.[119]. Nevertheless, it must be highlighted that the actinobacterial diversity associated with our samples should be much higher given that only about 1% of microbial life is culturable. In this regard, culture-independent techniques like metagenomics analysis or 16S rRNA metabarcoding are very important to allow a more comprehensive vision of the microbial community of a given sample[33].

Two actinobacterial isolates obtained in this study (strains C_017_1 and C_017_5; **Figure 11** and **Table 5**) affiliated to the genera *Microbacterium*, appear to be two strains of a new species, considering the 98.7% 16S rRNA cut-off value to discriminate between species[138].

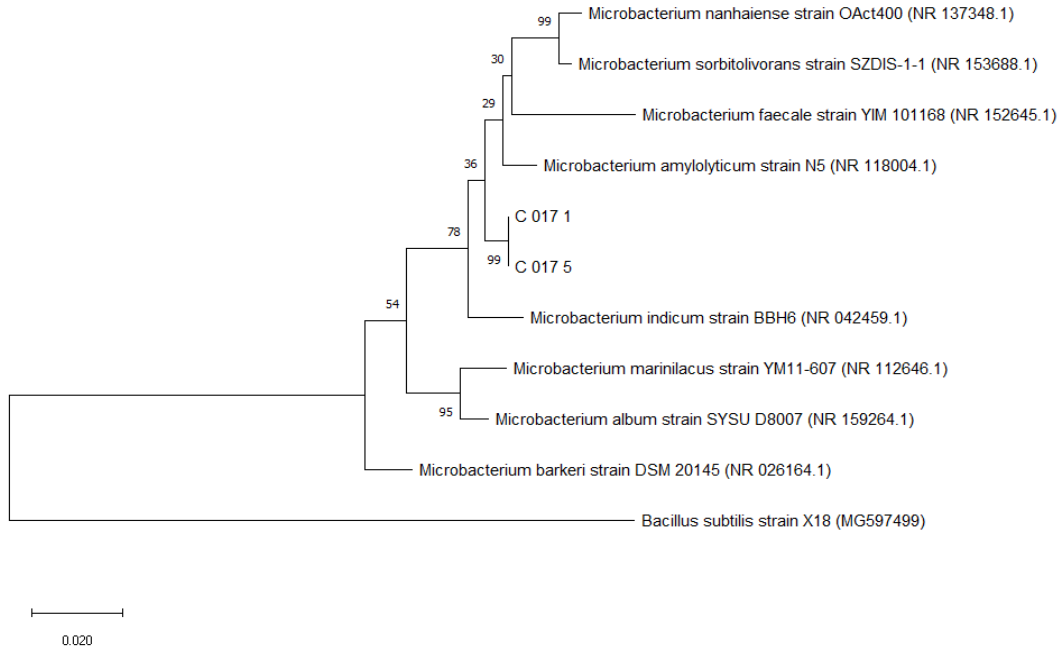

Figure 11. Maximum Likelihood phylogenetic tree using 16S rRNA gene sequences of the two putative new actinobacterial strains isolated from the deep-sea coral *Lophelia pertusa*, together with closely related type strains from GenBank. The tree was generated using 1375 bp and 1000 bootstraps. Numbers at nodes represent percentage bootstrap. Numbers in parenthesis correpond to GenBank accession numbers. *Bacillus subtilis* was used as an outgroup.

This finding is particularly important in the light that new actinobacterial strains may produce secondary metabolites with original chemical structures, which may be promising for the discovery of novel compounds with important pharmaceutical applications[29,34]. However, to confirm this new taxonomy, deeper studies are necessary, as the analysis of 16S rRNA gene similarity is not enough by itself to propose a new species.

Table 5. Phylogenetic identification of the actinobacterial strains isolated from the nine deep-sea samples analyzed in this study, according to the 16S rRNA database from NCBI BLAST tool

Sample	Isolate	Selective medium	Closest species	Query cover (%)	Similarity (%)	Accession number
#020	S_020_2	M1	*Tsukamurella tyrosinosolvens* strain DOS-1a	100	99.93	MK788236.1
#020	S_020_3	M1	*Brevibacterium aureum* strain Enb17	99	99.93	AY299093.1
#020	S_020_4	M1	*Tsukamurella tyrosinosolvens* strain DSM 44234	100	99.86	NR_042801.1

#020	S_020_5	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.86	NR_153678.1
#020	S_020_6	NPS	*Tsukamurella tyrosinosolvens* strain DSM 44234	100	99.93	NR_042801.1
#020	S_020_8	NPS	*Tsukamurella tyrosinosolvens* strain DSM44234	100	99.93	NR_042801.1
#020	S_020_9	M1	*Tsukamurella strandjordii* strain ATCC BAA-173	100	99.14	NR_025113.1
#020	S_020_10	M1	*Tsukamurella tyrosinosolvens* strain DSM44234	100	99.78	NR_042801.1
#020	S_020_13	NPS	*Brevibacterium aureum* strain Enb17	100	99.79	AY299093.1
#021	S_021_1	M4	*Tsukamurella tyrosinosolvens* strain DSM 44234	100	99.93	NR_042801.1
#021	S_021_2	NPS	*Brevibacterium aureum* strain Enb17	100	99.79	AY299093.1
#021	S_021_3	NPS	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.35	NR_153678.1
#023	S_023_3	NPS	*Tsukamurella tyrosinosolvens* strain DSM 44234	100	99.86	NR_042801.1
#023	S_023_4	NPS	*Microbacterium aerolatum* strain NG-T15	100	99.64	KF844053.1
#001	S_001_1	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.86	NR_153678.1
#001	S_001_2	M1	*Brevibacterium siliguriense* strain DBS-LAZ-11/17	100	98.99	MG231266.1
#001	S_001_4	M1	*Microbacterium aerolatum* strain NG-T15	100	100	KF844053.1
#001	S_001_5	M1	*Tsukamurella tyrosinosolvens* strain DSM 44234	100	99.78	NR_042801.1
#001	S_001_10	M1	*Microbacterium ginsengiterrae* strain DCY37	100	98.85	NR_116483.1
#001	S_001_11	M1	*Brevibacterium sediminis* strain GCMCC 1.15472	100	99.86	NR_153678.1
#001	S_001_12	NPS	*Tsukamurella tyrosinosolvens* strain DSM44234	100	99.78	NR_042801.1
#001	S_001_15	NPS	*Brevibacterium picturae* strain LMG 22061	100	99.54	NR_025614.1
#026	S_026_1	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	99	99.71	NR_153678.1
#026	S_026_2	M1	*Microbacterium aerolatum* strain NG-T15	100	99.71	MT433875.1
#026	S_026_3	M1	*Microbacterium oxydans* strain DSM 20578	100	99.78	NR_044931.1
#026	S_026_4	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.86	NR_153678.1
#026	S_026_5	M1	*Microbacterium aerolatum* strain NG-T15	100	99.93	KF844053.1
#026	S_026_6	M1	*Microbacterium aerolatum* strain NG-T15	100	99.78	KF844053.1
#026	S_026_7	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.85	NR_153678.1
#026	S_026_8	NPS	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.57	NR_153678.1
#026	S_026_9	NPS	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.93	NR_153678.1
#026	S_026_10	M4	*Brevibacterium aureum* strain Enb17	100	100	AY299093.1
#026	S_026_13	M4	*Microbacterium aerolatum* strain NG-T15	100	99.71	KF844053.1
#026	S_026_15	NPS	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.86	NR_153678.1

#026	S_026_16	NPS	*Microbacterium aerolatum* strain NG-T15	100	99.78	KF844053.1
#026	S_026_17	NPS	*Microbacterium ginsengiterrae* strain DCY37	100	98.84	NR_116483.1
#026	S_026_19	M4	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.43	NR_153678.1
#014	C_014_1	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.86	NR_153678.1
#014	C_014_6	M4	*Microbacterium ginsengiterrae* strain DCY37	100	98.71	NR_116483.1
#014	C_014_7	NPS	*Rhodococcus qingshengii* strain JCM 15477	100	99.93	NR_043535.1
#014	C_014_9	NPS	*Brachybacterium rhamnosum* strain H-6S	100	99.06	NR_042109.1
#014	C_014_10	NPS	*Brevibacterium siliguriense* strain DSM 23676	100	99	LT629766.1
#014	C_014_11	NPS	*Microbacterium aerolatum* strain NG-T15	100	99.64	KF844053.1
#014	C_014_12	NPS	*Tsukamurella tyrosinosolvens* strain DSM44234	100	100	NR_042801.1
#014	C_014_13	NPS	*Tsukamurella tyrosinosolvens* strain DOA-a1	100	99.86	MK788236.1
#014	C_014_16	M4	*Rhodococcus erythropolis* strain TG-2	100	99.71	MN922941.1
#017	C_017_1*	M1	*Microbacterium amylotyticum* strain N5	99	97.90	NR_118004.1
#017	C_017_2	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.64	NR_153678.1
#017	C_017_3	NPS	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.86	NR_153678.1
#017	C_017_5*	NPS	*Microbacterium amylolyticum* strain N5	99	98.05	NR_118004.1
#017	C_017_6	M1	*Microbacterium aerolatum* strain 263XY4	100	99.78	KF954552.1
#017	C_017_7	M4	*Brevibacterium aureum* strain Enb17	100	100	AY299093.1
#025	C_025_1	M1	*Leucobacter komagatae* strain IFO 15245	100	99.71	NR_114929.1
#025	C_025_2	M1	*Micrococcus luteus* strain NCTC 2665	100	99.43	NR_075062.2
#025	C_025_3	NPS	*Tsukamurella tyrosinosolvens* strain DOS 1-a	100	100	MK788236.1
#025	C_025_4	M1	*Microbacterium aerolatum* strain NG-T15	100	99.50	KF844053.1
#025	C_025_6	NPS	*Brevibacterium aureum* strain Enb17	100	99.64	AY299093.1
#025	C_025_7	NPS	*Microbacterium aerolatum* strain 263XY4	100	99.42	KF954552.1
#025	C_025_9A	M4	*Microbacterium aerolatum* strain NG-T15	100	99.86	KF844053.1
#025	C_025_11	NPS	*Brevibacterium aureum* strain Enb17	100	99.93	AY299093.1
#004	Sed_004_2	M1	*Microbacterium ginsengiterrae* strain DCY37	100	98.92	NR_116483.1
#004	Sed_004_7	NPS	*Leucobacter komagatae* strain IFO15245	100	98.85	NR_114929.1
#004	Sed_004_12	NPS	*Micrococcus luteus* strain AA6-2	100	100	KU663666.1
#004	Sed_004_13	NPS	*Brachybacterium paraconglomeratum* strain LMG 19861	100	99.35	NR_025502.1
#004	Sed_004_22	M1	*Brevibacterium sediminis* strain CGMCC 1.15472	100	99.72	NR_153678.1
#004	Sed_004_27	NPS	*Microbacterium aerolatum* strain NG-T15	100	99.78	KF844053.1
#004	Sed_004_28	M4	*Tsukamurella tyrosinosolvens* strain DSM 44234	100	100	NR_042801.1
#004	Sed_004_31	NPS	*Streptomyces aculeolatus* strain NBRC14824	100	99.93	NR_041166.1

*putatively new species

3.2. Antimicrobial activity potential of the isolated deep-sea Actinobacteria

Fifty-nine actinobacterial crude extracts were tested for their antimicrobial activity using the disc diffusion method. The results revealed that 2 actinobacterial extracts had activity against at least one of the following reference strains: *S. aureus* (n=1), *B. subtilis* (n=1) and *C. albicans* (n=1). These isolates were affiliated with *Brevibacterium* (n=1) and *Brachybacterium* (n=1) genera. The diameter of the inhibition halos obtained, as well as the MIC values determined are presented in **Table 6**.

Table 6. Actinobacteria isolated from the deep-sea samples with antimicrobial activity

Isolates	Taxonomic identification	Zone of inhibition (mm)			MIC (µg mL^{-1})		
		S. aureus	***B. subtilis***	***C. albicans***	***S. aureus***	***B. subtilis***	***C. albicans***
S_026_1	*Brevibacterium sediminis* strain CGMCC 1.15472				1000	1000	ND*
C_014_9	*Brachybacterium rhamnosum* strain H-6S				ND	ND	1000

*ND – Not determined.

No halo	<1 cm	1-2 cm

The diameter of the inhibition halos produced by the active extracts ranged between <1 cm and 2 cm, while the respective MIC values were 1000 µg mL^{-1}(Table 6). The extract S_026_1, derived from the strain *Brevibacterium sediminis*, inhibited the growth of the Gram-positive bacteria *S. aureus* and *B. subtilis*. *Brevibacterium* strains have been previously reported to exhibit antimicrobial activity. Meena *et al.* (2019) recovered *Brevibacterium* species from deep-sea sediments collected at Barren Island, displaying antimicrobial activity against Gram-positive and Gram-negative bacteria.[139] In another study, Kiran *et al.* (2014) isolated a *Brevibacterium aureum* strain from a marine sponge collected at the South coast of India, with activity against *Streptococcus* sp., *S. aureus*, *B. subtilis*, *E. coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterococcus faecalis,* and *C. albicans*[140].

The organic extract recovered from *Brachybacterium* sp. strain C_014_9, inhibited the growth of *C. albicans*. Antimicrobial activity against this yeast has also been observed by Kiran *et al.* (2014), with the species *Brachybacterium paraconglomeratum*, recovered from a marine sponge[141]. Apart from *C. albicans*, this species also inhibited the growth of *S. aureus*, *B. subtilis*, *E. coli*, *Streptococcus* sp., *Klebsiella pneumoniae*, *Micrococcus luteus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Enterococcus faecalis*[141].

Even though, in this study, only two actinobacterial strains exhibited antimicrobial activity, it is well known that many microorganisms affiliated with the actinobacterial genera recovered from our deep-sea samples have antimicrobial properties. For instance, Graça *et al.* (2015) recovered various *Microbacterium* isolates from marine sponges collected at Gettysburg and Ormonde Peaks (200 km WSW off Cape St. Vincent, Portugal), which were bioactive against *C. albicans* and *B. subtilis*[142]. Santos *et al.* (2019) recovered a marine *Microbacterium* strain with antibacterial activity against Methicillin-resistant *S. aureus* (MRSA)[143]. *Micrococcus* has one of the smallest genomes among Actinobacteria, and has very few genes associated to the production of secondary metabolites.[143] Even though this genus has a reduced ability to produce bioactive molecules, Santos *et al.* (2019) reported *Micrococcus* strains with antifungal activity against *C. albicans* as well as other fungi[143]. In fact, a few new antimicrobial metabolites have been recovered from this genus[144]. For instance, Eltamany *et al.* (2014) described the isolation of a new antibacterial xanthone from a *Micrococcus* strain isolated from a marine sponge, which exhibited activity against *Enterococcus faecalis* and *S. aureus*[144]. In addition, the compound 2,4,4′-trichloro-2′-hydroxydiphenylether, produced by the same genus, exhibited bioactivity against *Enterococcus faecalis* and *S. aureus*[144]. Moreover, Vollbrecht *et al.* (1999) reported that some *Tsukamurella* strains are producers of oligosaccharide lipids that are able to inhibit the growth of Gram-negative and Gram-positive bacteria, as well as of fungi[145].

It is worth to emphasize that *Streptomyces* sp. are one of the most diverse and common Actinobacteria genera to be recovered from the marine environment[33]. In addition, they are the most fruitful producers of antimicrobial compounds among Actinobacteria, with new compounds being frequently discovered[28,29,33,34,70,113,114]. However, in the present study, only one *Streptomyces* strain was isolated and, therefore, this may explain the low number of extracts with antimicrobial activity against the reference strains tested.

3.3. Cytotoxic activity of the isolated Actinobacteria

Cytotoxicity assays address the effect of a compound or extract on cell morphology, its ability to attach to surfaces, changes in the growth rate, cell death and disintegration[146].

At the moment, there is a lack of studies focusing on finding new cytotoxic molecules derived from marine Actinobacteria[31]. In order to evaluate the cytotoxic potential of the crude extracts from the actinobacterial strains isolated in the present study, the MTT assay was performed using two cancer cell lines (HepG2 and T47-D). The cell line hCMEC/D3 was also exposed to the extracts in order to investigate

their general toxicity. The percentage of cellular viability for each cell line exposed to each extract was determined after 24 h and 48 h of exposure.

From the 59 extracts tested, 23 extracts from *Brevibacterium*, *Tsukamurella*, *Microbacterium*, *Micrococcus*, *Rhodococcus* and *Leucobacter* strains, showed statistically significant cytotoxic activity against at least one of the cell lines tested when compared to the negative control (DMSO). Twenty of them reduced the cellular viability of T-47D cells, 8 decreased the viability of HepG2 cells and 19 also showed activity in hCMEC/D3 cells (**Figures 12**, **13** and **14**).

After 24 h of exposure, six organic extracts obtained from *Brevibacterium* (S_020_1, S_020_3 and S_026_1), *Microbacterium* (C_017_1 and C_025_4) and *Leucobacter* (C_025_1) strains, reduced significantly the viability of T-47D cells (**Figure 12a**).

After 48 h, a total of twenty extracts belonging to the strains *Brevibacterium* (S_020_1, C_014_1, S_020_3, S_026_1, S_001_1 and S_020_11), *Tsukamurella* (S_001_5, S_020_4, S_020_2, S_020_8 and S_020_6), *Microbacterium* (Sed_004_2, C_017_1, C_025_4, S_026_6 and S_001_10), *Micrococcus* (C_025_2 and Sed_004_12), *Rhodococcus* (C_014_7), and *Leucobacter* (C_025_1), exhibited cytotoxic activity against the T-47D cell line (**Figure 12b**). The extract S_026_1 notably reduced the cell viability in ca. 50% after 48 h of extract exposure. In addition, the extract C_017_1, obtained from a potential new species, reduced significantly the cell viability of T-47D cells, after 24 h and 48 h of exposure. This result is particularly interesting because, as it was discussed earlier, new actinobacterial species are a promising source of novel compounds with potential apllications in the pharmaceutical field[29,34].

Liver cancer cell lines, in particular, have been widely used for cytotoxicity studies and to investigate other cellular processes[147]. HepG2 has been the most studied cell line for hepatocellular carcinoma-related research[147]. After 24 h of exposure of this cell line to the organic extracts, only the extract of strain C_025_1 significantly decreased the viability of HeG2 cells when compared to the solvent control (**Figure 13a**).

After 48 h, the extract C_025_1 continued to exhibit cytotoxicity against the HepG2 cell line, but 7 additional actinobacterial extracts, obtained from *Brevibacterium* (S_020_3, S_026_1 and S_001_2), *Microbacterium* (C_017_1 and C_025_4) and *Micrococcus* (C_025_2 and Sed_004_12) species, also reduced significantly the viability of this cell line (**Figure 13b**). All of these extracts, except for the extract from strain S_001_2, have also reduced the cell viability of the breast carcinoma cell line T-47D. The

extract from the strain C_017_1 was able to decrease significantly the viability of HepG2 cells after 48 h and, among the extracts tested, the crude extract from strain S_026_1 appears to be the most cytotoxic for this cell line.

In order to investigate the general toxicity of the crude extracts, a non-tumor cell line was tested. Eight extracts belonging to the genera *Brevibacterium* (S_020_1, C_014_1, S_020_3, and S_026_1), *Microbacterium* (Sed_004_2, C_025_4 and S_026_3) and *Leucobacter* (C_025_1) decreased the viability of hCMEC/D3 cell line after 24 h of exposure (**Figure 14a**).

Nineteen crude extracts derived from *Brevibacterium* (S_020_1, C_014_1, S_020_3, C_017_2, S_026_1 and S_020_11), *Microbacterium* (Sed_004_2, C_017_1, C_025_4, S_026_3, S_026_6 and S_001_10), *Tsukamurella* (S_001_5, S_020_4, S_020_2 and S_020_8), *Micrococcus* (C_025_2), *Leucobacter* (C_025_1) and *Rhodococcus* (C_014_7) strains, were able to cause a decrease in the cellular viability of hCMEC/D3 cells after 48 h of exposure, indicating that most of the bioactive extracts exhibited general cytotoxicity (**Figure 14b**).

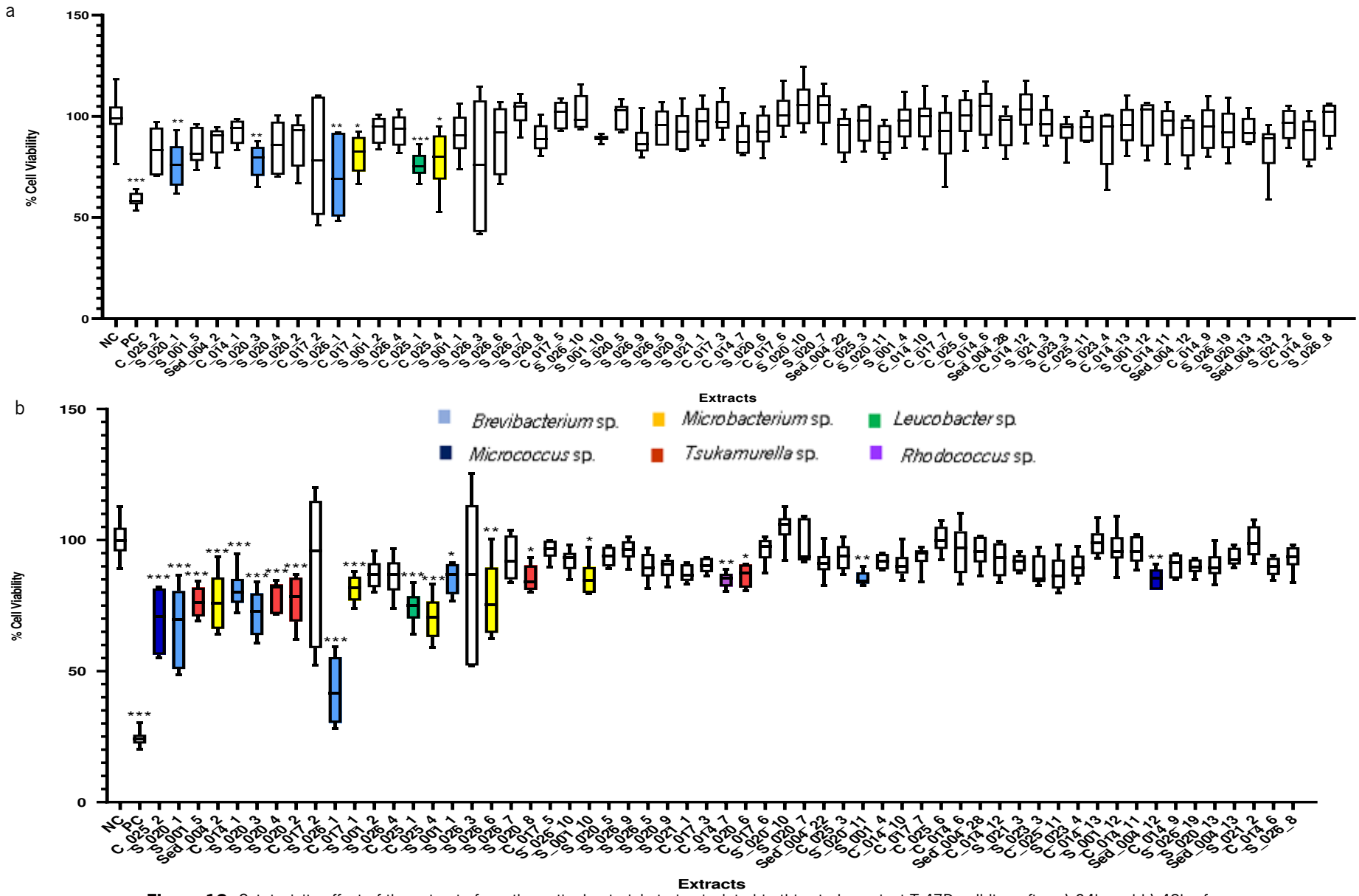

Figure 12. Cytotoxicity effect of the extracts from the actinobacterial strains isolated in this study against T-47D cell line after a) 24h and b) 48h of exposure. P value: 0.033(*), 0.002 (**), <0.001 (***).

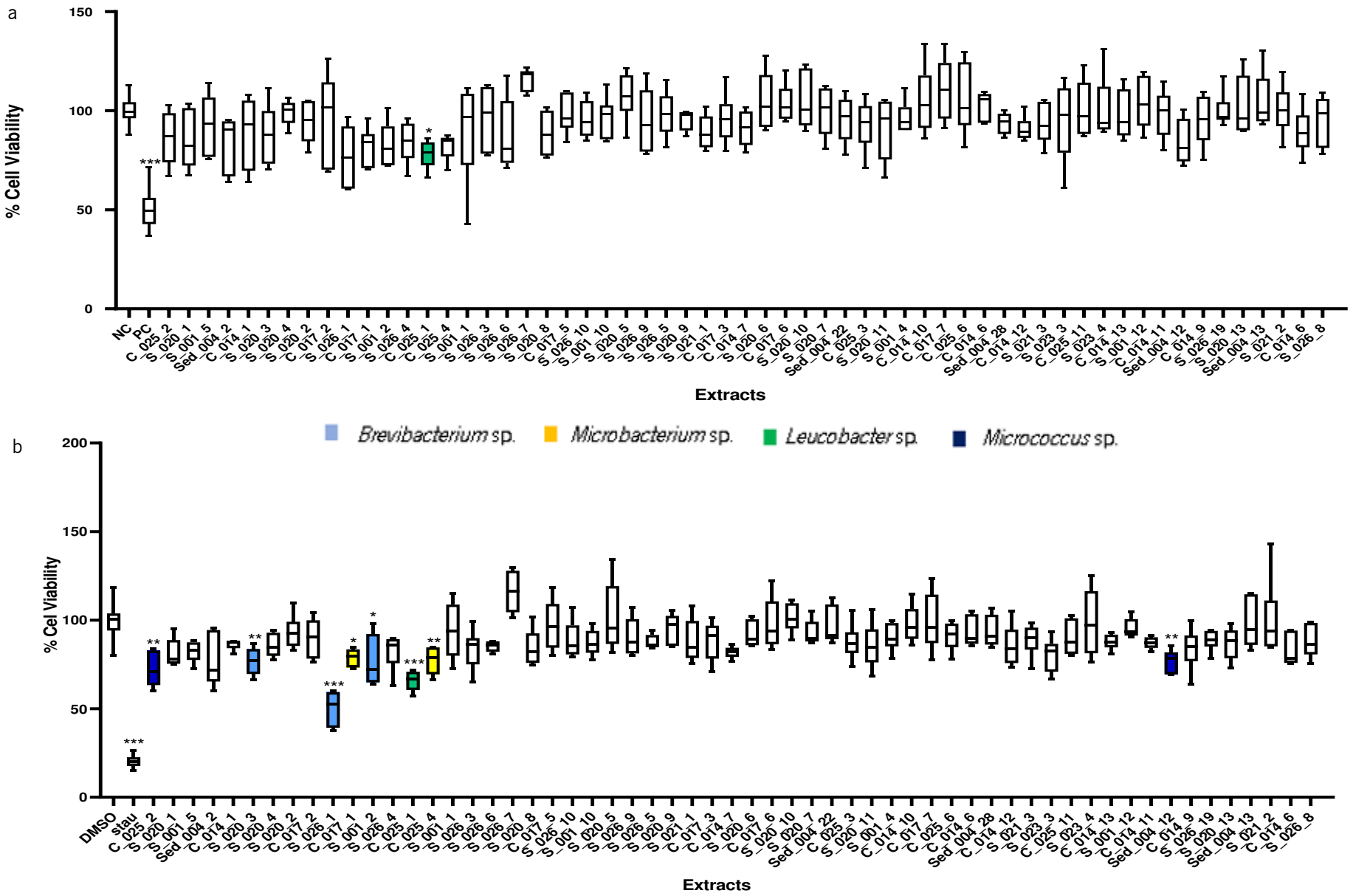

Figure 13. Cytotoxicity effect of the extracts from the actinobacterial strains isolated in this study against HepG2 cell line after a) 24h and b) 48h of exposure. P value: 0.033(*), 0.002 (**), <0.001 (***).

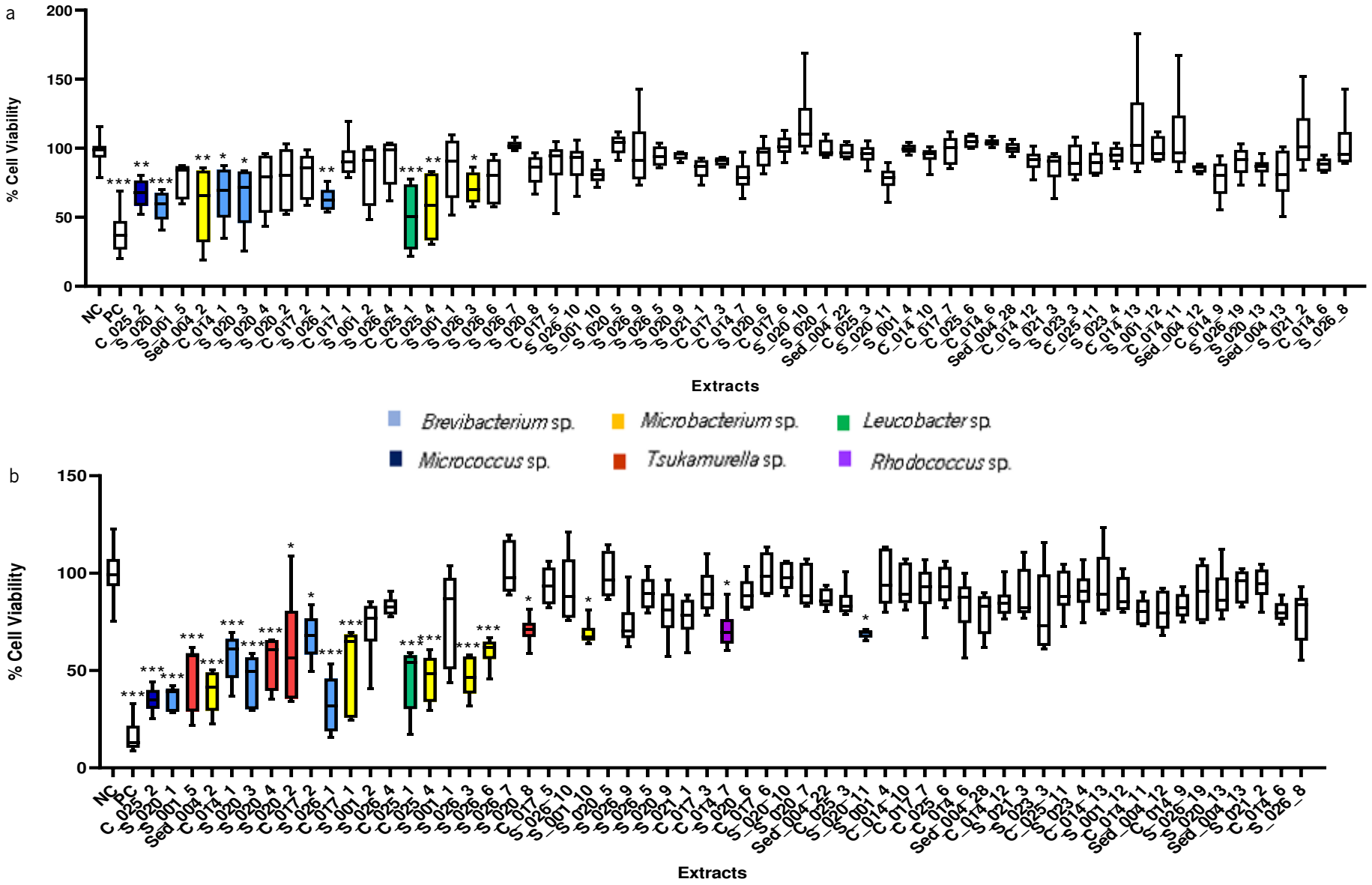

Figure 14. Cytotoxicity effect of the extracts from the actinobacterial strains isolated in this study against hCMEC/D3 cell line after a) 24h and b) 48h of exposure. P value: 0.033(*), 0.002 (**), <0.001 (***).

RESULTS AND DISCUSSION

From the 59 actinobacterial extracts tested for cytotoxic activity, the extract obtained from the strain Sed_004_12 was the only one that was able to decrease the viability of both human cancer cell lines after 48 h of exposure, but did not affect the viability of hCMEC/D3 cells. In addition, the extract from strain S_001_2 showed bioactivity exclusively against HepG2 cells, while the extracts derived from strains S_001_1 and S_020_6 showed cytotoxicity only against T-47D cells, not affecting the viability of the hCMEC/D3 cell line.

The capacity of deep-sea Actinobacteria to produce compounds with cytotoxic activity has been reported before. Peng *et al.* (2015) recovered cytotoxic compounds from a *Micrococcus* strain isolated from a deep-sea sediment collected on the Western Pacific Ocean[148]. *Microbacterium* strains, isolated from a deep-sea sediment collected in the south-west Indian Ocean, were found to produce Microbacterins A and B, belonging to the class of peptides named peptaibols, with potent cytotoxic activity against a panel of human tumor cell lines[149].

Our findings suggest that marine rare deep-sea Actinobacteria, such as the genera identified in this study, namely, *Brevibacterium*, *Tsukamurella*, *Microbacterium*, *Micrococcus*, *Rhodococcus* and *Leucobacter*, are a promising source of anticancer compounds that may help tackling cancer-related disorders.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

4. CONCLUSIONS AND FUTURE WORK

It is well established that Actinobacteria represent an important group from a biotechnological point of view. They produce natural compounds with a wide range of applications, several of which are commonly used for medical purposes.

In this study, we aimed to investigate the actinobacterial diversity of nine deep-sea samples, including sponges, corals and sediments, which were collected at the South Coast of Madeira Archipelago. The 16S rRNA gene analysis revealed 68 isolates associated to the genera *Brevibacterium* (25), *Microbacterium* (20), *Tsukamurella* (14), *Leucobacter* (2), *Micrococcus* (2), *Rhodococcus* (2), *Brachybacterium* (2) and *Streptomyces* (1). In addition, the isolates C_017_1 and C_017_5 were found to potentially represent new *Microbacterium* species, as their 16S rRNA gene sequences showed less than 98.7% similarity with database species.

Up to the moment, 59 actinobacterial extracts were tested for their bioactive potential. Two organic extracts, recovered from a *Brevibacterium* and a *Brachybacterium* strains, exhibited antimicrobial activity against *B. subtilis*, *S.aureus* or *C. albicans*, with a MIC value of 1000µg mL^{-1}.

Cytotoxicity assays revealed 23 actinobacterial extracts capable of reducing the cellular viability of the human cancer cell lines HepG2 and T-47D, though many of them also presented activity in the non-tumor cell line, hCMEC/D3, showing a general cytotoxicity action. These extracts were derived from *Brevibacterium*, *Microbacterium*, *Tsukamurella*, *Micrococcus*, *Leucobacter* and *Rhodococcus* strains. Four organic extracts (Sed_004_12, S_001_1, S_001_2 and S_020_6) are particularly interesting as they exhibited cytotoxic activity against at least one of the human cancer cell lines tested, but did not reduce significantly the viability of hCMEC/D3 cells.

In conclusion, this study shows that the 9 deep-sea samples collected at the South Coast of Madeira archipelago presented some diversity in terms of actinobacterial genera, being little colonized in terms of cultivable *Streptomyces* species and having a higher abundance of rare actinobacterial genera. The low number of *Streptomyces* isolates recovered in this study may justify the reduced number of strains exhibiting antimicrobial activity. On the contrary, a high number of actinobacterial strains exhibited cytotoxic activity, though many of these strains seem to exert a general cytotoxic action. Nonetheless, these activities are very interesting, and it will be very important to investigate what is (are) the bioactive compound(s) responsible for them in order to find out if new molecules are being produced.

In order to conclude this work and determine if the bioactive strains are producing novel compounds, there are some future tasks that need to be done. First, the antimicrobial and cytotoxic activities of the actinobacterial strains that were not tested under the scope of the present study need to be screened. Secondly, the extracts exhibiting bioactivity will need to be chemically analyzed to determine if new secondary metabolites are responsible for the observed activities. Regarding this task, dereplication will be a crucial process as it enables a rapid identification of known compounds[150]. Compounds with no hits in the dereplication process that may explain the observed bioactivities, indicating potential new molecules, will have to be submitted to a round of analytical methods, that will include chromatographic and nuclear magnetic resonance (NMR) techniques, to sequentially separate and purify the chemical constituents of the organic extracts and achieve the chemical structures of the compound(s).

CHAPTER 5

REFERENCES

5. REFERENCES

1. Rolain, J., Abat, C., Jimeno, M., Fournier, P. & Raoult, D. Do we need new antibiotics? *Clin. Microbiol. Infect.* **22**, 408–415 (2016).

2. Willey, J., Sherwood, L. M. & Woolverton, C. J. Antimicrobial Chemotherapy. in *Prescott, Harley, and Klein's Microbiology* 835–858 (2008).

3. Munita, J. M. & Arias, C. A. Mechanisms of Antibiotic Resistance. *Microbiol Spectr.* **4**, 1–37 (2016).

4. Thomas, C. M. & Nielsen, K. M. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat. Rev. Microbiol.* **3**, 711–721 (2005).

5. Willey, J., Sherwood, L. M. & Woolverton, C. J. Microbial Genetics: Mechanisms of Genetic Variation. in *Prescott, Harley, and Klein's Microbiology* 345–349 (2008).

6. Pendleton, J. N., Gorman, S. P. & Gilmore, B. F. Clinical relevance of the ESKAPE pathogens. *Expert Rev. Anti. Infect. Ther.* **11**, 297–308 (2013).

7. Vivas, R., Barbosa, A., Dolabela, S. S. & Jain, S. Multidrug-Resistant Bacteria and Alternative Methods to Control Them: An Overview. *Microb Drug Resist.* **25**, 890–908 (2019).

8. World Health Organization. Cancer. (2018). Available at: https://www.who.int/news-room/fact-sheets/detail/cancer. (Accessed: 17th October 2019)

9. Schernhammer, E. S., Zhang, Y. & Devore, E. E. Sleep Disturbance/Misalignment. *Encyclopedia of Cancer, 3rd Edition* 1–8 (2018).

10. Patel, A. V & Rees-Punia, E. Causes of Cancer: Physical Inactivity. *Encyclopedia of Cancer* 1–6 (2018).

11. Park, Y. Obesity and Cancer: Epidemiological Evidence. *Encyclopedia of Cancer* **3**, 88–97 (2019).

12. Romieu, I. Dietary Factors and Cancer. *Encyclopedia of Cancer* 1–11 (2018).

13. Smith, J. W. & Groopman, J. D. Aflatoxins. *Encyclopedia of Cancer* 1–14 (2018).

14. Song, M. Aging and Cancer. *Encyclopedia of Cancer* 44–52 (2019).

15. Anastasiadi, Z., Lianos, G. D., Ignatiadou, E., Harissis, H. V & Mitsis, M. Breast cancer in young women: an overview. *Updates Surg.* **69**, 313–317 (2017).

16. Marengo, A., Rosso, C. & Bugianesi, E. Liver Cancer: Connections with Obesity , Fatty Liver, and Cirrhosis. *Annu. Rev. Med.* **67**, 1–15 (2016).

17. Saitta, C., Pollicino, T. & Raimondo, G. Obesity and liver cancer. *Ann. Hepatol.* 1–6 (2019).

18. Demain, A. L. & Vaishnav, P. Natural products for cancer chemotherapy. *Microb. Biotechnol.* **4**, 687–699 (2011).

19. Wright, G. D. Natural Product Reports Opportunities for natural products in 21 st century antibiotic discovery. *Nat. Prod. Rep.* **34**, 694–701 (2017).

20. Katz, L. & Baltz, R. H. Natural product discovery: past, present, and future. *J. Ind. Microbiol. Biotechnol.* **43**, 155–176 (2016).

21. Genilloud, O. Natural products discovery and potential for new antibiotics. *Curr. Opin. Microbiol.* **51**, 81–87 (2019).

22. Verma, E., Chakraborty, S., Tiwari, B. & Mishra, A. K. Antimicrobial Compounds From Actinobacteria: Synthetic Pathways and Applications. in *New and Future Developments in Microbial Biotechnology and Bioengineering* (eds. Gupta, V. & Rodriguez-Couto, S.) **101**, 277–296 (Elsevier B.V., 2018).

23. Sharma, P., Dutta, J. & Thakur, D. Future Prospects of Actinobacteria in Health and Industry. in *New and Future Developments in Microbial Biotechnology and Bioengineering* (eds. Gupta, V. & Rodriguez-Couto, S.) 305–324 (Elsevier B.V., 2018).

24. Lewin, G. R. *et al.* Evolution and Ecology of Actinobacteria and Their Bioenergy Applications. *Annu. Rev. Microbiol.* **70**, 235–254 (2016).

25. Barka, E. A. *et al.* Taxonomy, Physiology and Natural Products of Actinobacteria. *Microbiol. Mol. Biol. Rev.* **80**, 1–44 (2016).

26. Srilekha, V., Krishna, G., Seshasrinivas, V. & Charya, M. A. S. Antibacterial and anti-inflammatory activities of marine Brevibacterium sp. *Res. Pharm. Sci.* **12**, 283–289 (2017).

27. Busi, S. & Pattnaik, S. S. *Current Status and Applications of Actinobacteria in the Production of Anticancerous Compounds*. *New and Future Developments in Microbial Biotechnology and Bioengineering* (Elsevier B.V., 2018).

28. Manivasagan, P., Venkatesan, J., Sivakumar, K. & Kim, S. K. Marine actinobacterial metabolites: Current status and future perspectives. *Microbiol. Res.* **168**, 311–332 (2013).

29. Subramani, R. & Sipkema, D. Marine Rare Actinomycetes: A Promising Source of Structurally Diverse and Unique Novel Natural Products. *Mar. Drugs* **17**, (2019).

30. de Lima Procópio, R. E., da Silva, I. R., Martins, M. K., de Azevedo, J. L. & de Araújo, J. M. Antibiotics produced by Streptomyces. *Brazilian J. Infect. Dis.* **16**, 466–471 (2012).

31. Shams, S., Anjum, K., Qamar, S. & Akhter, N. Emerging biopharmaceuticals from marine actinobacteria. *Environ. Toxicol. Pharmacol.* **49**, 34–47 (2017).

32. Sayed, A. M. *et al.* Extreme environments: microbiology leading to specialized metabolites. *J. Appl. Microbiol.* **128**, 630–657 (2020).

33. Subramani, R. & Aalbersberg, W. Marine actinomycetes: An ongoing source of novel bioactive metabolites. *Microbiol. Res.* **167**, 571–580 (2012).

34. Dhakal, D., Pokhrel, A. R., Shrestha, B. & Sohng, J. K. Marine Rare Actinobacteria: Isolation, Characterization, and Strategies for Harnessing Bioactive Compounds. *Front. Microbiol.* **8**, (2017).

35. Mohammadipanah, F., Atasayar, E., Heidarian, S. & Wink, J. Glycomyces sediminimaris sp. nov., a new species of actinobacteria isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* **68**, 2357–2363 (2018).

36. Chen, R.-W. *et al.* Rubrobacter indicoceani sp. nov., a new marine actinobacterium isolated from Indian Ocean sediment. *Int. J. Syst. Evol. Microbiol.* **68**, 3487–3493 (2018).

37. Ng, Z. Y., Fang, B.-Z., Li, W.-J. & Tan, G. Y. A. Marinitenerispora sediminis gen. nov., sp. nov., a member of the family Nocardiopsaceae isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* **69**, 3031–3040 (2019).

38. Li, L., Wang, J., Zhou, Y.-J., Lin, H.-W. & Lu, Y.-H. Streptomyces reniochalinae sp. nov. and Streptomyces diacarni sp. nov., from marine sponges. *Int. J. Syst. Evol. Microbiol.* **69**, 99–104 (2019).

39. Oh, M., Kim, J.-H., Yoon, J.-H., Schumann, P. & Kim, W. Cellulosimicrobium arenosum sp. nov., Isolated from Marine Sediment Sand. *Curr. Microbiol.* **75**, 901–906 (2018).

40. Ramaprasad, E. V. V, Mahidhara, G., Sasikala, C. & Ramana, C. V. Rhodococcus electrodiphilus sp. nov., a marine electro active actinobacterium isolated from coral reef. *Int. J. Syst. Evol. Microbiol.* **68**, 2644–2649 (2018).

41. Claverías, F. *et al.* Corynebacterium alimapuense sp. nov., an obligate marine actinomycete isolated from sediment of Valparaíso bay, Chile. *Int. J. Syst. Evol. Microbiol.* **69**, 783–790 (2019).

42. Li, L., Zhu, H.-R., Xu, Q.-H., Lin, H.-W. & Lu, Y.-H. Micromonospora craniellae sp. nov., isolated from a marine sponge, and reclassification of Jishengella endophytica as Micromonospora endophytica comb. nov. *Int. J. Syst. Evol. Microbiol.* **69**, 715–720 (2019).

43. Li, L., Zhang, D., Tang, W.-Z., Lin, H.-W. & Lu, Y.-H. Geodermatophilus marinus sp. nov., isolated from the marine sponge Leucetta chagosensis. *Int. J. Syst. Evol. Microbiol.* **69**, 2966–2971 (2019).

44. Qu, Z. *et al.* Actinoplanes sediminis sp. nov., isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* **68**, 71–75 (2018).

45. Phongsopitanun, W. *et al.* Streptomyces verrucosisporus sp. nov., isolated from marine sediments. *Int. J. Syst. Evol. Microbiol.* **66**, 3607–3613 (2016).

46. Veyisoglu, A. *et al.* Micromonospora orduensis sp. nov., isolated from deep marine sediment. *Antonie Van Leeuwenhoek* **113**, 397–405 (2020).

47. Wang, S., Zhou, Y. & Zhang, G. Nocardioides flavus sp. nov., isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* **66**, 5275–5280 (2016).

48. Zhang, G., Wang, L., Li, J. & Zhou, Y. Pseudonocardia profundimaris sp. nov., isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* **67**, 1693–1697 (2017).

49. Terahara, T. *et al.* Streptomyces otsuchiensis sp. nov., a biosurfactant-producing actinobacterium isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* **69**, 3740–3744 (2019).

50. Li, L., Xu, Q.-H., Wang, X.-T., Lin, H.-W. & Lu, Y.-H. Actinomadura craniellae sp. nov., isolated from a marine sponge in the South China Sea. *Int. J. Syst. Evol. Microbiol.* **69**, 1207–1212 (2019).

51. Nouioui, I. *et al.* Actinoalloteichus fjordicus sp. nov. isolated from marine sponges:

phenotypic, chemotaxonomic and genomic characterisation. *Antonie Van Leeuwenhoek* **110**, 1705–1717 (2017).

52. Carro, L. *et al.* A study of three bacteria isolated from marine sediment and description of Micromonospora globispora sp. nov. *Syst. Appl. Microbiol.* **42**, 190–197 (2019).

53. Phongsopitanun, W. *et al.* Micromonospora fluostatini sp. nov., isolated from marine sediment. *Int. J. Syst. Evol. Microbiol.* **65**, 4417–4423 (2015).

54. Afonso de Menezes, C. B. *et al.* Williamsia spongiae sp. nov., an actinomycete isolated from the marine sponge Amphimedon viridis. *Int. J. Syst. Evol. Microbiol.* **67**, 1260–1265 (2017).

55. Román-Ponce, B. *et al.* Six novel species of the obligate marine actinobacterium Salinispora, Salinispora cortesiana sp. nov., Salinispora fenicalii sp. nov., Salinispora goodfellowii sp. nov., Salinispora mooreana sp. nov., Salinispora oceanensis sp. nov. and Salinispora vitie. *Int. J. Syst. Evol. Microbiol.* **70**, 4668–4682 (2020).

56. Thawai, C., Rungjindamai, N., Klanbut, K. & Tanasupawat, S. Nocardia xestospongiae sp. nov., isolated from a marine sponge in the Andaman Sea. *Int. J. Syst. Evol. Microbiol.* **67**, 1451–1456 (2017).

57. Jiang, Z. *et al.* Kocuria subflava sp. nov., isolated from marine sediment from the Indian Ocean. *Antonie Van Leeuwenhoek* **108**, 1349–1355 (2015).

58. Silva, F. S. P. *et al.* Streptomyces atlanticus sp. nov., a novel actinomycete isolated from marine sponge Aplysina fulva (Pallas, 1766). *Antonie Van Leeuwenhoek* **109**, 1467–1474 (2016).

59. Souza, D. T. *et al.* Saccharopolyspora spongiae sp. nov., a novel actinomycete isolated from the marine sponge Scopalina ruetzleri (Wiedenmayer, 1977). *Int. J. Syst. Evol. Microbiol.* **67**, 2019–2025 (2017).

60. Li, L., Gui, Y.-H., Xu, Q.-H., Lin, H.-W. & Lu, Y.-H. Spongiactinospora rosea gen. nov., sp. nov., a new member of the family Streptosporangiaceae. *Int. J. Syst. Evol. Microbiol.* **69**, 427–433 (2019).

61. de Menezes, C. B. A. *et al.* Williamsia aurantiacus sp. nov. a novel actinobacterium producer of antimicrobial compounds isolated from the marine sponge. *Arch. Microbiol.* **201**, 691–698 (2019).

62. Veyisoglu, A., Cetin, D., Inan Bektas, K., Guven, K. & Sahin, N. Streptomyces ovatisporus sp. nov., isolated from deep marine sediment. *Int. J. Syst. Evol. Microbiol.* **66**, 4856–4863 (2016).

63. He, Y.-Q. *et al.* Actinomarinicola tropica gen. nov. sp. nov., a new marine actinobacterium of the family Iamiaceae, isolated from South China Sea sediment environments. *Int. J. Syst. Evol. Microbiol.* **70**, 3852–3858 (2020).

64. Das, L., Deb, S. & Das, S. K. Glutamicibacter mishrai sp. nov., isolated from the coral Favia veroni from Andaman Sea. *Arch. Microbiol.* **202**, 733–745 (2020).

65. Intra, B. *et al.* Micromonospora pelagivivens sp. nov., a new species of the genus Micromonospora isolated from deep-sea sediment in Japan. *Int. J. Syst. Evol. Microbiol.*

70, 3069–3075 (2020).

66. Zhang, G., Wang, L., Li, J. & Zhou, Y. Amycolatopsis albispora sp. nov., isolated from deep-sea sediment. *Int. J. Syst. Evol. Microbiol.* **66**, 3860–3864 (2016).

67. Zhang, G., Wang, L., Xie, F., Pei, S. & Jiang, L. Nesterenkonia salmonea sp. nov. and Nesterenkonia sphaerica sp. nov., isolated from the Southern Atlantic Ocean. *Int. J. Syst. Evol. Microbiol.* **70**, 923–928 (2020).

68. Chen, R.-W. *et al.* Rubrobacter tropicus sp. nov. and Rubrobacter marinus sp. nov., isolated from deep-sea sediment of the South China Sea. *Int. J. Syst. Evol. Microbiol.* **70**, 5576–5585 (2020).

69. Zhou, Y., Pei, S., Xie, F., Gu, L. & Zhang, G. Saccharopolyspora coralli sp. nov. a novel actinobacterium isolated from the stony coral Porites. *Int. J. Syst. Evol. Microbiol.* **70**, 3241–3246 (2020).

70. Valliappan, K., Sun, W. & Li, Z. Marine actinobacteria associated with marine organisms and their potentials in producing pharmaceutical natural products. *Appl Microbiol Biotechnol* **98**, 7365–7377 (2014).

71. Liang, Y. *et al.* Bioactive Polycyclic Quinones from Marine Streptomyces sp. 182SMLY. *Mar. Drugs* **14**, 10 (2016).

72. Lacret, R. *et al.* New ikarugamycin derivatives with antifungal and antibacterial properties from Streptomyces zhaozhouensis. *Mar. Drugs* **13**, 128–140 (2014).

73. Chen, H., Cai, K. & Yao, R. A new macrolactam derivative from the marine actinomycete HF-11225. *J. Antibiot. (Tokyo).* **71**, 477–479 (2018).

74. Huang, Y. *et al.* A new medermycin analog from the marine-derived actinomycetes Streptomyces sp. ZS-A45. *J. Asian Nat. Prod. Res.* **21**, 826–831 (2019).

75. Paulus, C. *et al.* New natural products identified by combined genomics-metabolomics profiling of marine Streptomyces sp. MP131-18. *Sci. Rep.* **7**, 42382 (2017).

76. Choi, B.-K., Lee, H.-S., Kang, J. S. & Shin, H. J. Dokdolipids A-C, Hydroxylated Rhamnolipids from the Marine-Derived Actinomycete Actinoalloteichus hymeniacidonis. *Mar. Drugs* **17**, (2019).

77. Kalinovskaya, N. L. *et al.* The Antitumor Antibiotics Complex of Aureolic Acids from the Marine Sediment-associated Strain of Streptomyces sp. KNIM 9048. *Nat. Prod. Commun.* **12**, 571–577 (2017).

78. Zhang, Y.-M. *et al.* Ergosterols from the Culture Broth of Marine Streptomyces anandii H41-59. *Mar. Drugs* **14**, (2016).

79. Sproule, A. *et al.* Terrosamycins A and B, Bioactive Polyether Ionophores from Streptomyces sp. RKND004 from Prince Edward Island Sediment. *Mar. Drugs* **17**, (2019).

80. Ortiz-López, F. J. *et al.* New 3-Hydroxyquinaldic Acid Derivatives from Cultures of the Marine Derived Actinomycete Streptomyces cyaneofuscatus M-157. *Mar. Drugs* **16**, (2018).

81. Zhang, D., Shu, C., Lian, X. & Zhang, Z. New Antibacterial Bagremycins F and G from the Marine-Derived Streptomyces sp. ZZ745. *Mar. Drugs* **16**, (2018).

82. Ibrahim, A. H. *et al.* New Cytotoxic Cyclic Peptide from the Marine Sponge-Associated Nocardiopsis sp. UR67. *Mar. Drugs* **16**, (2018).

83. Zhen, X. *et al.* A New Analogue of Echinomycin and a New Cyclic Dipeptide from a Marine-Derived Streptomyces sp. LS298. *Mar. Drugs* **13**, 6947–6961 (2015).

84. Cao, D. D. *et al.* Antimicrobial lavandulylated flavonoids from a sponge-derived actinomycete. *Nat. Prod. Res.* **34**, 413–420 (2020).

85. Yang, C.-L. *et al.* Strepchazolins A and B: Two New Alkaloids from a Marine Streptomyces chartreusis NA02069. *Mar. Drugs* **15**, (2017).

86. Gong, T. *et al.* Tetrocarcin Q, a New Spirotetronate with a Unique Glycosyl Group from a Marine-Derived Actinomycete Micromonospora carbonacea LS276. *Mar. Drugs* **16**, (2018).

87. Pérez-Bonilla, M. *et al.* Phocoenamicins B and C, New Antibacterial Spirotetronates Isolated from a Marine Micromonospora sp. *Mar. Drugs* **16**, (2018).

88. Chen, M.-H. *et al.* Isolation, purification and identification of two new alkaloids metabolites from marine-derived Verrucosispora sp. FIM06025. *Nat. Prod. Res.* **33**, 2897–2903 (2019).

89. Yan, J.-X. *et al.* Madurastatin D1 and D2, Oxazoline Containing Siderophores Isolated from an Actinomadura sp. *Org. Lett.* **21**, 6275–6279 (2019).

90. Iniyan, A. M., Sudarman, E., Wink, J., Kannan, R. R. & Vincent, S. G. P. Ala-geninthiocin, a new broad spectrum thiopeptide antibiotic, produced by a marine Streptomyces sp. ICN19. *J. Antibiot. (Tokyo).* **72**, 99–105 (2019).

91. Gao, M.-Y. *et al.* A new naphthalenepropanoic acid analog from the marine-derived actinomycetes Micromonospora sp. HS-HM-036. *J. Asian Nat. Prod. Res.* **19**, 930–934 (2017).

92. Cheng, C. *et al.* Isolation of Petrocidin A, a New Cytotoxic Cyclic Dipeptide from the Marine Sponge-Derived Bacterium Streptomyces sp. SBT348. *Mar. Drugs* **15**, (2017).

93. Wang, J.-N., Zhang, H.-J., Li, J.-Q., Ding, W.-J. & Ma, Z.-J. Bioactive Indolocarbazoles from the Marine-Derived Streptomyces sp. DT-A61. *J. Nat. Prod.* **81**, 949–956 (2018).

94. Jiang, Y.-J., Li, J.-Q., Zhang, H.-J., Ding, W.-J. & Ma, Z.-J. Cyclizidine-Type Alkaloids from Streptomyces sp. HNA39. *J. Nat. Prod.* **81**, 394–399 (2018).

95. Rodríguez, V. *et al.* Anthracimycin B, a Potent Antibiotic against Gram-Positive Bacteria Isolated from Cultures of the Deep-Sea Actinomycete Streptomyces cyaneofuscatus M-169. *Mar. Drugs* **16**, (2018).

96. Hamed, A. *et al.* N-Acetylborrelidin B: a new bioactive metabolite from Streptomyces mutabilis sp. MII. *Z. Naturforsch. C.* **73**, 49–57 (2018).

97. Zhou, S.-Y., Zou, Y.-L., Wang, G.-W., Liao, Z.-H. & Chen, M. Two new compounds from a marine-derived Streptomyces sp. *J. Asian Nat. Prod. Res.* **19**, 1172–1176 (2017).

98. Gui, C. *et al.* Antimicrobial Spirotetronate Metabolites from Marine-Derived Micromonospora harpali SCSIO GJ089. *J. Nat. Prod.* **80**, 1594–1603 (2017).

99. Chen, E. *et al.* Mathermycin, a Lantibiotic from the Marine Actinomycete Marinactinospora thermotolerans SCSIO 00652. *Appl. Environ. Microbiol.* **83**, (2017).

100. Takehana, Y. *et al.* Fradiamine A, a new siderophore from the deep-sea actinomycete Streptomyces fradiae MM456M-mF7. *J. Antibiot. (Tokyo).* **70**, 611–615 (2017).

101. Thi, Q. V. *et al.* Secondary Metabolites from an Actinomycete from Vietnam's East Sea. *Nat. Prod. Commun.* **11**, 401–404 (2016).

102. Cao, D. D. *et al.* Antimicrobial Lavandulylated Flavonoids from a Sponge-Derived Streptomyces sp. G248 in East Vietnam Sea. *Mar. Drugs* **17**, (2019).

103. Hu, C. *et al.* Neoantimycins A and B, Two Unusual Benzamido Nine-Membered Dilactones from Marine-Derived Streptomyces antibioticus H12-15. *Molecules* **22**, (2017).

104. Zhang, Y., Zhang, C., Wang, K., Chen, G. & Sun, P. New Avermectin Analogues from a Mutant Streptomyces avermectinius Strain. *Chem. Biodivers.* **14**, (2017).

105. Fu, S. *et al.* Secondary metabolites from marine-derived Streptomyces antibioticus strain H74-21. *Nat. Prod. Res.* **30**, 2460–2467 (2016).

106. Nong, X.-H., Wei, X.-Y. & Qi, S.-H. Pteridic acids C-G spirocyclic polyketides from the marine-derived Streptomyces sp. SCSGAA 0027. *J. Antibiot. (Tokyo).* **70**, 1047–1052 (2017).

107. Huang, P. *et al.* Anti-MRSA and anti-TB metabolites from marine-derived Verrucosispora sp. MS100047. *Appl. Microbiol. Biotechnol.* **100**, 7437–7447 (2016).

108. Thi, Q. V. *et al.* Antimicrobial Metabolites from a Marine-Derived Actinomycete in Vietnam's East Sea. *Nat. Prod. Commun.* **11**, 49–51 (2016).

109. Wang, Q. *et al.* Neo-actinomycins A and B, natural actinomycins bearing the 5H-oxazolo[4,5-b]phenoxazine chromophore, from the marine-derived Streptomyces sp. IMB094. *Sci. Rep.* **7**, 3591 (2017).

110. Sarmiento-Vizcaíno, A. *et al.* Paulomycin G, a New Natural Product with Cytotoxic Activity against Tumor Cell Lines Produced by Deep-Sea Sediment Derived Micromonospora matsumotoense M-412 from the Avilés Canyon in the Cantabrian Sea. *Mar. Drugs* **15**, (2017).

111. Braña, A. F. *et al.* Lobophorin K, a New Natural Product with Cytotoxic Activity Produced by Streptomyces sp. M-207 Associated with the Deep-Sea Coral Lophelia pertusa. *Mar. Drugs* **15**, (2017).

112. Ward, A. C. & Bora, N. Diversity and biogeography of marine actinobacteria. *Curr. Opin. Microbiol.* **9**, 279–286 (2006).

113. Kamjam, M., Sivalingam, P., Deng, Z. & Hong, K. Deep Sea Actinomycetes and Their Secondary Metabolites. *Front. Microbiol.* **8**, (2017).

114. Bull, A. T. & Stach, J. E. M. Marine actinobacteria: new opportunities for natural product search and discovery. *TRENDS Microbiol.* **15**, (2007).

115. Mahmoud, H. M. & Kalendar, A. A. Coral-Associated Actinobacteria: Diversity, Abundance, and Biotechnological Potentials. *Front. Microbiol.* **7**, 204 (2016).

116. Kellogg, C. A. Microbiomes of stony and soft deep-sea corals share rare core bacteria. *Microbiome* **7**, 90 (2019).

117. Lawler, S. N. *et al.* Coral-associated bacterial diversity is conserved across two deep-sea Anthothela species. *Front. Microbiol.* **458**, 1–18 (2016).

118. Sarmiento-Vizcaíno, A. *et al.* Pharmacological Potential of Phylogenetically Diverse Actinobacteria Isolated from Deep-Sea Coral Ecosystems of the Submarine Avilés Canyon in the Cantabrian Sea. *Microb. Ecol.* **73**, 338–352 (2017).

119. Xu, D. *et al.* Bioprospecting Deep-Sea Actinobacteria for Novel Anti-infective Natural Products. *Front. Microbiol.* **9**, 787 (2018).

120. Kennedy, J. *et al.* Evidence of a putative deep sea specific microbiome in marine sponges. *PLoS One* **9**, (2014).

121. Jackson, S. A. *et al.* Diverse and abundant secondary metabolism biosynthetic gene clusters in the genomes of marine sponge derived Streptomyces spp. Isolates. *Mar. Drugs* **16**, 67 (2018).

122. Xu, D. *et al.* Nocardiopsistins A-C: New angucyclines with anti-MRSA activity isolated from a marine sponge-derived Nocardiopsis sp. HB-J378. *Synth. Syst. Biotechnol.* **3**, 246–251 (2018).

123. Hameş-Kocabaş, E. E. & Uzel, A. Isolation strategies of marine-derived actinomycetes from sponge and sediment samples. *J. Microbiol. Methods* **88**, 342–347 (2012).

124. Girão, M. *et al.* Actinobacteria Isolated From Laminaria ochroleuca: A Source of New Bioactive Compounds. *Front. Microbiol.* **10**, 1–13 (2019).

125. Xin, Y., Kanagasabhapathy, M., Janussen, D., Xue, S. & Zhang, W. Phylogenetic diversity of Gram-positive bacteria cultured from Antarctic deep-sea sponges. *Polar Biol.* **34**, 1501–1512 (2011).

126. Williams, S. E. *et al.* The Bristol Sponge Microbiome Collection: A Unique Repository of Deep-sea Microorganisms and Associated Natural Products. *Antibiotics* **9**, 1–18 (2020).

127. Yakimov, M. M. *et al.* Phylogenetic survey of metabolically active microbial communities associated with the deep-sea coral Lophelia pertusa from the Apulian plateau, Central Mediterranean Sea. *Deep Sea Res. Part I Oceanogr. Res. Pap.* **53**, 62–75 (2006).

128. Zhang, G., Cao, T., Ying, J., Yang, Y. & Ma, L. Diversity and novelty of actinobacteria in Arctic marine sediments. *Antonie Van Leeuwenhoek* **105**, 743–754 (2014).

129. Ulanova, D. & Goo, K.-S. Diversity of actinomycetes isolated from subseafloor sediments after prolonged low-temperature storage. *Folia Microbiol. (Praha).* **60**, 211–216 (2015).

130. Chen, P. *et al.* Diversity, Biogeography, and Biodegradation Potential of Actinobacteria in the Deep-Sea Sediments along the Southwest Indian Ridge. *Front. Microbiol.* **7**, 1340 (2016).

131. Ettoumi, B. *et al.* Diversity, ecological distribution and biotechnological potential of Actinobacteria inhabiting seamounts and non-seamounts in the Tyrrhenian Sea. *Microbiol. Res.* **186–187**, 71–80 (2016).

132. da Silva, M. A. C. *et al.* Phylogenetic identification of marine bacteria isolated from deep-sea sediments of the eastern South Atlantic Ocean. *Springerplus* **2**, 127 (2013).

133. Chen, P. *et al.* Brevibacterium sediminis sp. nov., isolated from deep-sea sediments from the Carlsberg and Southwest Indian Ridges. *Int. J. Syst. Evol. Microbiol.* **66**, 5268–5274 (2016).

134. Verma, P. *et al.* Complex bacterial communities in the deep-sea sediments of the Bay of Bengal and volcanic Barren Island in the Andaman Sea. *Mar. Genomics* **31**, 33–41 (2017).

135. Zhang, H., Zhang, W., Jin, Y., Jin, M. & Yu, X. A comparative study on the phylogenetic diversity of culturable actinobacteria isolated from five marine sponge species. *Antonie Van Leeuwenhoek* **93**, 241–248 (2008).

136. Zhang, X.-Y. *et al.* Diversity and antibacterial activity of culturable actinobacteria isolated from five species of the South China Sea gorgonian corals. *World J. Microbiol. Biotechnol.* **29**, 1107–1116 (2013).

137. Williams, S. E. *et al.* The Bristol Sponge Microbiome Collection: A Unique Repository of Deep-Sea Microorganisms and Associated Natural Products. *Antibiotics* **9**, 509 (2020).

138. Yarza, P. *et al.* Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat. Rev. Microbiol.* **12**, 635–645 (2014).

139. Meena, B., Anburajan, L., Vinithkumar, N. V., Kirubagaran, R. & Dharani, G. Biodiversity and antibacterial potential of cultivable halophilic actinobacteria from the deep sea sediments of active volcanic Barren Island. *Microb. Pathog.* **132**, 129–136 (2019).

140. Seghal Kiran, G., Anto Thomas, T., Selvin, J., Sabarathnam, B. & Lipton, A. P. Optimization and characterization of a new lipopeptide biosurfactant produced by marine Brevibacterium aureum MSA13 in solid state culture. *Bioresour. Technol.* **101**, 2389–2396 (2010).

141. Kiran, G. S., Sabarathnam, B., Thajuddin, N. & Selvin, J. Production of Glycolipid Biosurfactant from Sponge-Associated Marine Actinobacterium Brachybacterium paraconglomeratum MSA21. *J. Surfactants Deterg.* **17**, 531–542 (2014).

142. Graça, A. P. *et al.* The antimicrobial activity of heterotrophic bacteria isolated from the marine sponge Erylus deficiens (Astrophorida, Geodiidae). *Front. Microbiol.* **6**, 389 (2015).

143. Santos, J. D. *et al.* Bioactivities and Extract Dereplication of Actinomycetales Isolated From Marine Sponges. *Front. Microbiol.* **10**, (2019).

144. Eltamany, E. E. *et al.* New antibacterial xanthone from the marine sponge-derived Micrococcus sp. EG45. *Bioorg. Med. Chem. Lett.* **24**, 4939–4942 (2014).

145. Vollbrecht, E., Rau, U. & Lang, S. Microbial conversion of vegetable oils into surface-active di-, tri-, and tetrasaccharide lipids (biosurfactants) by the bacterial strain Tsukamurella spec. *Lipid/Fett* **101**, 389–394 (1999).

146. Miret, S., De Groene, E. M. & Klaffke, W. Comparison of in vitro assays of cellular toxicity in the human hepatic cell line HepG2. *J. Biomol. Screen.* **11**, 184–193 (2006).

147. Arellanes-Robledo, J., Hernández, C., Camacho, J. & Pérez-Carreón, J. I. In Vitro Models of HCC. *Liver Pathophysiol.* 563–579 (2017).

148. Peng, K. *et al.* Secondary metabolites from a deep-sea-derived actinomycete Micrococcus sp. R21. *Zhongguo Zhongyao Zazhi* **40**, 2367–2371 (2015).

149. Liu, D. *et al.* Microbacterins A and B, new peptaibols from the deep sea actinomycete microbacterium sediminis sp. nov. YLB-01(T). *Org. Lett.* **17**, 1220–1223 (2015).

150. Mohamed, A., Nguyen, C. H. & Mamitsuka, H. Current status and prospects of computational resources for natural product dereplication: a review. *Brief. Bioinform.* 1–13 (2015).