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DAS GRUTAS AO MAR PROFUNDO: A REVELAR OS TESOUROS DOS ACTINOMYCETES EM AMBIENTES EXTREMOS

FROM CAVES TO THE DEEP SEA: REVEALING THE TREASURES OF ACTINOMYCETES IN EXTREME ENVIRONMENTS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica da Professora Doutora Maria de Fátima Carvalho, Investigadora no Centro interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) da Universidade do Porto e o Professor Doutor Newton Carlos Marcial Gomes, Investigador Principal no Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro

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palavras-chave **Actinobacteria, OSMAC, Produtos naturais, Compostos antimicrobianos, Grutas, Mar profundo,**

Resumo

O surgimento da resistência antimicrobiana, juntamente com a ausência de novas descobertas de antibióticos, apresenta um importante desafio para a saúde global no século XXI, à medida que estirpes bacterianas multirresistentes se tornaram comuns em hospitais. As actinobactérias destacam-se como uma fonte promissora de novos antibióticos, uma vez que este diverso grupo de bactérias é conhecido por produzir uma vasta gama de compostos bioativos, com uma especial prevalência de agentes antimicrobianos. As actinobactérias que habitam ambientes extremos exibem uma diversidade taxonómica e genética significante, oferecendo grande potencial para a descoberta de novas vias biossintéticas para a síntese de compostos bioativos. Este trabalho foca-se no estudo de actinobactérias provenientes de ambientes extremos, especificamente do mar profundo e de grutas, em termos da sua biodiversidade e capacidade para produzir novos compostos antimicrobianos.

A abordagem OSMAC, que significa uma estirpe muitos compostos, representa uma estratégia promissora para desbloquear clusters de genes biossintéticos adormecidos e descobrir novos compostos naturais. No primeiro estudo apresentado nesta tese, quatro condições OSMAC foram aplicadas a sete estirpes de actinobactérias previamente isoladas de amostras de mar profundo dos arquipélagos dos Açores e Madeira, com o objetivo de investigar se essas condições poderiam despoletar a produção de compostos antimicrobianos potencialmente novos. Essas condições incluíram a adição ao meio de cultura de: i) um lisado de *E. coli*; ii) sobrenadante resultante do crescimento de *E. coli*; iii) uma solução de N-acetilglucosamina; e iv) uma solução de cloreto de lantânio (III). Os extratos resultantes da abordagem OSMAC foram testados quanto à sua atividade antimicrobiana contra *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 25241) e *Candida albicans* (ATCC 10231). O ensaio antimicrobiano permitiu identificar um extrato de *Streptomyces xinghaiensis* C_003.1.9 cultivado sem condição OSMAC, capaz de inibir o crescimento de *S. aureus* e *B. subtilis*. Os extratos de actinobactérias foram submetidos a análises metabólicas, incluindo a desreplicação baseada em espectrometria de massa e análise de redes moleculares. Estes resultados revelaram a presença de onze compostos naturais conhecidos com relevância farmacêutica e um possível novo composto, no extrato de *Microbacterium ginsengiterrae* C_014 6 (principal composto m/z 747.304), obtido com a adição de uma solução de N-acetilglucosamina. Os resultados obtidos reforçam a necessidade de continuar este estudo, garantindo se estão sendo sintetizados compostos genuinamente novos, e avançando para a etapa subsequente de elucidação química destes compostos.

O segundo estudo apresentado nesta tese teve como objetivo o isolamento e a identificação taxonómica de actinobactérias provenientes da gruta de Vjetrenica, na Bósnia-Herzegovina. Foram obtidos 111 isolados de actinobactérias, utilizando os meios seletivos de cultura SCN e TWYE. Onze géneros diferentes foram identificados, com a maior representatividade de isolados pertencendo ao género *Streptomyces*. Este estudo também identificou três possíveis novas estirpes pertencentes aos géneros *Streptomyces* e *Sphaerisporangium.* Esses resultados sugerem que as grutas são uma excelente fonte de actinobactérias, incluindo novas taxonomias, e um excelente ambiente para a exploração de novos compostos bioativos produzidos por estes microrganismos.

Keywords **Actinobacteria, OSMAC, Natural products, Antimicrobial compounds, Caves, Deep-sea**

abstract

The emergence of antimicrobial resistance, along with the absence of new antibiotic discoveries, presents a major global health challenge in the 21st century, as multidrug-resistant bacterial strains have become common in hospitals. Actinobacteria stand out as a promising source for novel antibiotics since this diverse group of bacteria is known to produce a wide range of bioactive compounds, notably characterized by a significant prevalence of antimicrobial agents. Actinobacteria inhabiting extreme environments exhibit significant taxonomic and genetic diversity, offering great potential for the discovery of new biosynthetic pathways for synthesizing bioactive compounds. This work focuses on the study of actinobacteria derived from extreme environments, namely from the deep sea and caves, in terms of their biodiversity and capacity to produce novel antimicrobial compounds.

The One Strain Many Compounds (OSMAC) approach represents a promising strategy unlocking dormant biosynthetic gene clusters and discovering new natural compounds. In the first study presented in this thesis, four OSMAC conditions were applied to seven actinobacterial strains previously isolated from deep-sea samples collected at the Azores and Madeira archipelagos, with the objective of investigating if these conditions could trigger the production of potentially novel antimicrobial compounds. These conditions included the addition to the culture medium of: i) an *E. coli* lysate; ii) the supernatant resulting from *E. coli* growth; iii) a solution of N-acetylglucosamine and iv) a solution of lanthanum (III) chloride. The extracts resulting from the OSMAC approach were tested for their antimicrobial activity against *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 25241) and *Candida albicans* (ATCC 10231). The antimicrobial assay allowed the identification of an extract from *Streptomyces xinghaiensis* C_003.1.9 grown under no OSMAC condition, capable of inhibiting the growth of *S. aureus* and *B. subtilis*. The actinobacterial extracts were subjected to metabolomic analyses, including mass spectrometry-based dereplication and molecular networking analyses. These results revealed the presence of eleven known natural compounds with pharmaceutical relevance and a possible new compound, from the extract of *Microbacterium ginsengiterrae* C_014 6 (major compound m/z 747.304), obtained under the addition of N-acteylglucosamine solution. The results obtained reassure the need to continue this study, ensuring if genuinely novel compounds are being synthesized, and advancing towards the subsequent stage for the chemical elucidation of these compounds.

The second study presented in this thesis aimed at the isolation and taxonomic identification of actinobacteria from the Vjetrenica Cave, in Bosnia-Herzegovina. A total of 111 actinobacterial isolates were obtained, using SCN and TWYE as selective culture media. Eleven different genera were identified, with the largest fraction of isolates belonging to the *Streptomyces* genus. This study also led to the identification of three possible novel strains of the genera *Streptomyces* and *Sphaerisporangium*. These results suggest that caves are an excellent source of actinobacteria, including novel taxonomies, and are an excellent environment for the exploration of novel bioactive compounds produced by these microorganisms.

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Chapter 1

Introduction

In the last times, the challenge of antibiotic resistance and the crisis in discovering new treatments have stimulated developments in strategies for uncovering natural compounds that can combat life-threatening infections brought by multidrug-resistant pathogens (Álvarez-Martínez et al., 2020). The extensive use of antibiotics to manage infections in human and animal diseases, as well as in agriculture, has created unprecedented circumstances for the dissemination of resistance factors within bacterial populations (Mancuso et al., 2021). As a result, pathogenic bacterial strains with resistance to many of the currently available antibiotics are now consistently identified (Brown & Wright, 2016). Antimicrobial resistance, coupled with the sluggish pace of new antibiotics discoveries, has emerged as a significant global health challenge in the 21st century (Goel et al., 2021).

Since the discovery of actinomycin and streptomycin, soil-dwelling actinobacteria, and most prominently those belonging to the genus *Streptomyces*, have been the main source of natural products with clinical relevance (Santos-Aberturas & Vior, 2022). Actinobacteria constitute one of the most diverse and ubiquitous group of microorganisms in nature and remain a key source for pharmaceutical compounds discovery (Hu et al., 2020). These microorganisms have an exceptional biosynthetic potential, most of which remains unknown, as shown since the entry into the genomic era. They thrive in complex and challenging ecosystems, engaging in diverse interactions with other organisms, where the production of secondary metabolites plays a key role (Santos-Aberturas & Vior, 2022).

Bioprospecting underexploited environments characterized by strong selecting factors has been a viable alternative to find novel strains from unique microbial communities, but also to discover new natural products (Genilloud, 2017).

1.1 Phylum Actinomycetota

Actinomycetota represent one of the largest taxonomic units among the major lineages currently recognized within the Bacteria domain (Barka et al., 2016). Members of this phylum include Gram-positive bacteria with a high guanine and cytosine content (65-75%) in their DNA, giving them more stability when exposed to high temperatures (Gray et al., 2011). Members of this phylum, hereinafter referred to in this thesis as actinobacteria, display the most significant morphological differentiation among gram-positive bacteria (Li et al., 2016). These microorganisms exhibit a wide variety of morphologies, differing in

terms of the presence or absence of a substrate or aerial mycelium, the colour of the mycelium, the production of diffusible pigments, and the structure and appearance of their spores. (Barka et al., 2016; Miao & Davies, 2010).

1.2 Taxonomic Classification of actinobacteria

The taxonomic classification of actinobacteria has changed over time with the evolution of molecular tools. Based on 16S rRNA gene phylogenetic relationships, the phylum Actinomycetota comprises 6 classes, 35 orders and 69 families (https://lpsn.dsmz.de/phylum/actinomycetota).

The 16S rRNA is the central structural component of the bacterial and archaeal 30S ribosomal subunit and is required for the initiation of protein synthesis and correct stabilization of codon-anticodon pairing at the A-site of the ribosome during mRNA translation. Due to the functional constancy and highly conserved nature of the 16S rRNA gene, it has been an important phylogenetic marker in the classification of new microorganisms (Jay & Inskeep, 2015). Gene similarity in 16S rRNA has been considered a significant molecular marker in the taxonomy of prokaryotes due to it being universal, relatively stable, and highly conserved. Consequently, analysis of the 16S rRNA sequences has been kept as a preferable approach for the classification of bacterial isolates (Mohammadipanah & Dehhaghi, 2017). For the 16S rRNA gene similarity, a threshold of 98.7% has been established for the delineation of new species (Stackebrandt, 2006).

1.3 Environmental distribution of actinobacteria

Actinobacteria are ubiquitous in the world, their habitats range from terrestrial, aquatic (including marine and freshwater environments), tidal flat ecosystems, and even extreme environments, like deserts, hot springs, salt lakes, caves and the dee-sea (Qin et al., 2016; Rachid & Doğruöz Güngör, 2023; Rateb et al., 2018). In addition, these microorganisms can establish symbiotic associations with a variety of organisms, such as marine sponges, tunicates, ants, termites, among others (Kurtbóke, 2017).

Deep-sea Actinobacteria

Marine habitats are a rich source of diverse and mostly uncharacterised actinobacteria (Ngamcharungchit et al., 2023). There are many different environmental conditions and oceanographic parameters characteristic of the deep-sea. Pressure increases by 1 atm for every 10 m below sea level, reaching more than 1000 atm in the deepest part of the trenches. Temperatures taper off rapidly with increasing depth down to ~2ºC. Lower temperatures reduce the rates of biochemical reactions, consequently, species inhabiting deep-sea have to adapt their biochemical machinery to cope with such pressures and temperatures (Skropeta, 2008).

The deep-sea is poorly investigated in terms of microbial diversity due to the difficulty in accessing it (Bull et al., 2005). The deep-sea environment comprises a collection of heterogenous habitats including abyssal, plain, hydrothermal vents, continental margins, cold water corals, seamounts, hadal zones and oxygen minimum zones that nest numerous life forms (Paulus, 2021). To ensure survival in this habitat, deep-sea organisms had to develop unique biochemical, metabolic and physiological capabilities, which may also translate into the production of new metabolites absent in terrestrial microorganisms (Kamjam et al., 2017). Studies indicate that deep-sea ecosystems contain a wide range of unique actinobacteria and that these microorganisms are a source of new molecules (Siro et al., 2023). With the breakthrough of technological barriers associated with deep-sea actinobacteria isolation strategies, such as sample collection and cultivation under standard laboratory conditions, more deep-sea actinobacteria and their natural products have been identified (Kamjam et al., 2017). Different families of actinobacteria have been isolated from deep-sea samples, such as Pseudonocardiaceae, Brevibacteriaceae, Rubrobacteraceae, Micrococcaceae, Micromonosporaceae, Streptomycetacae, Microbacteriaceae, Lamiaceae, Nocardiopsaceae and Miltoncostaeaceae (Siro et al., 2023).

Actinobacteria from caves

Though caves have been studied for hundreds of years, their microbiomes are generally unexplored and overlooked. With a combination of unique conditions including high humidity, relatively low and stable temperature, and limited nutrients, caves are expected to shelter novel microorganisms with biotechnological benefits (Barton, 2006). Members of

actinobacteria are reported to be a dominant microbial population of caves ecosystems, with 47 species belonging to 30 different genera of actinobacteria having been reported from cave and cave-related habitats (Rangseekaew & Pathom-Aree, 2019).

The incredibly starved nature of subsurface habitats presumably stimulates unique strategies of indigenous microbiomes, among which secondary metabolism might be one of the key features enabling life in such challenging environments (Bhullar et al., 2012). The observation that actinobacteria, which are among the most prolific producers of secondary metabolites, are also abundant in limestone caves is not likely a coincidence. Therefore, actinobacteria isolated from oligotrophic environments, particularly the rare representatives of the phylum, are expected to own a unique metabolome that could potentially be an important source of novel natural products (Lavoie et al., 2017).

1.4 Actinobacteria as sources of bioactive compounds

Actinobacteria are of great importance in the field of biotechnology, producing a plethora of bioactive secondary metabolites, including many of the clinically used antibiotics and other natural products with pharmaceutical, industrial or agricultural relevance (Jose et al., 2021). These microorganisms are known to produce compounds with a vast range of activities like antimicrobial, anticancer and antiviral compounds, immunosuppressants, herbicides, among others, making them an attractive source of drugs (János, 2005; Newman & Cragg, 2007). Secondary metabolites have been isolated from marine organisms such as *Salinispora* spp., arctic *Streptomyces nitrosporeus*, desert-dwelling *Streptomyces* spp. and species that live in the microbiomes of animals, insects and plants (Jensen et al., 2007; Sayed et al., 2020; A. Yang et al., 2013). Several examples of novel bioactive compounds isolated from actinobacteria are listed in Table 1.1.

Secondary metabolites are mainly produced in the late exponential and/or stationary phases of growth by narrow taxonomic groups of organisms, of which actinobacteria are included. These metabolites show unusual and varied chemical structures, and are often formed as mixtures of closely related members of a chemical family (Moo-Young, 2011). Since these secondary metabolites have no primary functions, for example, as energy source, cell synthesis, or genetic information, understanding the regulation processes is crucial in order to optimize and manipulate the production processes (Hassan et al., 2019). In nature,

secondary metabolites are produced as a survival strategy of the microorganism (Moo-Young, 2011).

Table 1.1 Examples of novel bioactive compounds isolated from actinobacteria. Adapted from Jose et al., (2021) review. a- antibacterial (MIC ≤20 μg mL1); b- antifungal (MIC ≤20 μg/mL); c- cytotoxic or anticancer (IC50 ≤50 μg/mL); D-anti-inflammatory; e- antiplasmodial; f- others (EC50 ≤10 μg/mL).

Microorganism	**Compound**	**Bioactivity**	**Reference**
Nocardia lactamdurans	B-lactam	Antibiotic (a)	(Iacovelli et al., 2020)
Streptomyces griseus	Streptomycin	Antibiotic	(Ishigaki et al., 2017)
Streptomyces rimosus	Oxytetracycline	Antibiotic	(Yin et al., 2015)
Streptomyces lavenduligriseus	5-glycidyl filipin III	Antifungal (b)	(J. Yang et al., 2016)
Actinoalloteichus hymeniacidonis **179DD-027**	Dokdolipid B	Anticancer (c)	(Choi et al., 2019)
Micromonospora yangpuensis **DSM 45.577**	Yangpumicin A	Anticancer	(Yan et al., 2017)
Streptomyces violaceoruber **YIM 101.131**	Violacin A	Anti-inflammatory (d)	(Tokala et al., 2018)
Streptomyces **sp. CMBMQ030**	Naseseazine C	Antiplasmodial (e)	(Shende et al., 2020)
Micromonospora **sp.**	Manzamine A	Antimalarial (f)	(Waters et al., 2014)
Streptomyces koyangensis	Neoabyssomicins F & G	Antiviral	(Jose et al., 2021)
Streptomyces **sp. CB01388**	Herbicidin L	Anti-cryptosporidium	(Ahmed et al., 2018)
Streptomyces **sp. AM-2504**	Virantmycins B	Antiviral	(Cong et al., 2019)
Streptomyces **sp. KIB-H1289**	Lorneic acids F	Antityrosinase	(Yu et al., 2020)
Streptomyces **sp. SBT345**	Ageloline A	Antioxidant	(Cheng et al., 2020)
Streptomyces **sp. SSC21**	Suncheonosides A-D	Antidiabetic	(Shin et al., 2015)
Streptomyces sp. RAN54	Pyrozazone	Neuroprotective	(Bao et al., 2018)

1.5 The OSMAC approach

Actinobacteria are versatile secondary metabolite producers with great application in industries. Nonetheless, the advancement of genomic sequencing techniques has revealed the presence of a large fraction of silent biosynthetic gene clusters in the genomes of these microorganisms, meaning that they are not expressed under laboratory conditions (Abdelmohsen et al., 2015). Triggering the expression of these silent clusters can unlock the

chemical diversity they control, allowing the discovery of novel molecules of both medical and biotechnological interest (Romano et al., 2018).

The OSMAC strategy (One Strain Many Compounds) has proven to be an effective approach to awake and upregulate silent genes within individual strains, leading to the discovery of promising new molecules (Bode et al., 2002). This strategy is based on the alteration of easily accessible cultivation parameters, for example, medium composition, physical-chemical parameters, as temperature and pH, addition of chemical elicitors, co-cultivation, among others, to increase the number of secondary metabolites available from one microbial source (Fig. 1.1). Applying this strategy by altering the fermentation conditions of a given microorganism can expand its genetic expression, increasing its potential as a producer of unusual specialized metabolites that can be exploited (Y. Q. Zhang et al., 2022)

The variation of the culture medium, such as changes in nutrient regimes, and physical parameters like temperature or pH directly affects metabolism (Ngamcharungchit et al., 2023). The utilisation of chemical elicitors also represents a potent approach capable of unlocking the expression of cryptic biosynthetic gene clusters in actinobacteria (Zong et al., 2022). Many of the microbial interactions that elicit the production of bioactive secondary metabolites are underpinned by complex chemical ecology mechanisms relying on diffusible molecules. Classical examples of such mechanisms are autoinducer molecules that modulate quorum-sensing communication processes. Autoinducers are generally used to recognize population cell density while in turn regulating various cellular processes, for example for antibiotic production (Romano et al., 2018b). Another commonly used method is co-cultivation, which is attained by the cultivation of two or more microbial strains. Within their natural environment, actinobacteria are part of diverse microbial communities that include archaea, bacteria, fungi, protists and viruses. These intricate microbial communities have evolved specific interactions, where small molecules, such as secondary metabolites play a pivotal role in mediating relationships between microbial species (symbionts or competitors), including triggering the production of antibiotics (Gray et al., 2011). In the One Strain Many Compounds (OSMAC) methodology, innovative techniques are applied to

awaken dormant gene cluster, consequently elevating the yield of secondary metabolites in microorganisms by adjusting their growth environments (Romano et al., 2018) (Figure 1.1).

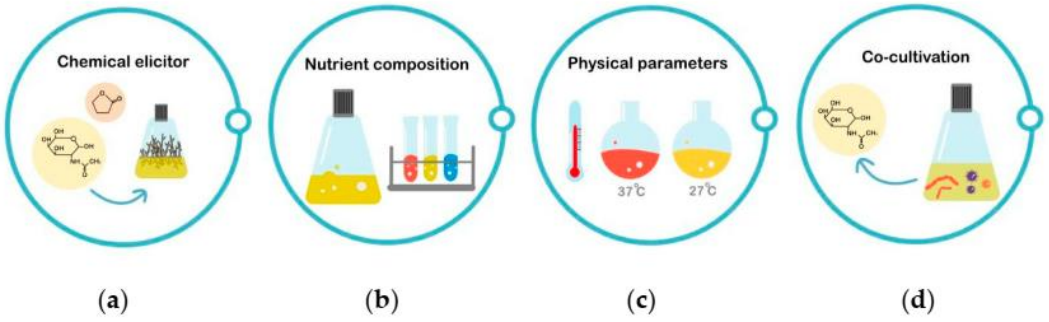

Figure 1.1 The OSMAC methodology (Figure from (Ngamcharungchit et al., 2023)). (a) Chemical elicitors can trigger silent compounds, (b) changing nutrient regimes, (c) altering physical parameters such as temperature, and (d) Co-cultivation. These methods can improve and trigger the production of many secondary metabolites

1.6 Aim and outline of this Thesis

Actinobacteria are renowned for their prolific production of natural products, including a multitude of commercially available antibiotics. With the urgent need to address antibiotic resistance, the discovery of novel antimicrobial compounds from actinobacteria remains a critical frontier in modern medicine. In this way, the bioprospection of extreme environments, which are often less explored, offers a promising avenue for the discovery of new natural products. In this context, the work presented in this master's thesis aimed to study actinobacteria from extreme environments, namely from deep-sea and cave ecosystems, in order to investigate their diversity and potential to produce bioactive compounds with antimicrobial activity.

This thesis is organized into 4 chapters. Chapter 1 consists of a general introduction of the thesis subject, where several aspects related to actinobacteria are described, mainly focusing on deep-sea and cave actinobacteria, production of secondary metabolites and respective bioactivities, and approach to the OSMAC strategy as a way to enhance the discovery of new bioactive compounds. Chapter 2 comprises a study where the OSMAC approach was applied to seven deep-sea actinobacterial strains as a strategy to trigger the production of new antimicrobial compounds. Chapter 3 covers the isolation and taxonomic identification of actinobacteria from a cave in Bosnia. Chapter 4 consists in a general discussion and conclusions about this work. Finally, Chapter 5 comprises the references present in this thesis.

Chapter 2

Screening antimicrobial activity of deep-sea actinobacteria using the OSMAC approach

2. Introduction

The oceans, accounting for over 70% of the surface of the Earth, represent a prolific source of microorganisms with distinct structural, physiological and chemical diversities. The deep-sea biosphere represents 95% of the oceans' microbial biomass, and a source of unique strains that remain to be targeted for drug discovery (Surajit et al., 2006). Life in the deep-sea involves exposure to high hydrostatic pressures and low temperatures, requiring its inhabitants to adapt their genetic, biochemical and physiological processes, presenting unique challenges in terms of gene regulation, structure and function of proteins and other cellular components, as well as metabolism and physiology (Skropeta, 2008). Thus, the exploration of unique ecological niches, like the deep-sea, may lead to the discovery of novel natural products (Ngamcharungchit et al., 2023).

Actinobacteria are a large and diverse group of bacteria known to produce a wide range of secondary metabolites, many of which with relevant biological activities, including antimicrobial, anticancer, antiviral or immunosuppressant activities (Alwali & Parkinson, 2023). These microorganisms alone account for more than half of the antibiotics derived from the microbial world (Bérdy, 2012). The remarkable capacity of actinobacteria to synthesize potent antimicrobial compounds offers a valuable resource to help tackling the global health problem of multidrug resistance. As the prevalence of multi-antibiotic-resistant pathogens continues to rise, there is a growing need for innovative antibiotics to effectively combat this escalating crisis (Miethke et al., 2021).

Actinobacteria inhabiting unique environments with different environmental constraints are considered resources for novel compounds to be investigated in drug discovery programs (Hussain et al., 2020). New marine actinobacteria have been discovered, and some produce chemical diversity not observed in terrestrial actinobacteria, like the genus *Salinispora*, which has an obligate requirement for seawater for growth (Baltz, 2008; Fenical & Jensen, 2006). Nonetheless, many interesting bioactive compounds await to be discovered, since they cannot be produced, or are produced at very low levels when grown in laboratory conditions. This limitation is likely attributed to the absence of the specific chemical triggers that these bacteria encounter within their environments (Alwali & Parkinson, 2023).

In the One Strain-Many Compounds (OSMAC) methodology, new approaches are employed to activate silent gene clusters, thereby enhancing the production of secondary metabolites

in microorganisms by modifying their growth conditions (Romano et al., 2018). Through these alterations, the OSMAC approach can stimulate the synthesis of new or low-yield secondary metabolites. By employing an OSMAC strategy, Gamaleldin et al. (2020) identified the production of Desferrioxamine B, a compound with potential to remove excess iron from patients with transfusion-dependent hemoglobin disorders, and Bafilomycin D, an antitumor, anti-inflammatory and anti-viral compound, both produced by actinobacteria.

In light of the above, the work presented in this chapter aimed to apply the OSMAC strategy to seven actinobacterial isolates previously obtained from several deep-sea samples collected at the Madeira and Azores archipelagos, with the ultimate goal of searching for new compounds with antimicrobial activity.

2.2. Materials and Methods

2.2.1 Actinobacterial strains

Seven actinobacterial strains previously isolated from several deep-sea samples were used for this experiment. The taxonomy of these strains, along with details regarding their isolation sources, is indicated in Table 2.1.

Table 2.1 Information relative to the actinobacterial strains used in this study

Strain	Taxonomic identification	Geographic origin	Marine matrix	Type of collection	Depth (m)	Isolation medium	Growth medium	Campaign
Sed_044 18	*Brevibacterium antiquum*	Azores	Sediment	ROV	2076	[a)]M1	M1	[c)]M150 BIODIAZ
C_192 3	*Microbacterium aerolatum*	Azores	Coral	ROV	1535-1557	M1	M1	M150 BIODIAZ
C_014 6	*Microbacterium ginsengiterrae*	Madeira	Coral	Lula1000 manned submersible	804	M1	M1	[d)]Deep_Madeira 2019
C_003 1.9	*Streptomyces xinghaiensis*	Madeira	Coral	Biobox	1980	[b)]MA	MA	[e)]OOM2018_LUSO
C_084 6	*Micromonospora* sp.	Madeira	Coral	Biobox	600	MA	MA	OOM2018_LUSO
C_063 8	*Salinispora goodfellowii*	Azores	Coral	Agassiz Trawl	1501-1516	M1	MA	M150 BIODIAZ

S_021 1	*Tsukamurella tyrosinosolvens*	Madeira	Sponge	Lula1000 manned submersible	463	M1	M1	Deep_Madeira 2019

a) Composition of medium M1: 10 g starch, 4 g yeast extract, 2 g peptone, 15 g agar and 1L seawater.

b) Composition of medium MA: 55,2 g marine agar and 1 L deionized water.

c) Financed by DFG-Senatskommission für Ozeanographie.

d) Financed by Direção Regional do Ordenamento do Território-DROTA, Madeira.

e) Organized by Observatório Oceânico da Madeira.

2.2.2 OSMAC strategies used and growth conditions

Each actinobacterial isolate selected for this study was grown under four different conditions, consisting in the addition to the culture medium of: (i) lysate of *Escherichia coli*; (ii) supernatant from an *Escherichia coli* culture; (iii) N-acetylglucosamine and (iv) lanthanum (III) chloride. All strains were grown in 100 ml Erlenmeyer flasks containing 30 ml of the respective liquid culture medium, under the different OSMAC conditions detailed below. The inoculum of the selected actinobacterial strains was obtained by cultivating each of them in the respective agar medium. A loopful of each isolate was then used to inoculate the 100 ml Erlenmeyer flasks. The flasks were incubated in an orbital shaker (Model 210, Comecta SA, Barcelona, Spain) at 28ºC, 100 rpm in the dark for 5 days. After this period, 0.5g of Amberlite® XAD16N resin (Sigma-Aldrich, MO, United States) was added to each Erlenmeyer, to adsorb the metabolites, and the flasks were incubated for an additional period of two days (Ribeiro et al., 2020).

For each OSMAC condition, two sets of controls were also established, one consisting in uninoculated cultures and another consisting of cultures grown under standard conditions, i.e., without the application of any OSMAC condition.

2.2.2.1 Escherichia coli lysate

E. coli strain ATCC 25922, pre-grown on Mueller-Hinton agar (MH), was used to inoculate 2 litre Erlenmeyer flasks, containing 1 liter of Mueller Hinton culture medium (MH, Liofilchem, Roseto d. Abruzzi, Italy). The flasks were then incubated at 37ºC for a duration of 3 days with continuous agitation at 100 rpm. After this incubation period, the culture was harvested and subsequently centrifuged at 3046 g for 10 minutes to separate the *E. coli* biomass pellet from the supernatant. The biomass pellet was subjected to lyophilization under vacuum conditions (0.058 mbar) at -55 ºC for 2 days. Finally, 1.5 grams of the

lyophilized *E. coli* biomass were added per litre of culture medium and the mixture was autoclaved to produce an *E. coli* lysate.

2.2.2.2 *E. coli* supernatant

To obtain *E. coli* supernatant, *E. coli* ATCC 25922 was grown in MH culture medium using the previously mentioned procedure. The collected supernatant was initially frozen at -80ºC for 24 hours and subsequently subjected to lyophilization under vacuum conditions (0.058 mbar) at -55ºC for 4 days. To prepare the culture media, 1.5 grams of the lyophilized supernatant were added to each litre of culture medium, followed by sterilization in an autoclave.

2.2.2.3 Solution of N-acetylglucosamine

A solution of N-acetylglucosamine (Santa Cruz Biotechnology, Inc., Dallas, United States) at 400 mM was prepared in deionized water (Rigali et al., 2008). This solution was filtered through a 0.22 µm sterile filter and added to the respective culture medium. The resulting concentration of the compound in the medium was 50 mM.

2.2.2.4 Solution of Lanthanum (III) chloride

A solution of Lanthanum (III) chloride (Merck KGaA, Darmstadt, Germany) was prepared in deionized water at a concentration of 200 mM (Xu et al., 2018). This solution was filtered through a 0.22 µm sterile filter and added to the respective culture medium. The resulting concentration of the compound in the medium was 2 mM.

2.2.3 Preparation of crude extracts

After the incubation period of the OSMAC cultures and the respective controls, the content of each flask was centrifuged at 3600 g for 10 minutes. The resulting supernatant was removed, and the pellet was washed twice with distilled water. The obtained pellet (consisting of biomass and resin) was freeze-dried for two days and extracted with a 1:1 (v/v) mixture of acetone and methanol. The pellet was immersed in the mixture (30 mL) for 30 minutes, with constant agitation. The liquid phase was collected in round bottom flasks and was filtered through a Whatman No 1 filter paper. The process was repeated twice and the extract was dried in a rotary evaporator and transferred to 8 mL vials. The organic extract

used for the antimicrobial assay was then dissolved in DMSO (DMSO ≥ 99.9%, Sigma-Aldrich, Mo, United States) to obtain stock solutions with final concentrations of 1 and 10 mg/mL.

3.2.4 Screening of Antimicrobial Activity

Antimicrobial activity of the actinobacterial crude extracts was assessed through the agar-based disk diffusion method, using five reference microorganisms: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 25241) and *Candida albicans* (ATCC 10231). The bacterial strains were grown on Mueller-Hinton agar (MH) (Liofilchem, TE, Italy), while the yeast, *C. albicans*, was grown on Sabouraud Dextrose agar (SD) (Liofilchem, TE, Italy). After growth, the microorganisms were suspended in their respective broth media and the turbidity of the cultures was adjusted to 0.5 McFarland standard (OD625= 0.08-0.13). These standardized suspensions were used to inoculate MH or SD agar plates, by evenly streaking the plates with a swab soaked in the inoculum cultures. Subsequently, 6 mm antibiotic blank discs (MH, Liofilchem, Roseto d. Abruzzi, Italy), were placed on the surface of the inoculated plates and loaded with 15 µL of the crude extract at the concentration of 1 mg/mL. As positive controls, 15 µL of enrofloxacin (1 mg/mL) were used for the bacterial strains, while 15 µL of nystatin (1 mg/mL) was used for the yeast. The negative control consisted of 15 µL of DMSO. All assays were conducted in duplicate. The agar plates were then incubated at 37 °C for 24 h, after which the presence of inhibition halos was examined and the respective diameters were measured.

2.2.5 Dereplication and Molecular Network Analysis

All extracts were submitted to metabolomic analysis, namely MS-based dereplication and molecular networking analyses. The extracts were resuspended in methanol at a concentration of 1 mg/mL and used for LC-HRESIMS/MS analysis on a system composed of a Dionex Ulimate 3000 HPLC coupled to a qExactive Focus Mass spectrometer controlled by XCalibur 4.1 software (Thermo Fisher Scientific, MA United States). The chromatographic step was carried out in an ACE UltraCore 2.5 Super C18 column (75 mm x 2.1 mm, Avantor, Radnor, Pennsylvania). Five microliters of each extract were injected and separated using a gradient from 99.5% eluent A (95% water, 5% methanol, 0.1 formic

acid, v/v) and 0.5% eluent B (95% isopropanol, 5% methanol, 0.1 formic acid, v/v) to 10% eluent A and 90% eluent B, over 9.5 min and then maintained for 6 min before returning to initial conditions. The UV absorbance of the eluate was monitored at 254 nm and at Full MS scans, at the resolutions of 70.000 full-width at half maximum (FWHM) (range of 150-200 m/z), followed by data-dependent MS2 (ddMS2, Discovery mode) at the resolution of 17.500 FWHM (isolation window used was 3.0 amu and the normalized collision energy was 35) was carried out.

The raw data obtained from the dereplication of the extracts was converted to mzML format and submitted to the dereplication analysis tools of the Global Natural Products Social Molecular Networking platform (GNPS), including the Insilico Peptidic Natural Product DEREPLICATOR, DEREPLICATOR VarQuest and DEREPLICATOR+, with default parameters, excluding ion mass precursor tolerance and fragment ion mass tolerance (set to 0.005 Da). For results annotated by the GNPS platform a threshold p-value $\leq 10^{-10}$ for DEREPLICATOR and DEREPLICATOR VarQuest, and a strict value of 15 for DEREPLICATER+ were applied to minimize false matches. Each annotation was manually checked to determine their association with compounds produced by actinobacteria and/or marine organisms (sponges and corals) and eliminated if they did not satisfy these criteria (Gurevich et al., 2018; Mohimani et al., 2017; Mohimani et al., 2018). For the DEREPLICATOR VarQuest, only putative masses in which their fragmented ions had a mass error between the range of 5 ppm when compared with the annotated fragments of natural products peptide table in the database, were considered. For the DEREPLICATER+, all the hits shown in the database were manually checked in the spectra from each corresponding extract, in Xcalibur™ software, and compared between the strain with the OSMAC condition and the respective controls.

To complement all dereplication data, molecular networking was performed using default parameters (except ion mass tolerance precursor, which was set to 0.02 Da and fragment ion mass tolerance, which was set to 0.02 Da, to account for high-resolution data). The resulting molecular network was visualized with Cytoscape v3.6.1 and searched for clusters of m/z data that were associated with a single extract. The HRESIMS/MS spectra corresponding to such data were annotated to clarify the likely masses of the compounds generating the clusters. These putative compound masses were searched in the Dictionary of NP (version 27.1) database (the range for accurate mass was 10 ppm). When hits from actinobacteria

and/or marine organisms (sponges and corals) were obtained, information regarding the biological activity of the compounds was retrieved from this database.

2.3 - Results

2.3.1 Antimicrobial Activity of Deep-Sea Actinobacteria Grown under OSMAC Conditions

The antimicrobial screening tests revealed that none of the OSMAC conditions examined proved effective in stimulating the production of secondary compounds with antimicrobial properties in the tested deep-sea actinobacteria. The only extract that showed bioactivity was derived from *Streptomyces xinghaiensis* C_003.1.9 grown in MA medium under standard conditions. This extract inhibited the growth of both *Staphylococcus aureus* and *Bacillus subtilis* (Fig. 2.1), producing inhibition halos of 10 mm and 12 mm, respectively.

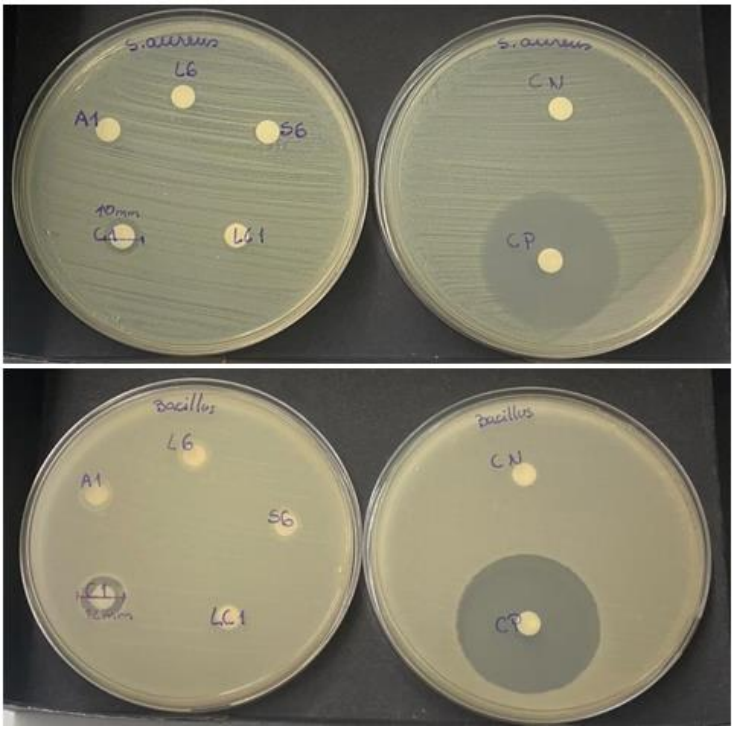

Figure 2.1 Antimicrobial activity of the actinobacterial extract derived from *S. xinghaiensis* strain C_003.1.9. The diameter of inhibition halos was determined by the agar disk diffusion method. The inhibition halos were observed in extract C1, derived from the growth of *S. xinghaiensis* in MA medium without the application of an OSMAC condition. On the right side of the image are shown the agar plates used as controls, being the negative control (CN) DMSO and the positive control (CP) enrofloxacin.

2.3.2 Dereplication analysis of the actinobacterial extracts

To understand if the OSMAC approaches employed in this study led to the differential production of secondary metabolites, all 44 extracts were dereplicated by LC-HRESIMS/MS and the raw data was subsequently analysed with the software Xcalibur^{TM}. The putative compound masses were used for three dereplication steps: Insilico Peptidic Natural Product Dereplicator, Dereplicator+ and by searching the predicted accurate mass against the Dictionary of NP database. After analysing all annotations related to natural products produced by actinobacteria and/or marine organisms, it was possible to find matches with some analogues of peptide metabolites and other natural products with antibacterial and cytotoxic activity. Tables 2.2 and 2.3 show the results obtained with the Insilico Peptidic Natural Product Dereplicator varquest and Dereplicator+, respectively.

Table 2.2 - Actinobacterial secondary metabolites potentially present in the tested extracts, according to the database Dereplicator +

Strain	Growth Condition	Compound	Score	Metabolite mass	FDR%	Adduct	Biological activity	Origin
***Microbacterium ginsengiterrae* C_14_6**	N-acetylglucosamine	Landomycin A	17	1068.46	0	M+2H	Angucycline antibiotic	Produced by *Streptomyces cyanogenus* S-136 (DSM 5087)
***Salinispora goodfellowii* C_063.8**	*E. coli* lysate	Salinilactam A	16	469.283	0	M+H	Bacteriostatic antibiotic drug	Produced by a strain of *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*)
***Brevibacterium antiquum* Sed_44_18**	Lanthanum (III) chloride	Demethylenenocardamine	17	586.33	0	M+H	Inhibits WNT[(a)] signalling	Produced by marine-derived *Streptomyces* sp. strain M1087 and soil-derived actinomycete CKK784

(a) Signal transduction pathways of cells to cell surface receptors.

Table 2.3 - Analogous peptides potentially present in the tested extracts, according to the database Dereplicator varquest

Strain	Growth Condition	Compound	Score	P-value	Peptide mass	FDR %	Biological activity	Origin
Microbacterium aerolatum C_192.3	N-acetylglucosamine	Sch 486058	10	4,70E-18	1530.76	5.88	Antibiotic	Produced by *Actinomyces* sp. SCC 2186
Brevibacterium antiquum Sed_44_18	M1 medium (No OSMAC condition)	Phakellistatin 2	10	1,40E-26	827.458	7.69	Cytotoxic	Constituent of the sponges *Phakellia costata* and *Stylotella aurantium*
Mricrobacterium sp. C_192.3	M1 **medium (No OSMAC** condition)	Rolloamide A	12	1,40E-32	763.463	9.09	Cytotoxic	Constituent of *Eurypon laughlini*
Microbacterium ginsengiterrae C_14_6	M1 **medium (No OSMAC** condition)	Callyaerin H	10	6,20E-28	1043.62	8.57	Cytotoxic	Isolated from *Callyspongia aerizusa*
Tsukamurella tyrosinosolvens S_21.1	M1 medium (No OSMAC condition)	ActinomycinPip	10	4,40E-23	1298.65	0	antibacterial and antitumor	Produced by *Streptomyces antibiotucs*
Tsukamurella tyrosinosolvens S_21.1	***E. coli*** lysate	Axinastatin 4	10	1,70E-26	806.469	7.5	Cytotoxic	Isolated from the sponge *Axinella cf. Carteri*
Micromonospora sp. C_84.6	Lanthanum (III) chloride	Antibiotic A54145	11	1,90E-23	1643.77	0	Depsipeptide antibiotic	Produced by *Streptomyces huawensiis*
Tsukamurella tyrosinosolvens S_21.1	Lanthanum (III) chloride	Phakellistatin_1	11	4,70E-30	827.458	10	Cytotoxic	Constituent of the sponges *Phakellia costata* and *Stylotella aurantium*

From the analysis of Tables 2.2 and 2.3, it is possible to observe that some growth conditions led to the differential expression of secondary metabolites known to be produced by actinobacteria. This is the case of the isolate *Micromonospora* sp. C_084.6 that produced the compound Antibiotic A54145, when grown in the presence of Lanthanum (III) chloride. This compound is a depsipeptide antibiotic, primarily described to be produced by *Streptomyces huawensiis*. The addition of *E. coli* lysate to the culture medium, led *Salinispora goodfellowii* C_063.8 to putatively produce the antibiotic Salinilactam A, known to be produced by a strain of *Saccharopolyspora erythraea*. The isolate *Brevibacterium antiquum* Sed_44.18 produced two secondary metabolites under different growth conditions. When grown in M1 culture medium under no OSMAC condition, the cytotoxic peptide Phakellistatin 2 was identified in the crude extract of this culture, while growth with Lanthanum (III) chloride led to the production of Demethylenenocardamine, a natural compound produced by a marine-derived species of *Streptomyces*, strain M1087, and a soil-derived actinomycete, strain CKK784, and known for its capacity to inhibit WNT signalling, a pathway that regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development (Komiya & Habas, 2008). Similarly, *Microbacterium aerolatum* strain C_192.3 produced the peptide Rolloamide A, a cytotoxic compound previously described from a marine sponge *Eurypon laughlini*, when cultivated in M1 medium without the application of any OSMAC condition, but the addition of N-acetylglucosamine to the culture medium, triggered the production of the antibiotic Sch 486058. For *Microbacterium ginsengiterrae* C_14.6, growth in M1 medium under standard conditions, i.e., under no OSMAC conditions, let to the identification in the respective crude extract of Callyaerin H, a peptide with a potent cytotoxic activity. However, when N-acetylglucosamine was added to the medium, the antibiotic Landomycin A was detected in the extract. Two of the four applied OSMAC conditions led *Tsukamurella tyrosinosolvens* strain S_021.1 to produce the cytotoxic agents, Axinastatin 4 and Phakellistatin 1. This was observed when this isolate was grown in the presence of *E. coli* lysate and lanthanum (III) chloride, respectively. In addition, when this strain was grown under no OSMAC conditions, a macrolide antibiotic with high antibacterial and antitumor activity, Actinomycin Pip, was detected by LC-HRESIMS/MS. In spite of most of the matched metabolites being recognised for their significant antimicrobial activities, these were not observed in our extracts.

Regarding the only isolate, which crude extract exhibited antimicrobial activity against *B. subtilis* (ATCC 6633) and *S. aureus* (ATCC 29213) when grown without the application of an OSMAC condition (*Streptomyces xinghaiensis* C_003.1.9), no compounds were identified in the GNPS database that could explain this activity, indicating the potential presence of a new secondary metabolite.

2.3.3 Molecular Networking Analysis

To complement the dereplication analysis, a molecular network was constructed using the workflow of the GNPS platform. We identified a total of eight clusters exclusive to a single extract from an OSMAC condition, which we designated as the primary criterion to enhance the likelihood that a cluster represents an unknown compound. Regarding the single-strain clusters, three were identified in the extract obtained from *Microbacterium ginsengiterrae* C_014 6, one in the extract from *Streptomyces xinghaiensis* C_003.1.9, one in the extract from *Micromonospora* sp. C_084.6, one in the extract from *Tsukamurella tyrosinosolvens* S_021.1 and another one in the extract *Microbacterium aerolatum* C_192.3, all obtained in the OSMAC condition addition of N-acetylglucosamine. It was also possible to find another single-strain cluster in the extract obtained from *Salinispora goodfellowii* C_063.8 when the addition of E. coli lysate was tested as an OSMAC condition.

The extracted ion chromatogram (EIC) for each m/z value in all single-strain clusters was evaluated to assess their relative abundance and search for the presence of shared adducts ions, such as hydrogen and sodium. After this analysis, the most abundant m/z values in each cluster were used to calculate an accurate mass to be used as a query in the Dictionary of NP database (version 27.1). For six clusters, the *m/z* 459.169, *m/z* 322.105, *m/z* 289.158, *m/z* 526.286 and *m/z* 539.198 values, match the exact masses of known natural products, such as Napyradomycin, Antibiotic FR900482, Trichostatin A, Tyrostatin, Opantamycin, respectively. For the cluster obtained from *Salinispora goodfellowii* C_063.8 (addition of *E. coli* lysate, as OSMAC condition), the *m/z* 470.29 value corresponds to the exact masses of the Micromonolactam and Salinilactam A compounds. For the cluster from the extract of *Microbacterium ginsengiterrae* C_014 6 (major compound m/z 747.304), no matches were identified in the dereplication process, suggesting that this cluster could potentially represent a novel metabolite. The chromatographic peak corresponding to the most abundant m/z value in the cluster indicated

that, under the employed OSMAC conditions, it is likely to be produced in substantial quantities, to allow for its isolation after scaling up (Fig. 2.2)

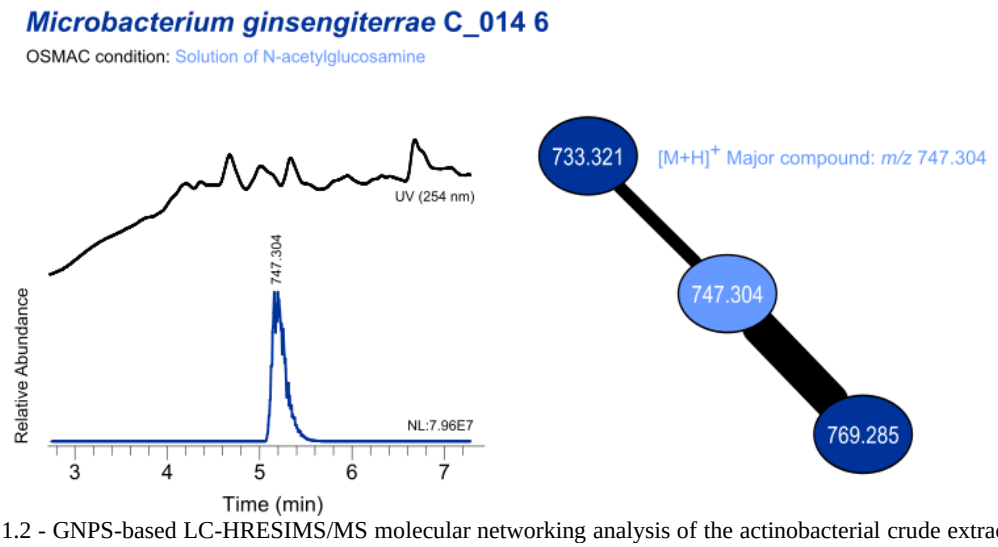

Figure 1.2 - GNPS-based LC-HRESIMS/MS molecular networking analysis of the actinobacterial crude extract from *Microbacterium gingengiterrae* C_014 6, obtained under the addition of N-acetylglucosamine solution, as OSMAC condition, which probably contains a new natural product. Single-strain molecular cluster represented by connected ellipses and marked with the corresponding *m/z* value. The major compound is determined from the UV chromatogram and EICs and is depicted in a distinct colour compared to the other nodes in the cluster. The NL (normalization level) value corresponds to the base peak intensity of the m/z value relative to the mass composition of the actinobacterial crude extract.

2.4 - Discussion

The rising prevalence of antibiotics resistance poses a significant threat to public health, being extremely urgent to discover new antibiotics that can be used as an alternative to combat infectious agents. In this respect, natural products of microbial origin are an important source of leads for the development of new antimicrobial compounds. Within the realm of bacteria, actinobacteria account for a significant market share in commercial antibiotics, making them an invaluable source for the development of these molecules (Jose & Jha, 2016). Nonetheless, a significant hurdle in the quest for novel secondary compounds produced by actinobacteria lies in the limited expression of their biosynthetic genes during standard laboratory cultivation (Zhang et al., 2016). In an attempt to circumvent this problem, the OSMAC approach was applied in this study to seven actinobacterial strains previously isolated from several deep-sea samples collected in the Azores and Madeira, in order to investigate if this strategy could trigger the production of potentially new antimicrobial compounds. The OSMAC strategies used took into account successful results reported in other studies. For example, the addition of N-acetylglucosamine to the culture medium led to the production of actinorhodin, an antibiotic produced by *Streptomyces coelicolor* A3 (2)(Magdalena et al., 2012). N-

acetylglucosamine is described to disrupt metabolic signals controlling antibiotic biosynthesis and trigger the production of natural compounds. The supplementation of the culture medium with the rare element, lanthanum (III) chloride, has been shown to enhance the production of secondary metabolites in several actinobacterial strains, leading to the production of compounds such as urauchimycin D, an antibiotic produced by a streptomycete isolate B1751 (Abdelmohsen et al., 2015; Zhang et al., 2022). We also employed two other OSMAC strategies, which consisted in the addition to the culture medium of the supernatant or dead cells of *E. coli*. In particular, the latter strategy is reported to induce the production of several compounds by actinobacteria, as is the case of undecylprodigiosin (Mavituna et al., 2016).

However, it was found that none of the OSMAC conditions applied caused the intended effect, with the only actinobacterial extract exhibiting antimicrobial activity being derived from a culture grown under standard conditions, i.e., without the application of an OSMAC condition. Nonetheless, the results of the dereplication process using LC-HRESIMS/MS revealed that some OSMAC approaches tested in this study led to the production of previously identified antibiotics and cytotoxic agents, which would not have been attainable by solely growing the tested strains on their regular culture medium, emphasizing the importance of adjusting culture conditions to promote the production of different secondary metabolites (Martín-Aragón et al., 2023). In spite of these compounds being detected in the extracts, this was not reflected in the antimicrobial assay, possibly because these compounds were produced in limited amounts, making them challenging to exhibit significant activity (Selim et al., 2021). Notably, the crude extract obtained from *Streptomyces xinghaiensis* strain C_003.1.9 with antimicrobial activity against *B. subtillis* (ATCC 6633) and *S. aureus* (ATCC 29213), when grown without the application of an OSMAC condition, showed no compounds in the dereplication process that could explain this bioactivity, being possible that it might be producing a new secondary metabolite.

The construction of a molecular network allows grouping together sets of spectra of related molecules to form clusters, even when the spectra do not correspond to known compounds (Wang et al., 2016). In the case of the crude extract from *Microbacterium ginsengiterrae* C_014 6, grown in the presence of N-acetylglucosamine, a unique cluster with a m/z 747.304 value was identified and no matches were found in the dereplication process, suggesting that this cluster can potentially represent a novel metabolite. Given

the relatively high relative abundance observed in the crude extract chromatogram, it appears feasible to pursue the isolation and elucidation of the chemical structure of this compound in the future. For the clusters identified in the other extracts, the respective masses were confirmed to match those of previously identified natural products contained in the Dictionary of NP database.

While the OSMAC approach adopted in this study did not lead to the identification of compounds with antimicrobial activity, the chemical dereplication and molecular network analysis of the extracts have shown several secondary metabolites produced with biological activity, and even potentially new compounds. Since actinobacteria are highly prolific in producing secondary metabolites with many bioactivities, including antibacterial, antifungal, anticancer, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malaria, antiviral and antioxidant, it would also be important to test the actinobacterial extracts produced in this study against these bioactivities.

While the OSMAC strategy implemented in this study did not yield the desired outcomes, its inherent value as a tool for discovering novel bioactive compounds remains unquestionable (Abdelmohsen et al., 2015). To further explore the potential of the actinobacterial isolates analysed in this study for producing new antimicrobial compounds, additional OSMAC approaches could be employed, including the addition to the culture medium of the supernatant or lysed biomass from different agents into the culture medium, aside from *E. coli*. Another highly promising avenue involves genome sequencing of these isolates to assess the presence of biosynthetic genes responsible for the production of novel molecules.

Chapter 3

Isolation and Taxonomic Identification of Actinobacteria from Vjetrenica Cave, Bosnia and Herzegovina

3.1 - Introduction

Spread all over the world, caves are characterised by limited availability of organic matter, low levels or even absence of light, variable levels of humidity, lack of or limited connectivity to the surface and low or high temperatures. Besides all these variable conditions that can be found in caves, these nutrient-limited ecosystems can also be impacted by the surface and groundwater, exposure to humans or other animals and the concentration of minerals on the rock surface (Ghosh et al., 2017). Many caves are associated with karst topography, which is formed by the dissolution of soluble bedrock, such as limestone, dolomite and gypsum, in areas where groundwaters are unsaturated with the minerals present in the surrounding host rock (Zhu et al., 2022).

Despite the challenging oligotrophic conditions found in caves, where nutrient concentrations are typically less than 2 mg per litre, bacteria are the dominant contributors to cave biodiversity and play a pivotal role in sustaining cave ecosystems. On average, their abundance reaches an impressive 10^6 cells per gram of rock (Barton and Jurado, 2007). The scarcity of nutrients and energy sources fosters an oligotrophic environment within these caves, where autotrophic bacteria undertake primary production, thereby sustaining the growth of various chemoorganotrophic microorganisms (Sanchez-Moral & Soler, 2001). Bacteria present under this oligotrophic environment often survive by modifying their metabolism (De Mandal et al., 2017). Cave microorganisms encompass a wide range of bacterial groups influenced by the cave's geology, soil or sediment characteristics.

Actinobacteria are renowned for their remarkable ability to produce an extensive array of secondary metabolites, many of which exhibit a multitude of biological activities (Selim et al., 2021). These microorganisms exhibit a broad distribution across terrestrial and aquatic environments, and they are notably abundant inhabitants of cave ecosystems as well (Ghosh et al., 2017). In spite of their abundance in caves, actinobacteria remain underexplored and poorly understood in terms of their community structure, diversity, biological activities, and mechanisms of environmental adaptation (Farda et al., 2022). These environments hold significant promise for the discovery of rare and novel actinobacterial species, offering exciting opportunities to unveil unique phylogenetic lineages and uncover new bioactive compounds with biological significance (Zada et al., 2022).

This chapter describes the isolation and taxonomic characterization of Actinobacteria inhabiting the Vjetrenica cave, in Bosnia- Herzegovina. The Vjetrenica cave, situated in the southern region of the Dinaric Karst, to the west of Popovo polje (Eastern Herzegovina), is a complex cave system stretching over 7,013.90 meters. Renowned as one of the world's most prominent biodiversity hotspots for cave-dwelling fauna, it serves as a type locality for 39 endemic taxa (Ozimec, & Lučić, 2010). As for December 2009, the cave's fauna inventory encompassed 219 taxa, comprising 37 protists and 182 animals, with 101 taxa adapted to cave life (comprising 49 troglobites and 52 sigobites) (Ozimec, & Lučić, 2010). An update to this inventory, published by Lučić (2019), revealed that Vjetrenica Cave now hosts more than 240 taxa, further underscoring its ecological significance and potential for discovery. Being one of the world's most prominent hotspots of biodiversity in terms of cave-dwelling fauna, this cave is also of great promise in what regards Actinobacteria biodiversity and its associated biotechnological potential. To the best of our knowledge there are no studies available on the isolation and identification of Actinobacteria within this remarkable cave system.

3.2 - Materials and Methods

3.2.1 - Sampling

Three different samples were collected from the Vjetrenica Cave, in August 2022. The first sample was a microbial biofilm present on the cave wall, close to the entrance of the Ponor Kovači cave (latitude 43°40'34.2"N; longitude 17°11'11.5"E), being named PK1. A sample of moonmilk was also collected in the lake Gornja Jakovljeva špilja (latitude 44°03'02.1"N; longitude 16°34'55.8"E), being named GJ1. Also, a black biofilm growing on a chair that has been in the cave for over a year was sampled near Hajdučki stol (latitude 42°50'45.21''N; longitude 17°59'1.68''N), being named V5. The samples were collected aseptically, frozen at -20 ºC, and transported and conserved at this temperature until its processing in the laboratory.

3.2.2 - Bacterial isolation

For actinobacterial isolation, 0.1 g of each sample was added separately to 500 µL of sterile saline solution and subjected to a pre-treatment consisting in incubation at 60 ºC in a water bath for 15 min. This pre-treatment aimed to inhibit undesired, fast-growing

bacteria and create suitable conditions for the selection of rare, slow-growing actinobacteria. Following pre-treatment, the samples were vortexed at maximum velocity for 1 min to ensure homogeneity. The samples were ten-fold diluted until 10^{-4} and an aliquot of 100 µl of each sample was spread on the surface of two different selective media: (i) Starch Casein Nitrate agar: starch, 10 g; casein, 0.3 g; KNO3, 2 g; NaCl, 2 g; K_2HPO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; FeSO4·7H2O, 0.01 g; agar, 17 g; dH_2O 1 L (Küster & Williams, 1964); and (ii) Tap Water Yeast Extract agar: yeast extract, 0.25 g; K_2HPO_4, 0.5 g; agar, 18 g; tap water 1 L (Coombs & Franco, 2003). The two media were supplemented with cycloheximide, nalidixic acid and nystatin, at 50 mgL^{-1} each, in order to inhibit the growth of fungi and Gram-negative bacteria. The plates were incubated for six weeks at a temperature of 28 °C. Every colony was purified in the medium where they were spotted. Pure cultures were cryopreserved at -80 ºC, in 20% (v/v) glycerol. The purified samples were also collected for DNA extraction, on collection tubes with 200 µL of TE buffer and kept at -20 ºC until extraction.

3.2.3 - Taxonomic identification of the isolates

All isolates were taxonomically identified through 16S rRNA gene sequencing. For each isolate, genomic DNA was extracted using the E.Z.N.A. Bacterial DNA kit (Omega, Biotek, Norcross, GA) according to the manufacturer's recommendation. The 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Weisburg et al., 1991). The PCR mixture (final volume of 10 µL) consisted in: 5 µL of MyTaq™ DNA Polymerase (Bioline, Meridian Bioscience), 1 µL of each primer, 2 µL of DNA template and 1 µL of nuclease-free water. The reaction was started with an initial denaturation at 95 ºC for 15 min followed by 30 cycles of denaturation at 94 ºC for 30 s, annealing at 48 ºC for 90 s, extension at 72 ºC for 90 s, and a final extension at 72 ºC for 10 min. PCR products were separated on a 1.4% agarose gel containing SYBR Safe (ThermoFisher Scientific, USA). Purification and sequencing of the samples was performed by GenCore, I3S (Instituto de Investigação e Inovação em Saúde, Portugal). The obtained 16S rDNA sequences were analysed using the Geneious software, version 2022.2.1. The taxonomic affiliation of the isolates was established using the 16S ribosomal RNA (bacteria and Archaea) database from NCBI BLAST tool

(https://blast.ncbi.nlm.nih.gov/Blast.cgi) and confirmed using the Identify tool from EzTaxon (http://www.ezbiocloud.net/).

For some strains, phylogenetic trees were constructed by aligning the sequences of each strain with their ten closest type strain sequences in Genbank, using the MUSCLE alignment tool from the Geneious software. The alignment was then used to generate a maximum likelihood phylogenetic tree with 1000 bootstraps, using the Molecular Evolutionary Genetics Analysis Program Version 11.0 (Kumar et al., 2018).

3.3 - Results

In this study, we isolated a total of 111 actinobacterial strains from the three samples collected from the Vjetrenica cave. Some examples of the morphological diversity of the isolates obtained from the analysed samples are shown in figure 3.1. These isolates exhibited typical morphologies of Actinobacteria, such as production of spores and hyphae, production of soluble pigments that impart colour to the culture medium, growth inside the agar, slow growth and rough colonies.

Most actinobacterial isolates identified by 16S sRNA gene sequencing belonged to the *Streptomyces* genus, however, strains affiliated with other genera (including some rare) were also identified, namely: *Micromonospora, Paenarthrobacter, Streptosporangium, Catellatospora, Nonomureae, Microbacterium, Afipia, Oerskovia, Sphaerisporangium* and *Rhodococcus,* as shown in figure 3.2.

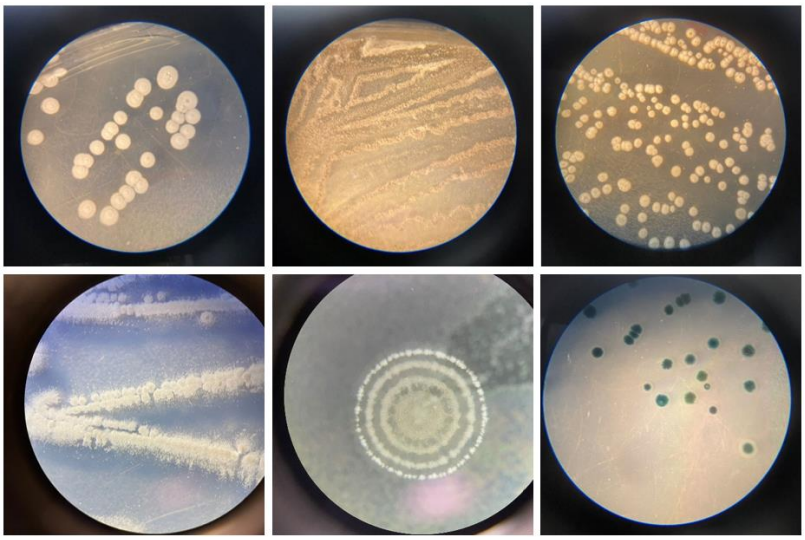

Figure 3.1 - Morphological diversity of some actinobacterial strains isolated from Vjetrenica cave samples.

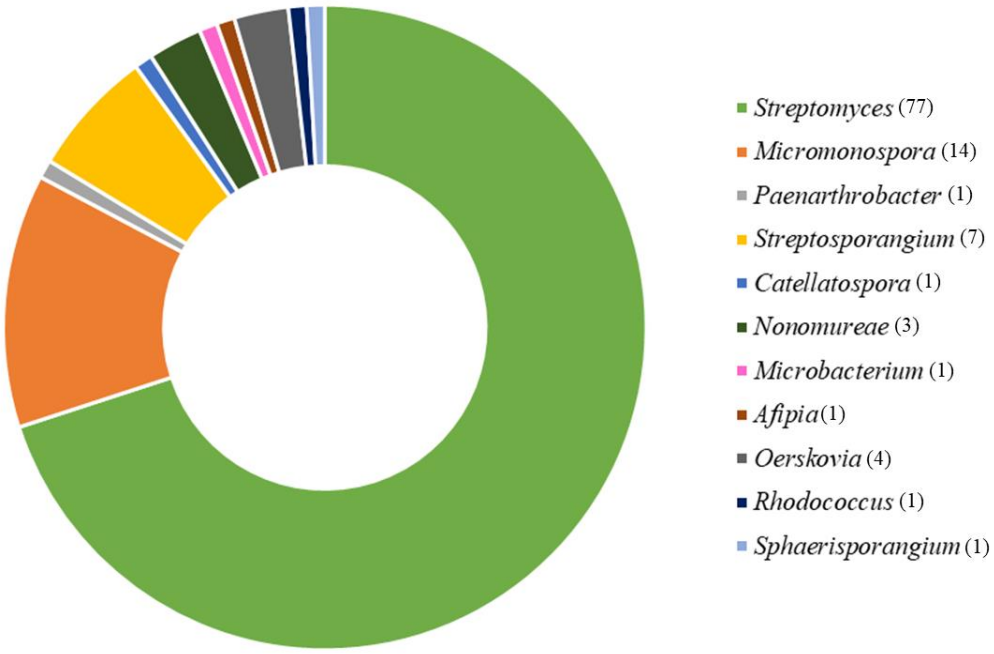

Figure 3.2- Abundance, in terms of genera, of actinobacterial strains isolated from the samples collected from the Vjetrenica cave. For each genus, numbers in brackets indicate the respective number of strains isolated.

Both SCN and TWYE media were effective in recovering actinobacterial strains, with the SCN medium recovering 73 strains and the TWYE medium leading to the isolation of 38 strains. The SCN medium allowed the growth of mainly *Streptomyces* species, whereas the TWYE medium was effective in recovering a higher diversity of genera, as shown in figure 3.3. In terms of the number of strains recovered from each of the three samples analysed, 66 strains were isolated from sample PK1, 35 strains were obtained from sample GJ1 and 11 strains were recovered from sample V5.

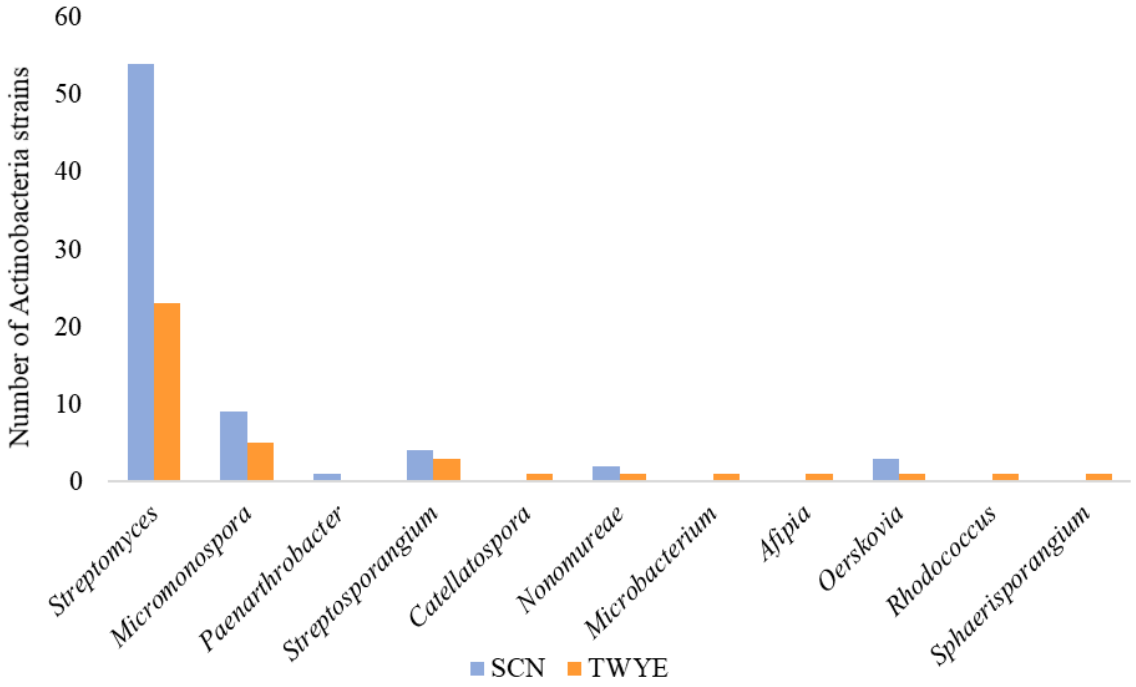

Figure 3.3 – Distribution, in terms of genera, of actinobacterial strains recovered from Vjetrenica cave samples, according to the selective culture media used in this study, SCN and TWYE.

Some genera were only retrieved from a particular sample. This is the case of the genera *Paenarthrobacter*, *Afipia*, *Catellatospora and Microbacterium*, which were only recovered from sample PK1, the genus *Oerskovia* that was only identified in sample GJ1 and the genera *Rhodococcus and Sphaerisporangium* that were only obtained from sample V5. The genera *Streptomyces*, *Micromonospora*, *Stresptosporangium* and *Nonomureae* were identified in at least two samples, as illustrated in figure 3.4.

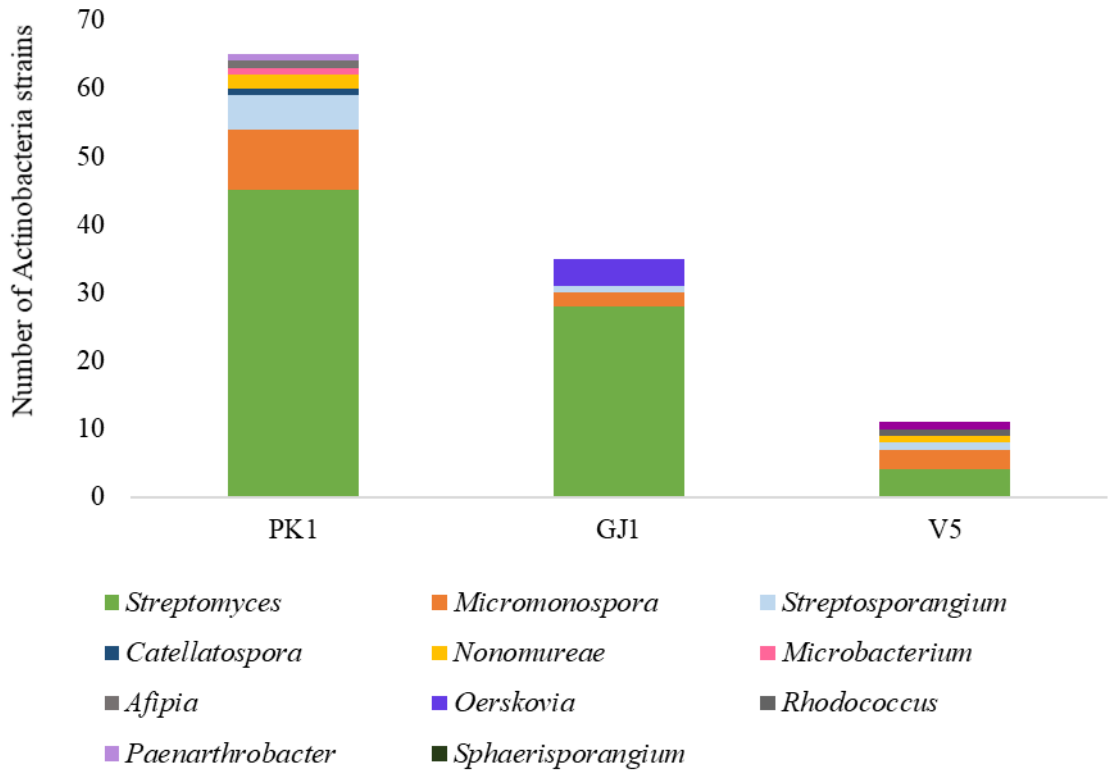

Figure 3.4 – Diversity of actinobacterial genera recovered from the three samples collected in the Vjetrenica cave, PK1, GJ1 and V5.

The taxonomic identification of each actinobacterial strain isolated in this study is indicated in table 3.1.

Table 3.1- Taxonomic identification of all the actinobacterial strains recovered from the three samples collected from the Vjetrenica cave

Strain code	Sample	Isolation Medium	Closest identification*	% similarity
P1	PK1	SCN	*Streptomyces* sp.	99.27
P2	PK1	SCN	*Streptomyces* sp.	99.12
P5	PK1	SCN	*Streptomyces daghestanicus*	99.71
P6	PK1	SCN	*Streptomyces cyaneofuscatus*	99.64
P7	PK1	SCN	*Streptomyces cyaneofuscatus*	99.93
P8	PK1	SCN	*Streptomyces* sp.	99.86
P10	PK1	SCN	*Streptomyces* sp.	99.85
P13	PK1	SCN	*Streptomyces albidoflavus*	99.78

Strain code	Sample	Isolation Medium	Closest identification*	% similarity
P15	PK1	TWYE	*Streptomyces* sp.	99.93
P16	PK1	TWYE	*Streptomyces* sp.	99.49
P17	PK1	TWYE	*Streptomyces advinii*	99.57
P19	PK1	TWYE	*Streptomyces exfoliatus*	99.71
P20	PK1	TWYE	*Streptomyces atroolivaceus*	99.5
P21	PK1	TWYE	*Streptomyces* sp.	99.43
P22	PK1	TWYE	*Streptomyces avidinii*	99.78
P23	PK1	TWYE	*Streptomyces exfoliatus*	99.64
P24	PK1	TWYE	*Streptomyces atroolivaceus*	99.57
P25	PK1	TWYE	*Streptomyces thermocarboxydus*	99.36
P27	PK1	TWYE	*Streptomyces* sp.	99.5
P28	PK1	TWYE	*Streptomyces zaomyceticus*	99.71
P31	PK1	TWYE	*Streptomyces zaomyceticus*	99.86
P32	PK1	TWYE	*Micromonospora palomenae*	99.41
P33	PK1	TWYE	*Catellatospora* sp.	99.55
P37	PK1	TWYE	*Nonomureae muscovyensis*	99.64
P41	PK1	TWYE	*Microbacterium oxydans*	99.19
P52	PK1	TWYE	*Micromonospora profundi*	99.85
P56	PK1	TWYE	*Streptomyces decoyicus*	100
P57	PK1	TWYE	*Streptomyces badius*	99.78
P58	PK1	TWYE	*Streptomyces thermocarboxydus*	99.43
P59	PK1	TWYE	*Streptomyces atratus*	99.5
P60	PK1	TWYE	*Streptomyces* sp.	99.42
P61	PK1	TWYE	*Streptosporangium carneum*	98.9
P62	PK1	TWYE	*Streptosporangium* sp.	99.36
P64	PK1	TWYE	*Micromonospora palomenae*	99.35
P65	PK1	TWYE	*Afipia broomeae*	95.18
P68	PK1	TWYE	*Streptomyces* sp.	99.42
P72	PK1	TWYE	*Streptomyces badius*	98.92

Strain code	Sample	Isolation Medium	Closest identification*	% similarity
P74	PK1	SCN	*Streptomyces* sp.	99.78
P75	PK1	SCN	*Streptosporangium jiaoheense*	99.21
P77	PK1	SCN	*Streptomyces umbrinus*	99.14
P78	PK1	SCN	*Streptomyces badius*	99.79
P79	PK1	SCN	*Micromonospora* sp.	99,35
P80	PK1	SCN	*Streptosporangium* sp.	99.71
P81	PK1	SCN	*Micromonospora* sp.	99.28
P85	PK1	SCN	*Micromonospora saelicesensis*	99.78
P86	PK1	SCN	*Nonomurea coxensis*	99.78
P87	PK1	SCN	*Streptomyces* sp.	99.78
P87	PK1	SCN	*Streptomyces* sp.	99.86
P88	PK1	SCN	*Micromonospora saelicesensis*	99.64
P90	PK1	SCN	*Streptomyces* sp.	99.78
P91	PK1	SCN	*Micromonospora citrea*	99.35
P92	PK1	SCN	*Streptomyces* sp.	98.62
P93	PK1	SCN	*Streptomyces sannanensis*	99.07
P96	PK1	SCN	*Streptomyces* sp.	99.64
P99	PK1	SCN	*Streptosporangium roseum*	99.64
P100	PK1	SCN	*Streptomyces* sp.	99.93
P102	PK1	SCN	*Streptomyces adustus*	99.64
P103	PK1	SCN	*Streptomyces atroolivaceus*	99.71
P104	PK1	SCN	*Streptomyces sannanensis*	99.06
P107	PK1	SCN	*Streptomyces cyaneofuscatus*	99.64
P108	PK1	SCN	*Streptomyces finlayi*	99.85
P109	PK1	SCN	*Streptomyces finlayi*	99.57
P111	PK1	SCN	*Paenarthrobacter* sp.	98.7
P114	PK1	SCN	*Micromonospora* sp.	99.35
P115	PK1	SCN	*Streptomyces griseus*	99.71
G5	GJ1	TWYE	*Oerskovia enterophila*	99.71
G22	GJ1	TWYE	*Streptomyces decoyicus*	99.5
G39	GJ1	SCN	*Streptomyces* sp.	98.86

Strain code	Sample	Isolation Medium	Closest identification*	% similarity
G40	GJ1	SCN	*Streptomyces decoyicus*	99.36
G42	GJ1	SCN	*Oerskovia merdavium*	99.71
G45	GJ1	SCN	*Streptomyces finlayi*	99.86
G46	GJ1	SCN	*Oerskovia* sp.	99.64
G49	GJ1	SCN	*Streptomyces* sp.	98.5
G50	GJ1	SCN	*Streptomyces decoyicus*	100
G52	GJ1	SCN	*Streptomyces maoxianensis*	99.57
G55	GJ1	SCN	*Streptomyces halstedii*	99.78
G58	GJ1	SCN	*Streptomyces lunælactis*	99.57
G59	GJ1	SCN	*Streptomyces* sp.	99
G61	GJ1	SCN	*Streptomyces* sp.	98.64
G63	GJ1	SCN	*Oerskovia enterophila*	99.57
G66	GJ1	TWYE	*Micromospora* sp.	99.57
G67	GJ1	SCN	*Streptosporangium roseum*	99.71
G72	GJ1	SCN	*Streptomyces atratus*	99.64
G74	GJ1	TWYE	*Streptomyces atratus*	99.29
G75	GJ1	SCN	*Streptomyces decoyicus*	99.57
G76	GJ1	SCN	*Streptomyces decoyicus*	99.71
G77	GJ1	SCN	*Streptomyces decoyicus*	98.79
G81	GJ1	SCN	*Streptomyces geldanamycininus*	99.71
G82	GJ1	SCN	*Streptomyces* sp.	98.93
G84	GJ1	SCN	*Streptomyces maoxianesis*	99.28
G85	GJ1	SCN	*Streptomyces* sp.	98.92
G86	GJ1	SCN	*Streptomyces decoyicus*	99.57
G87	GJ1	SCN	*Streptomyces decoyicus*	99.57
G88	GJ1	SCN	*Streptomyces decoyicus*	99.57
G89	GJ1	SCN	*Streptomyces maoxianensis*	99.36
G90	GJ1	SCN	*Micromonospora* sp.	99.06
G91	GJ1	SCN	*Streptomyces decoyicus*	100
G93	GJ1	SCN	*Streptomyces* sp.	98.92

Strain code	Sample	Isolation Medium	Closest identification*	% similarity
G94	GJ1	SCN	*Streptomyces* sp.	98.93
G95	GJ1	SCN	*Streptomyces decoyicus*	99.36
V11	V5	SCN	*Micromonospora kangleipakensis*	99.57
V12	V5	SCN	*Micromonospora haikouensis*	99.21
V15	V5	SCN	*Streptomyces antimycoticus*	100
V17	V5	SCN	*Streptomyces* sp.	99.35
V21	V5	TWYE	*Micromonospora palomenae*	99.28
V22	V5	TWYE	*Streptomyces avidinii*	99.78
V28	V5	TWYE	*Streptosporangium* sp.	99.71
V29	V5	TWYE	*Sphaerisporangium aureirubrum*	98.49
V35	V5	TWYE	*Rhodococcus erythropolis*	99.64
V57	V5	SCN	*Nonomuraea glycinis*	99.64
V60	V5	SCN	*Streptomyces endocoffeicus*	99.78

*According to 16S ribosomal RNA (Bacteria and Archea) data base from NCBI

According to the 16s rRNA gene similarity threshold for a new species of 98.7% (Stackebrandt & Schumann, 2006), three of the isolated actinobacterial strains (strains V29, G49 and G6) have the potential to constitute new species. According to figure 3.5, strain V29 clusters closely with *Sphaerisporangium aureirubrum* NEAU-GQTH1-3 (NR 145618.1), while figure 3.6 shows that strains G49 and G61 are possibly strains of the same species, as they cluster together in the same branch, sharing closer similarity to *Streptomyces paludis* GSSD-12.

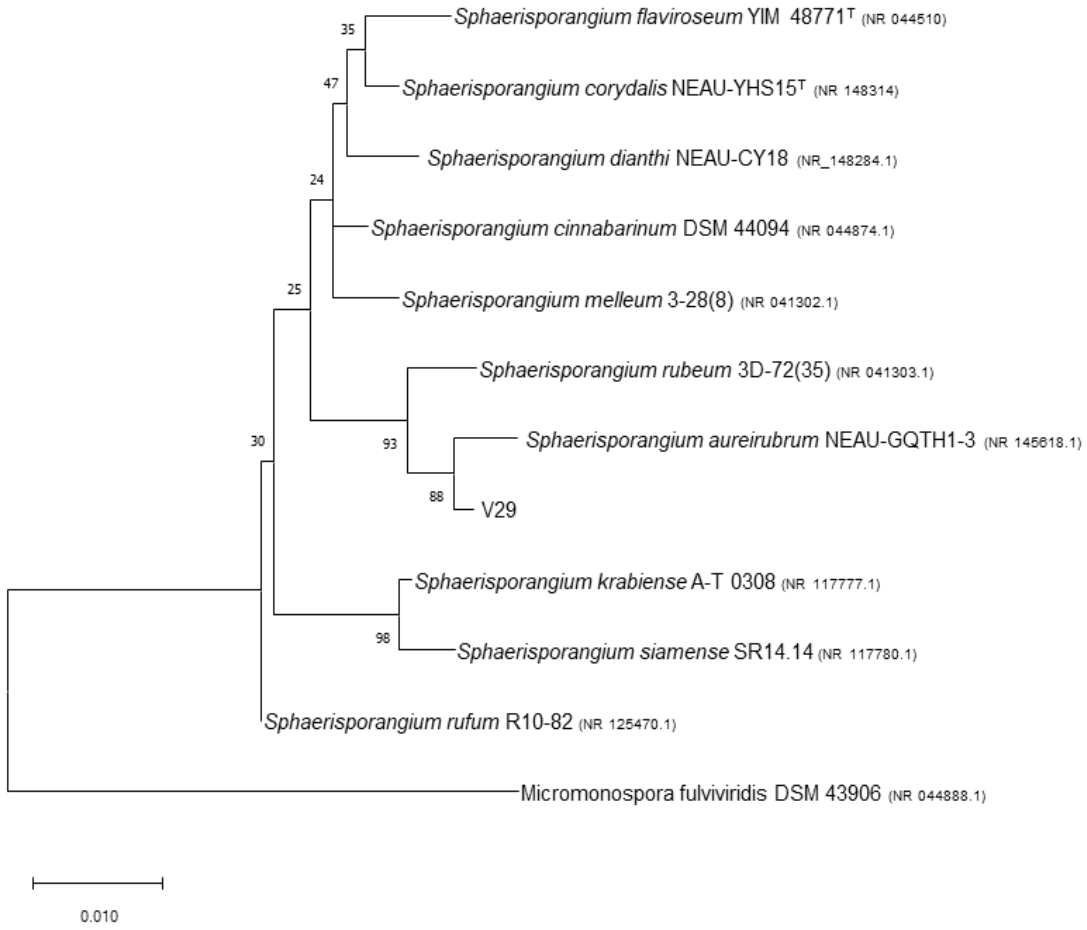

Figure 3.5 - Phylogenetic tree based on the ten type strains closest to strain V29, according to NCBI BLAST analysis. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. The phylogeny test used was the bootstrap analysis based on 1000 replicates to estimate branching order of the tree. This analysis involved 12 nucleotide sequences with 1417 bp and were conducted in MEGA 11 (Tamura et al., 2021). The GenBank accession numbers are indicated in parenthesis. *Micromonospora fulviviridis* DSM 43906 (NR 044888.1) was used as an outgroup. Scale bar corresponds to 0.010 substitutions per nucleotide position.

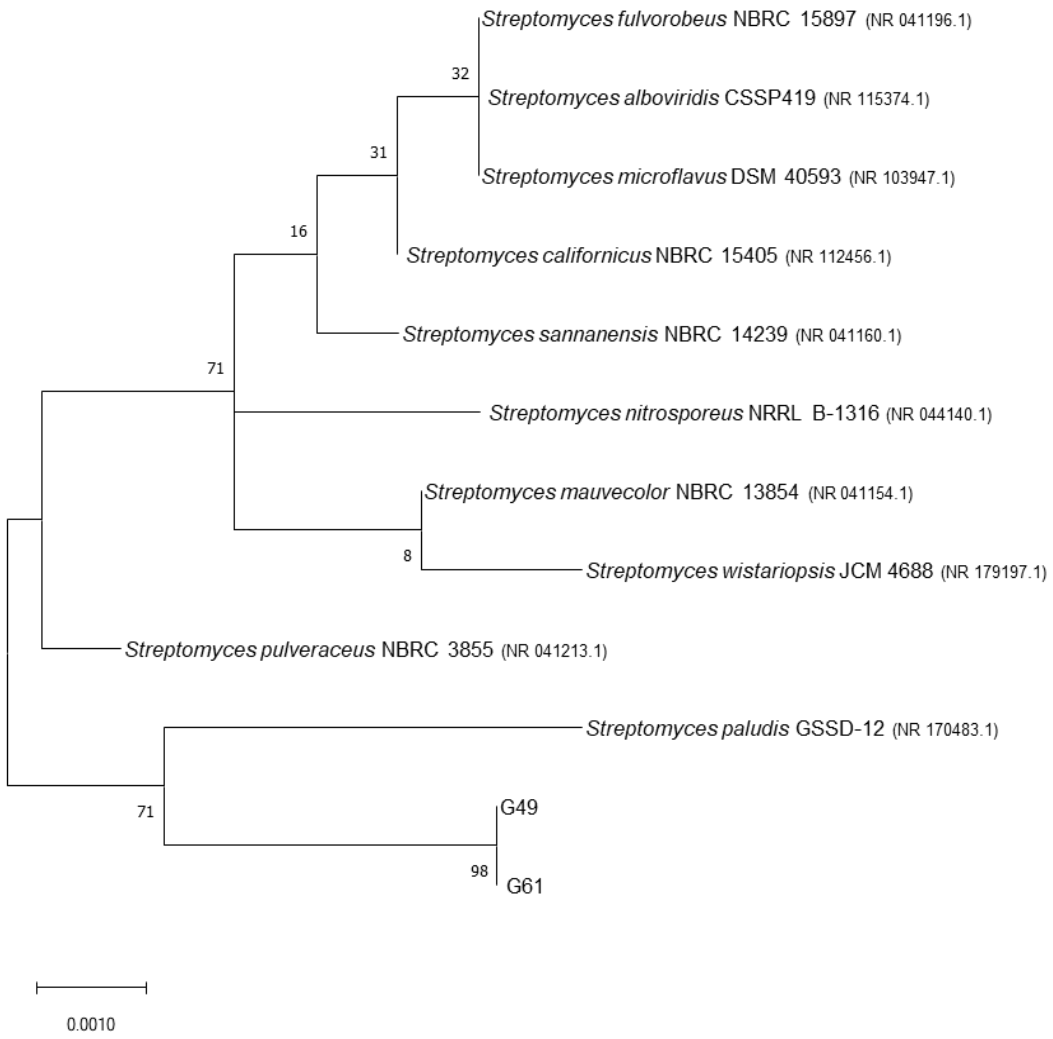

Figure 3.6 - Phylogenetic tree based on the ten type strains closest to strain G49 and G61, according to NCBI BLAST analysis. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. The phylogeny test used was the bootstrap analysis based on 1000 replicates to estimate branching order of the tree. This analysis involved 12 nucleotide sequences with 1492bp and were conducted in MEGA 11 (Tamura et al., 2021). The GenBank accession numbers are indicated in parenthesis. Scale bar corresponds to 0.010 substitutions per nucleotide position.

3.4 Discussion

Actinobacteria are among the most promising reservoirs of valuable biological compounds, having played a pivotal role in the development of numerous commercial drugs (De Simeis & Serra, 2021; Lee et al., 2020). As the interest in uncovering novel bioactive compounds continues to grow, the study of underexploited environments assumes increasing significance. This significantly enhances the potential for discovering previously undiscovered bioactive compounds (Karthikeyan et al., 2022). In this regard, cave dwelling actinobacteria are very promising, not only because caves hold several

environmental conditions suitable for the development of these microorganisms, such as the absence of direct sunlight, stable temperatures and humidity conditions (Mpakosi & Mironidou-Tzouveleki, 2023), but also because caves create selective pressure for the evolution of new biosynthetic genes, thus enhancing the prospects of discovering novel biologically active compounds (Rangseekaew & Pathom-Aree, 2019; Tiwari & Gupta, 2013). This unique habitat holds a high diversity of actinobacteria, and new genera are being described as more studies of biodiversity in this ecosystem rise, like the genera *Antricoccus, Beutenbergia, Knoellia, Lysinibacter, Spelaeicoccus, Hoyosella and Sphaerimonopora* that were isolated and described from caves and cave related habitats (Rangseekaew & Pathom-Aree, 2019).

Hence, the present study aimed to isolate and identify actinobacteria from the Vjetrenica cave, which is highly unexplored in terms of these microorganisms, with the ultimate goal of bioprospecting these microorganisms for the production of new natural compounds with biotechnological applications.

For this purpose, two culture media selective for actinobacteria were used, SCN and TWYE. SCN is a culture medium widely used for the cultivation of actinobacteria, especially of *Streptomyces*, while TWYE is an oligotrophic medium that better simulates the nutritional conditions of caves, allowing the growth of actinobacterial species more adapted to nutrients scarcity. Using these two media, we successfully isolated 110 actinobacterial strains, with the majority (70%) being affiliated to the genus *Streptomyces* that were recovered from the medium SCN. Microorganisms belonging to this genus have been frequently isolated from cave systems, indicating that *Streptomyces* are common inhabitants of caves (Farda et al., 2022). Nonetheless, the experimental approach adopted in this study also allowed the isolation of actinobacterial strains affiliated to ten other actinobacterial genera, some of them considered rare genera – *Micromonospora, Paenarthrobacter, Streptosporangium, Catellatospora, Nonomureae, Microbacterium, Afipia, Oerskvovia, Rhodococcus and Sphaerisporangium*. All these genera have been already identified in other caves, such as the Shaunghe Cave, in Asia, the Hampoeil Cave in Iran, Altamira and Tito Bustillo, in Spain or Pukzing Cave in India (Bastian et al., 2009; Lange-Enyedi et al., 2022; Mudgil et al., 2022; Nakaew et al., 2009), with the exception of the genus *Sphaerisporangium*, that has never been reported before in these habitats, making this the first study reporting its occurrence in caves. The TWYE medium was the most effective in recovering these rare genera of actinobacteria.

Cave associated actinobacteria have already proven to be a valuable source of new bioactive compounds. For instance, *Streptomyces tendae* strain HKI 0179, which was isolated from the ancient cave, the Grotta dei Cervi in Italy, has yielded compounds such as Cervimycin A, B, C, and D. These compounds exhibit remarkable activity against Gram positive bacteria, being effective even in some multi-drug-resistant strains (Herold et al., 2005). While a significant proportion of these remarkable compounds have been discovered within the *Streptomyces* genus, it is worth noting that other genera have also contributed to this achievement. An excellent example is the Hypogeamicins A, B, C and D, produced by *Nonomuraea specus*, which was isolated from the Hardin's cave system in Tennesse, USA. These compounds have shown cytotoxic properties against the colon cancer cell line TCT-1 and antimicrobial activity against *Bacillus subtilis* (Derewacz et al., 2014). The findings from these studies open great prospects for the discovery of novel bioactive compounds in the actinobacterial strains isolated in the present study.

Three potentially new actinobacterial strains belonging to the genera *Streptomyces* and *Sphaerisporangium* were identified in this study, despite two of them appearing to be the same species. These findings are always promising, since the discovery of new species significantly enhances the prospects of uncovering novel bioactive compounds, given that the evolution of the metabolism of these microorganisms is itself a speciation factor. The *Streptomyces* genus is still the largest current producer of new secondary metabolites, recognised for its capacity to produce antibiotics, antitumor compounds, biofilm inhibitors, antiparasitic compounds, and antioxidants, among other compounds (Laskaris & Karagouni, 2021), and thus, discovering new species of this genus is always exciting. On the other hand, the discovery in this study of new strains of the genus *Sphaerisporangium* marks a significant breakthrough, as it has been documented within a cave ecosystem for the very first time. This rare genus is still poorly studied in terms of its bioactive capacity, yet, a study conducted by Guo et al., (2022) revealed that its members have a high genetic potential to synthesize many new secondary metabolites. To substantiate that the three strains isolated in this study indeed represent novel actinobacterial species, additional research is imperative. To attain this confirmation, it becomes essential to conduct complete genome sequencing of these strains.

The next steps of this study will be to screen the collection of the 111 actinobacterial strains obtained from the three analysed cave samples, in terms of their bioactive properties and investigate their potential to produce new bioactive compounds, mainly

new antimicrobial compounds. Exploration of cave-associated actinobacteria and their bioactive compounds is still at an early stage. Ongoing and future research endeavours will serve to establish these microorganisms as prolific sources of novel bioactive compounds, offering valuable biotechnological benefits (Rangseekaew & Pathom-Aree, 2019).

Chapter 4

General Discussion and Conclusion

4.1 General Discussion

Antibiotic resistance and the lack of new antibiotics is a global problem. Natural compounds sourced from microorganisms play a pivotal role in the development of new antibiotics to combat drug-resistant bacteria. Actinobacteria are a vital source of bioactive compounds, and their ability to produce a wide array of secondary metabolites makes them one of the most extensively studied microorganisms among prokaryotes. Exploring actinobacteria inhabiting extreme environments holds great promise as a strategy for the discovery of new bioactive compounds. The deep sea and caves are great examples of extreme environments where the search for novel actinobacterial strains has proven effective, leading to the discovery of potentially unique bioactive compounds with pharmaceutical relevance.

The first work presented in this thesis (chapter 2), focused on the exploration of antimicrobial compounds within seven deep-sea actinobacterial strains, through the implementation of the OSMAC approach. This approach was selected as it has been shown to be an effective technique for awakening silent BGCs, with numerous studies demonstrating that its application can significantly expand the chemical portfolio offered by microorganisms. However, none of the 4 OSMAC conditions tested led to the detection of antimicrobial activity, with the only extract where this activity was identified being obtained from a culture of *S. xinghaiensis* C_003.1.9 that grew without applying any OSMAC condition. The next step in this work, was to understand if this extract could potentially contain new secondary metabolites responsible for the observed activity and also understand whether the applied OSMAC conditions could be triggering a differential expression of secondary metabolites in the seven actinobacteria isolates tested. For this, all extracts generated in the study were submitted to a dereplication process by LC-HRESIMS/MS, complemented by a molecular networking analysis. These detailed analyses revealed that the extract exhibiting antimicrobial activity did not contain any compounds that could account for this activity when compared to the GNPS database, indicating the potential presence of a new secondary metabolite. Additionally, the extract derived from *Microbacterium ginsengiterrae* C_014 6 grown in the presence of N-acetylglucosamine presented a cluster that also did not find any match in the dereplication process, indicating the potential presence of a novel metabolite. Although the results obtained with the application of the OSMAC strategy were not very productive, it was possible to identify two extracts of interest for future studies.

The second study introduced in this thesis (chapter 3) focused on the isolation and taxonomic characterization of actinobacteria inhabiting the Vjetrenica cave, in Bosnia-Herzegovina. This cave was found to be an exceptional reservoir of actinobacteria, with a total of 111 strains being isolated from the three analysed samples. In addition, this cave also revealed to be a source of new phylogenies, as three potentially new actinobacterial strains, belonging to the genera *Streptomyces* and *Sphaerisporangium*, were identified, despite two of them appearing to be the same species. The discovery of these new species is exciting since the *Streptomyces* genus is recognised to be the largest current producer of new secondary metabolites, and although still poorly studied in terms of bioactive capacity, the rare genus *Sphaerisporangium* was not documented before within a cave ecosystem. The genome sequencing of these strains will be pivotal in proving their new phylogeny. The exploration of cave environments seems, thus, to be a very promising strategy for the isolation of new actinobacteria and for the bioprospection of new secondary metabolites.

4.2 Conclusion

This thesis is focused on the application of different OSMAC strategies to investigate the antimicrobial potential of seven deep-sea actinobacteria strains and on the isolation and identification of actinobacteria from Vjetrenica Cave, in Bosnia-Herzegovina.

The results presented, indicated that, although the application of the OSMAC approach is a valuable tool for stimulating bioactivities and discovering new natural compounds, it was not particularly efficient with the seven actinobacterial strains tested. Nonetheless, it was possible to identify two extracts containing potentially novel secondary metabolites, one of them with antimicrobial activity, that deserve further studies in the future.

The Vjetrenica Cave proved to be a hotspot of actinobacteria, showing a significant diversity of these microorganisms. Overall, the genus *Streptomyces* represented 70% of all strains identified. This work also allowed the identification of three possible novel strains of the genera *Streptomyces* and *Sphaerisporangium*. These findings highlight the importance of exploring actinobacteria in cave environments, not only for unveiling their diversity, but also but also as a strategic approach to enhance the discovery of new bioactive molecules.

The promising results detailed in this thesis create opportunities for future research into the biotechnological potential and diversity of actinobacteria found in extreme environments.

Chapter 5
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