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DIVERSITY AND BIOACTIVE POTENTIAL OF ACTINOBACTERIA FROM THE MARINE SPONGE *HYMENIACIDON PERLEVIS*

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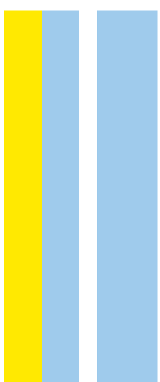

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DIVERSITY AND BIOACTIVE POTENTIAL OF ACTINOBACTERIA FROM THE MARINE SPONGE *HYMENIACIDON PERLEVIS*

Dissertation for the master's degree in Environmental Toxicology and Contamination submitted to the Abel Salazar Biomedical Sciences Institute from the Porto University.

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Abstract

Nowadays, human health faces several threats. Infections associated with multi-resistant bacteria and oncological diseases represent two of the health problems that cause most concern worldwide. For a more efficient combat of these pathologies, new therapeutic drugs need to be urgently found.

Natural products of microbial origin have been capturing an increasing attention from scientists and the pharmaceutical industry, as they are a rich source of bioactive molecules, many of them with recognized therapeutic properties.

Actinobacteria are microorganisms with high potential to produce secondary metabolites with important bioactivities. Due to the overexploration of terrestrial Actinobacteria, the scientific community has been looking at marine environments as a promising alternative for the bioprospection of novel bioactive molecules produced by these microorganisms.

Very few studies are available on the actinobacterial diversity associated with Portuguese marine environments. In this context, the present master thesis aimed to study the cultivable actinobacterial community associated with a marine sponge common in the Portuguese coast, *Hymeniacidon perlevis*, and investigate the potential of the retrieved isolates to produce compounds with antimicrobial and anticancer properties. To increase the success in the isolation of Actinobacteria, fragments of the sponge sample collected at Praia da Memória, in Northern Portugal, were subjected to 3 different pretreatments and inoculated on 3 selective culture media.

In total, 184 strains were isolated from the marine sponge, of which 119 were phylogenetically identified. 16S rRNA gene analysis revealed 82 isolates associated with the phylum Actinobacteria, with the largest fraction (52 strains) being affiliated with the genus *Streptomyces*. Isolates belonging to the genera *Gordonia*, *Nocardia*, *Nocardiopsis*, *Dietzia*, *Tsukamurella*, *Micromonospora*, *Micrococcus*, *Brachybacterium*, *Glutamicibacter*, *Rhodococcus* and *Paenoarthrobacter* were also recovered from the marine sponge *H. perlevis*.

Fifty-seven actinobacterial extracts were tested for their antimicrobial and anticancer activity. Antimicrobial activity was determined using the disk diffusion method and MIC was subsequently evaluated for extracts showing relevant activity. Seven extracts (all obtained from isolates of the genus Streptomyces) showed antimicrobial activity against *Bacillus subtilis* and/or *Candida albicans*, with MIC values between 15.62 and 125 μg mL^{-1}. Anticancer activity was tested in two human cancer cell lines (human liver cancer HepG2 and human colorectal cancer HCT-116) using the MTT method. Forty-one extracts were

able to reduce the cell viability of at least one cancer cell line. Of these, 6 extracts had a strong activity against the cell line HepG2, reducing its viability in more than 80% after 48 h of exposure, and 4 extracts reduced in more than 40% the viability of the cell line HCT-116.

As future work, it will be necessary to conclude the phylogenetic identification of the isolates that could not be identified during the timeframe of this thesis and to deeper assess the bioactive potential of all actinobacterial isolates. In addition, it will be necessary to conduct a chemical workflow to investigate the potential presence in the bioactive extracts of novel chemical entities.

In general, the results obtained in this master thesis allowed a better understanding of the diversity of Actinobacteria living in association with the marine sponge *H. perlevis*, distributed in the Portuguese coast. Some promising bioactivity results open up the possibility of finding novel compounds.

Keywords: marine actinobacteria, marine sponge, *Hymeniacidon perlevis*, bioactive compounds, antimicrobial, anticancer

Resumo

A saúde humana enfrenta atualmente várias ameaças. As infeções associadas a bactérias multirresistentes e doenças oncológicas constituem dois dos problemas mais preocupantes a nível mundial. Para um combate mais eficaz destas patologias é necessário encontrar urgentemente novos medicamentos.

Os produtos naturais de origem microbiana têm atraído cada vez mais a atenção dos cientistas e da indústria farmacêutica, uma vez que são uma fonte rica de moléculas bioativas, muitas delas com reconhecidas propriedades terapêuticas.

As actinobactérias são microrganismos que possuem um elevado potencial para produzir metabolitos secundários com importantes bioatividades. Devido à sobreexploração das actinobactérias terrestres, a comunidade científica tem olhado para os ambientes marinhos como uma alternativa promissora para a bioprospecção de novas moléculas bioativas produzidas por estes microrganismos.

Estudos associados com a diversidade de actinobactérias em ambientes marinhos portugueses são muito escassos na literatura. Neste contexto, a presente dissertação teve como objetivo estudar a comunidade de actinobactérias cultiváveis associada a uma esponja marinha comum da costa portuguesa, *Hymeniacidon perlevis*, e investigar o potencial dos isolados recuperados para produzir compostos com propriedades antimicrobiana e anticancerígena. De forma a aumentar o sucesso no isolamento de actinobactérias, fragmentos da amostra de esponja recolhida na Praia da Memória, no norte de Portugal, foram submetidos a três pré-tratamentos diferentes e inoculados em três meios de cultura seletivos.

No total, foram isoladas da esponja marinha 184 estirpes, das quais 119 foram identificadas filogeneticamente. A análise do gene 16S rRNA revelou 82 isolados associados ao filo Actinobacteria, com a maior fração destes pertencendo ao género *Streptomyces* (52 isolados). Isolados pertencentes aos géneros *Gordonia*, *Nocardia*, *Norcardiopsis*, *Dietzia*, *Tsukamurella*, *Micromonospora*, *Micrococcus*, *Brachybacterium*, *Glutamicibacter*, *Rhodococcus e Paenoarthrobacter* foram também recuperados da esponja marinha *H. perlevis*.

Cinquenta e sete extratos de actinobactérias foram testados quanto à sua atividade antimicrobiana e anticancerígena. A atividade antimicrobiana foi determinada usando o método de difusão em disco, seguido de posterior avaliação do MIC para os extratos que apresentaram atividade relevante. Sete extratos (todos obtidos de isolados do género *Streptomyces*) apresentaram atividade antimicrobiana contra *Bacillus subtilis* e/ou *Candida*

albicans, com valores de MIC entre 15.62 e 125 µg mL^{-1}. A atividade anticancerígena foi testada em duas linhas tumorais (carcinoma hepatocelular HepG2 e cancro do cólon e do reto HCT-116), usando o método do MTT. Quarenta e um extratos foram capazes de reduzir a viabilidade celular de pelo menos uma das linhas celulares testadas. Desses, 6 extratos apresentaram uma forte atividade contra a linha celular HepG2, reduzindo a sua viabilidade em mais de 80% após 48 h de exposição, enquanto 4 extratos reduziram em mais de 40% a viabilidade da linha celular HCT-116.

Como trabalho futuro, será necessário concluir a identificação filogenética dos isolados que não puderam ser identificados durante o período desta tese e avaliar mais profundamente o potencial bioativo de todos os isolados de actinobactérias. Além disso, será necessário realizar todo um trabalho químico para investigar a potencial presença nos extratos bioativos de novas entidades químicas.

De uma maneira geral, os resultados obtidos nesta tese de mestrado permitiram uma melhor compreensão da diversidade de actinobactérias que vivem em associação com a esponja marinha *H. perlevis*, distribuída na costa portuguesa. Alguns resultados promissores em termos de bioatividade abrem a possibilidade de encontrar novos compostos.

Palavras-chave: actinobactérias marinhas, esponja marinha, *Hymeniacidon perlevis*, compostos bioativos, antimicrobiano, anticancerígeno.

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1. Introduction

Microorganisms play a fundamental role in Earth's life. They decompose organic waste into nutrients, generating different molecules from organic matter that are reused in the ecosystems (Colwell, 1997). The microbial life is amazingly diverse and may be found in various conditions of pressure, salinity, pH, and temperature. Many microorganisms establish a symbiotic relationship with other organisms (Colwell, 1997). Their activities are mostly beneficial, however, pathogenic species also exist that may jeopardize human health.

Bacteria are one of the most diverse and abundant groups of microorganisms. They are ancestral living beings with an enormous capacity for adaptation, being capable of resisting environments with extreme conditions. The flexibility of these microorganisms to adapt to their surrounding environment may also have a negative side, especially in what concerns human health, and this is particularly notable in the case of the environmental presence of antibiotics, as it may lead to the development of bacterial resistance mechanisms that may compromise the efficacy of the drug (Undabarrena et al., 2016).

According to the World Health Organization, antibiotic resistance has become a world concern that needs immediate attention. This resistance is largely associated with the inappropriate use of antibiotics, with Portugal being one of the countries with the highest consumption of these drugs at the European level (Loureiro et al., 2016). In addition to multidrug-resistant bacterial infections, cancer continues to be one of the most serious human health pathologies (Ravikumar et al., 2012), representing one of the major challenges in medicine and pharmacology.

There is an urgent need to find new solutions for the treatment of these diseases, namely, to discover new antibiotics capable of killing multi-drug resistant microbial pathogens and new anticancer pharmaceuticals with improved efficacy. In this regard, Nature is an excellent source of novel pharmaceuticals. Currently, 50% of all pharmaceuticals used are directly derived from or inspired in natural products, i.e., products biologically produced (Sekurova et al., 2019). Natural products synthesised by microorganisms are a proven source of pharmacologically relevant compounds and of other bioactive compounds (Olano and Carmen 2009). About 45% of all bioactive compounds described to date are derived from the secondary metabolism of Actinobacteria, a bacterial phylum with a high biotechnological value (Arumugam et al. 2017; Jami et al. 2015; Jose et al., 2013).

Actinobacteria are commonly distributed across a vast range of habitats, including terrestrial and marine habitats. Terrestrial Actinobacteria have been intensively explored over the last

years (Jose et al., 2014*)*. The exhaustive exploration of this source of bioactive compounds has caused a dramatic decrease in the discovery of new bioactive molecules (Siddharth and Vittal, 2019), often leading to the re-isolation of known compounds (Meena et al. 2019). Oceans offer a great chemical wealth and their actinobacterial inhabitants are very scarcely explored, thus representing a promising source of novel bioactive compounds. In contrast to terrestrial Actinobacteria, marine Actinobacteria are exposed to very different environmental conditions in terms of nutrients available, light, oxygen concentration and pressure (Subramani and Sipkema, 2019), which are important factors for the evolution of new species and novel natural products (Mohseni et al.*,* 2013; Kasanah and Triyanto 2019).

1.1. Phylum Actinobacteria

The phylum Actinobacteria comprises a wide diversity of Gram-positive, aerobic and filamentous bacteria, typically having a high citosine+guanine content in their genomes that may vary between 50-70%, according to the species (Lawson, 2018; Priyanka *et al.* 2019; Sun *et al.* 2010). This phylum represents one of the largest taxonomic units within the domain Bacteria, showing a great wealth in terms of abundance and diversity of morphologies, physiologies and metabolic capabilities (Barka et al., 2016; Ventura et al. 2007). The phylogeny of their microorganisms, as well as the associated characteristics, have been well-reported over the years (Tischler et al.*,* 2019).

The taxonomy of this phylum was recently changed and now includes the classes *Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria* and *Thermoleophilia.* The vast majority of producers of bioactive compounds are integrated in the first class, which integrates the orders *Actinomycetales*, *Actinopolysporales*, *Bifidobacteriales*, *Catenulisporales*, *Corynebacteriales*, *Frankiales*, *Glycomycetales*, *Jiangellales*, *Kineosporiales*, *Micrococcales*, *Micromonosporales*, *Propionibacteriales*, *Pseudonocardiales*, *Streptomycetales* and *Streptosporangiales* (Ludwig et al. 2012). In this dissertation, the term "Actinobacteria" will be used to refer to members of the homonym class.

Actinobacteria have diverse morphologies that may vary from coccoid (e.g., *Micrococcus* spp.), rod-coccoid (e.g., *Arthrobacter* spp.), fragmenting hyphal forms (e.g., *Nocardia* spp.) to microorganisms with a highly differentiated branched mycelium (e.g., *Streptomyces* spp.) (Ventura et al. 2007). Many microorganisms have the capacity to reproduce through the production of spores that confer them the capacity to survive under harsh conditions (Passari et al., 2018).

These microorganisms are distributed by different ecological environments, including soils, marine environments and air, where they can occur as symbionts (e.g., *Frankia* spp.), pathogens (e.g., *Mycobacterium*, *Nocardia*, *Corynebacterim* spp.*)* or saprophytes (e.g., *Streptomyces* spp.) (Abdelmohsen et al. 2014a; Barka et al. 2016). They are also an important part of the gastrointestinal microbiome of many animals (e.g., *Bifidobacterium* spp.) (Ventura et al. 2007). Many of these microorganisms play a relevant ecological role, being capable of recycling nutrients, degrading complex polymers and, in some cases, removing organic and inorganic pollutants (Alvarez et al. 2017; Burghal et al.,2015; Mohseni et al. 2013).

1.2. Production of Bioactive Compounds by Actinobacteria

Natural products and their synthetic derivates are considered the most promising sources of new drugs, as natural chemical structures were designed along many years of evolution to interact with specific biological targets (Ziyat el al*.,* 2019). For this reason, many of these compounds have a high impact in human and animal health (Goodfellow and Fiedler, 2010).

Actinobacteria are largely recognized for their ability to produce bioactive compounds with high medicinal value (Jose et al., 2013; Meena et al. 2019). From the ca. 23 000 secondary metabolites reported to date, about 10 000 are produced by Actinobacteria (Sharma et al., 2019). The repertoire of compounds produced by these microorganisms include antiviral, antifungal, antitumor, antioxidant, anti-inflammatory, immunosuppressive and anti-parasitic compounds (Table 1) (Dholakiya et al. 2017). The genera *Streptomyces*, *Saccharopolyspora*, *Amycolatopsis*, *Micromonospora* and *Actinoplanes* are amongst the main representative genera of Actinobacteria producing commercially important bioactive molecules (Sharma et al., 2019).

The genome of Actinobacteria integrates large biosynthetic gene clusters coding to produce a wide variety of secondary metabolites. Some species, such as *Streptomyces coelicolor* and *Rhodococcus sp*., carry in their genome more than 20 biosynthetic gene clusters (Goodfellow and Fiedler 2010). Polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) are complex enzyme systems involved in the production of pharmaceutically relevant polyketides and nonribosomal peptides, respectively (Li et al. 2013).

The production of secondary metabolites depends on several parameters, such as nutrients available, pH, partial oxygen pressure (pO_2), temperature, agitation, mineral salts and metal ions available (Subramani and Sipkema 2019). The synthesis of these metabolites initiates when bacterial growth decreases or stops, so the production of secondary metabolites can

be supressed by freely available carbon sources, high levels of phosphorous and abundant nitrogen (Manivasagan et al. 2013). The production of secondary metabolites is correlated with microbial growth in the following way: (i) in the preparatory phase (lag phase), no secondary metabolites are synthesized; (ii) in the log phase, that corresponds to exponential growth, an intensive microbial growth occurs and the production of secondary metabolites is very limited; (iiii) a transition phase follows where poor microbial growth and synthesis of proteins occurs, and the secondary metabolism enzymes increase and start the production of secondary metabolites; (iv) the ultimate phase is the production phase, where the production of secondary metabolites is intensive (Bibb, 2005; Manivasagan et al. 2013).

Table 1 - Examples of secondary metabolites isolated from Actinobacteria (adapted from Sharma et al., 2019, Genilloud, 2018, Olano et al., 2009).

Biological Activity	Compound	Actinobacterial species	Reference
Antibacterial	Thiocoraline	*Micromonospora*	Romero et al. (1997)
	Rifamycin	*Salinospora arenicola*	Floss and Yu (2005)
	1,4-Dihydroxy-2-(3-hydroxybutyl)-9,10-anthraquinone 9,10-anthrac	*Streptomyces* sp.	Ravikumar et al. (2012)
	Asenjonamides A–C	*Streptomyces asenjonii*	Abdelkader et al. (2018)
	Teicoplanin	*Actinoplanes teichomyceticus*	Butler et al. (2014)
Cytotoxic	Pacificanones	*Salinospora pacifica*	Oh et al. (2008)
	Lucentamycins	*Nocardiopsis lucentensis*	Ji et al. (2007)
	Pyridinium	*Amycolatopsis alba*	Dasari et al. (2012)
	Tetrocarcin A	*Micromonospora chalcea*	Fang et al. (2008)
	Mitomycin C	*Streptomyces lavendulae*	August et al. (1996);
Antifungal	Bonactin	*Streptomyces* sp. *BD21-2*	Shumacher et al. (2003)
	Daryamides	*Streptomyces* sp. *CNQ-085*	Asolkar et al. (2006)
	Chandrananimycins	*Actinomadura* sp.	Maskey et al. (2003)
Antiviral	Benzastatin C	*Streptomyces nitrosporeus*	Lee et al. (2007)
Antiparasitic	Valinomycin	*Streptomyces* sp.	Pimentel-Elardo et al. (2010)

Immunosuppressive	Everolimus (derivative of Rapamycin)	*Streptomyces hygroscopoicus*	Chapman and Perry et al. (2004)
Anti-inflammatory	Diazepinomicin (ECO-4601)	*Micromonospora* sp.	Charan et al. (2004)
	Cyclomarin A	Streptmomyces sp.	Renner et al. (1999)
Antioxidant	Dermacozines A-G	*Dermacoccus* sp.	Abdel-Mageed et al. (2010)
	Lipocarbazoles	*Tsukamurella pseudospumae*	Schneider et al. (2009)

1.3. Actinobacterial Compounds with Antimicrobial Activity

The discovery of new antibiotics is an urgent issue. The intensive utilization, misuse and wrong disposal of antibiotics is leading to the emergence of multi-drug resistant microbial pathogens, which has been accompanied by a decline in the discovery of new antibacterial drugs by the pharmaceutical industry (Li and Webster, 2018).

Ten classes of antibiotics have been reported to be exclusively produced by Actinobacteria: polyene macrolides, oligomycin-type large-membered macrolides, daunomycin-type anthracyclines, nigericin-type polyether antibiotics, nonactin-type cyclopolylactones, aminoglycosides, anthracyclines, streptothricins, actinomycins and quinoxaline-peptides (Subramani and Sipkema 2019). Currently, Actinobacteria derived antibiotics, such as daptomycin, erythromycin, fosfomycin, lincomycin, neomycin, streptomycin and tetracycline are substances with a high commercial importance (Jose et al., 2013). Within the class Actinobacteria, the genus *Streptomyces* is one of the major producers of antibiotics (Manivasagan et al., 2014a). It is estimated that between 1950 and 1970, 60% of the antibiotics isolated from Actinobacteria and in commercial use were solely produced by microorganisms of this genus (Subramani and Sipkema 2019).

Apart from antibacterial agents, Actinobacteria have also been reported to produce compounds with antifungal activity, such as N-(2-hydroxyphenyl)-2-phenazinamine (NHP), isolated from *Nocardia dassonville*, and with significant activity against *C. albicans*, Amphotericin B, also with activity against *C. albicans*, and synthetized by *Streptomyces nodosus,* and Yatakemycin, produced by a *Streptomyces* strain AO 356, with ability to inhibit the growth of *Aspergillus fumigatus* and *C. albicans* (Danasekaran et al., 2012).

1.4. Actinobacterial Compounds with Anticancer Activity

Nowadays, cancer is the second cause of human mortality. In 2018 this disease was responsible for the death of 9.6 million people (WHO, 2019). The incidence of this serious

disease is increasing in the world population due to several factors, but changes in nutrition, lifestyle and environmental pollution are believed to have a strong correlation with many cases of the pathology (Sithranga et al., 2010). New pharmaceuticals for the treatment of this disease will always be an important fighting mechanism and, therefore, it is crucial to discover new drugs. In recent decades, natural compounds have gained significant importance for the treatment of cancer, and many of this anticancer compounds are derived from Actinobacteria (Ravikumar et al., 2012). An illustrative example of an important anticancer agent produced by Actinobacteria is Doxorubicin, an anthracycline drug produced by a soil *Streptomyces*, *Streptomyces peucetius*, and currently marketed for the treatment of solid tumours and haematological neoplasms (Sekurova et al., 2019). Another relevant example is Salinosporamide A, a bicyclic γ-lactam-β-lactone proteasome inhibitor, produced by the marine Actinobacteria *Salinospora tropica*, that is under clinical trials for its ability to combat multiple myeloma, solid tumours, and lymphoma (Potts and Lam, 2010).

1.5. Marine Actinobacteria

Oceans occupy more than 70% of the Earth's surface and constitute a vast reservoir of various forms of life (Singh et al. 2013). The marine environment is known to be an immense source of microbial biodiversity with a vast biochemical and molecular wealth (Dharmaraj, 2010; Prudhomme *et al.* 2008). Several studies have shown that the marine environment is an emerging source of structurally diverse natural products with innovative biotechnological applications, namely of new drugs for treating diseases that need immediate attention (Goodfellow and Fiedler, 2010; Kasanah and Triyanto 2019). Diverse marine actinobacterial species capable of producing biologically active compounds, including antimicrobial, anti-tumour, anti-malarial, antioxidant and anti-inflammatory compounds have been discovered in this environment (Jayaprakashvel, 2012). For example, Abyssomycin C, a polycyclic polyethylene antibiotic synthetized by a marine strain of *Verrucosispora*, Diazepinomicin, an alkaloid produced by a *Micromonospora* strain, with antitumor, antimicrobial and anti-inflammatory therapeutic potential, and Streptochlorin, obtained from a marine *Streptomyces* (*Streptomyces roseolilacinus*), exhibiting antiangiogenic, anti-tumour, anti-inflammatory and antibiotic activities (Hassan and Shaikh 2017).

Actinobacteria are a representative group of marine microbial communities (Bull and Stach, 2007), but for decades they were strictly considered terrestrial microorganisms, and when isolated from marine sources they were perceived to be derived from resistant spores of terrestrial species (Jensen et al. 2005). Nonetheless, there are currently strong evidences that support the existence of indigenous species of marine Actinobacteria (Bredholt et al. 2008; Manivasagan et al. 2014b). Examples of actinobacterial genera recovered from

marine environments include *Salinispora*, *Marinospora*, *Micromonospora, Salinobacterium, Marinophilus* and *Streptomyces* (Dharmaraj, 2010). In the marine environment, Actinobacteria are mainly present in sediments but these microorganisms may also be distributed in the planktonic form along the water column or be associated with diverse marine organisms, such as sponges, fishes, molluscs, corals and seaweeds (Abdelmohsen et al., 2014b; Hassan and Shaikh 2017; Genilloud 2018) (Fig. 1).

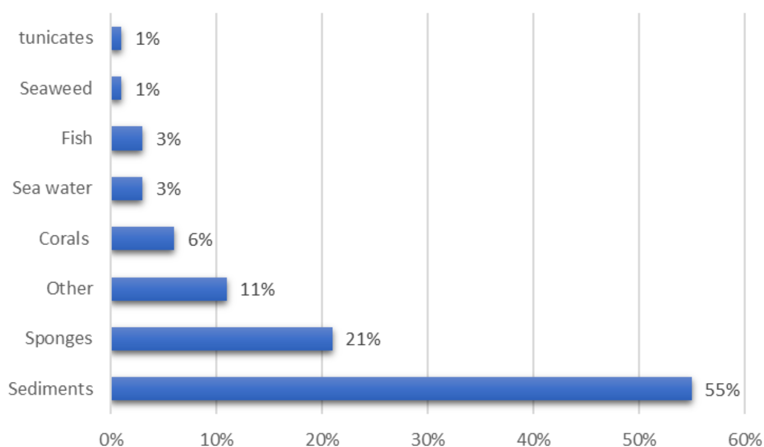

Figure 1. Distribution of Actinobacteria in the marine environment (a total of 10 400 16S rRNA gene sequences of marine Actinobacteria obtained until 2014 were used for the construction of the Figure). Adapted from Abdelmohsen et al. (2014a).

1.6. Marine Sponges

Marine sponges are an ancient group of multicellular ocean animals that are believed to have formed about 600 million years ago (Montalvo et al. 2005). These invertebrate organisms are sessile filter-feeders and have in their surface tiny pores where water enters and circulates across a chain of channels (Thomas *et al.,* 2010). They are affiliated with the phylum Porifera and may be distributed from intertidal to deep zones in the ocean, colonizing in great abundance tropical oceans and also appearing with some frequency in temperate waters (Thomas *et al.,* 2010).

Marine sponges have been a prominent target of research not only due to their ecological importance but also to the production of many bioactive compounds, including alkaloids, terpenes, polyketides, fatty acids and peptides (Bell, 2008). These compounds may be involved in the defence system of the sponge or have ecological functions, but many of them also have a powerful biomedical action (Paul et al., 2019).

A very relevant attribute of these marine organisms is their ability to establish a tremendous variety of symbiotic relationships with several microorganisms, like Cyanobacteria,

Actinobacteria, Fungi, Chloroflexi, Bacteroidetes, Algae and Dinoflagellates (Abdelmohsen et al. 2010; Alex et al. 2013;Xi et al., 2012). It is estimated that 60% of the sponge biomass is microorganisms (Gandhimathi et al. 2008). Some studies have shown that sponges collected from different geographical areas have a similar microbiota, suggesting associations with specific bacterial symbionts (Kuo, 2019). Such symbiotic relationships are possible and have so much success due to the capacity of marine sponges to promote a favourable environment for microbial establishment (Yang et al., 2015). Microbial communities of sponges are involved in various biological processes, such as photosynthesis, nitrogen cycle, sulphate reduction, carbon fixation, with all of these mechanisms having influence in the sponge metabolism (Yang et al., 2015). Additionally, these microbial symbionts also cooperate to defend their host against competitors, predators and microbial pathogens, through the production of diverse bioactive compounds (Brinkmann et al., 2018).

Several studies have shown that Actinobacteria are abundant symbionts of marine sponges (Zhang et al. 2006). Genera like *Actinoalloteichus*, *Micromonospora*, *Nocardiopsis*, *Nocardia* and *Rhodococcus* have been identified in marine sponges (Yang *et al.*, 2015). The abundance of Actinobacteria in marine sponges vary with sponges species but, in some sponges, it can reach 20% of the total microorganisms present (Xi et al., 2012).

The detection of biosynthetic genes encoding polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) in Actinobacteria isolated from marine sponges, suggests a rich presence of bioactive compounds with antibacterial, antifungal, antiparasitic, antimalarial, immunomodulatory, anti-inflammatory, antioxidant and anticancer activities (Abdelmohsen et al. 2014b; Xi et al*.,* 2012).

The presence of unique bacterial communities in marine sponges, many of them novel entities as proven by 16S rRNA gene analysis (Montalvo et al. 2005), indicates that the study of actinobacterial communities of marine sponges is a promising way for the discovery of new drugs.

1.6.1. The marine sponge *Hymeniacidon perlevis*

The intertidal marine sponge *Hymeniacidon perlevis,* belonging to the genera *Demospongiae,* is very abundant and common in the Portuguese coast (Alex et al., 2013; Regueiras et al., 2019). This species is known to be distributed from the Atlantic to the Mediterranean coast, inhabiting hard surfaces that are graved into the sediment and in shells and some marine invertebrates such as nematodes, annelids and crustaceans may also be associated to this species. The species is typically orange to red in colour and has

an uneven surface of smooth texture and compressible consistency from which often emerge small projections (Fig. 2) (Soest et al., 2011).

The actinobacterial community associated with specimens of this sponge distributed by the Portuguese coast has never been studied before.

Figure 2. The marine Sponge *Hymeniacidon perlevis* (from Regueiras et al., 2019).

1.7. Aim and outline of this thesis

Novel pharmaceuticals are immediately needed to combat serious diseases, such as infections by multiresistant bacteria and cancer. Natural compounds produced through the secondary metabolism of marine Actinobacteria have proven to be a promising source of molecules with therapeutic properties that may play a significant role in human health.

This MSc thesis had as objective to study the cultivable actinobacterial community associated with the marine sponge *Hymeniacidon perlevis*, collected in a beach, in northern Portugal, and investigate the antimicrobial and anticancer potential of the isolated actinobacterial strains. A collection of 184 microbial strains previously isolated from this sponge under the scope of another MSc thesis (Ribeiro, 2017) was used as the basis for this study.

This thesis is arranged in five sections. The first section introduces the theme of the thesis, where the phylum Actinobacteria and its biotechnological value is described, focusing particularly on marine Actinobacteria. Section 2 describes in detail the materials and methods used to perform this study and is followed by sections 3 and 4 where the results obtained are, respectively, presented and discussed. The last section includes the main conclusions of the work.

2. Materials and Methods

As referred in the preceding chapter, a collection of 184 microbial strains previously isolated from the sponge *H. perlevis* (Ribeiro, 2017) was used for the accomplishment of the work presented in this thesis. The methodology associated with the sampling of the sponge and subsequent actinobacterial isolation will be included here for a better contextualization of all the work executed.

2.1. Sampling and bacterial Isolation

A sample of the marine Sponge *H. perlevis* was collected in the intertidal zone of Praia da Memória, in Matosinhos, Portugal (41°13.840157'N, 8°43.331863'W). The sample was immersed in water from its natural habitat, transported to the laboratory in a cooling box and processed within a period of 2 hours from the time of collection. The sponge was identified through the analysis of its morphological characteristics by an experienced researcher, as belonging to the species *Hymeniacidon Perlevis.*

In the laboratory, the sponge was washed under sterile conditions with sterile seawater in order to remove loose particles. Then, three pieces, with ca. 1.5 g each, were cut and homogenized separately in a sterile mortar with 2 mL of sterile seawater. The macerated fragments were placed in Falcon tubes of 15 mL and submitted to one of the following pretreatments: incubation in a microwave (2450 MHz) for 3 min at 120 W (pretreatment 1); incubation in a water bath at 60 ºC for 30 min (pretreatment 2) and incubation in an oven at 120 ºC for 60 minutes (pretreatment 3). The treated samples were ten-fold diluted to 10^{-4} and an aliquot of 100 µL of each dilution was inoculated on the following selective culture media: M1 agar (10 g of soluble starch, 4 g of yeast extract, 2 g of peptone and 17 g of agar, per litter of seawater); M2 agar (6 mL of glycerol, 1 g of arginine, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$ and 18 g of agar, per liter of seawater: deionized water in the ratio of (90:10); Starch-Casein-Nitrate agar (10 g of soluble starch, 0.3 g of casein, 2 g of K_2HPO_4, 2 g of KNO_3, 2 g of NaCl, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_20$ and 17 g of agar, per liter of seawater: deionized water in the ratio of 60:40. All media were supplemented with cycloheximide (50 mgL^{-1}), nystatin (50 mgL^{-1}) and nalidixic acid (50 mgL^{-1}) to inhibit the growth of fungi and Gram-negative bacteria. The plates were incubated at 28 ºC for a period up to four months. During incubation, the plates were periodically observed and all colonies with different morphological characteristics were picked and re-streaked various times in the same selective culture medium until obtainment of axenic cultures.

For cryopreservation purposes, each isolate was grown in 5 mL of liquid medium, M1, M2 or SCN, according to the isolation medium where the strain was isolated, at 25 ºC, 100 rpm, for one week. Each isolate was cryopreserved at -80 ºC in 30% (v/v) glycerol.

2.2. Taxonomic Identification of isolated strains

Microbial biomass for DNA extraction was obtained from liquid cultures previously grown for cryopreservation purposes. Samples of 2 mL were collected from the cultures, centrifuged at 7.000 g for 5 min and the resulting pellet was used for DNA extraction. Genomic DNA was extracted using E.Z.N.A® bacterial Kit (Omega Bio-Tek, Norcross, GA), according to manufacturer's instructions. A few modifications steps were introduced to the extraction protocol: (i) as a first step, samples were submitted to a preheating at 95 °C for 10 min, followed by incubation on ice for 10 min; (ii) in the step of lysozyme addition, samples were incubated at 37 °C for 30 min, instead of 10 min as described in the protocol; (iii) in the optional step to facilitate cell lysis, two Zirconia beads (2.3 mm diameter) were added along with the glass beads and the samples were vortexed for 10 min; (iv) incubation with proteinase K was made with a stock at 10 mg mL^{-1} and was extended up to 2h; (v) centrifugation speed described in the protocol was increased in all steps from 10.000 g to 13.000 g; (vi) in the final step of DNA elution, 25 µL of elution buffer were added to the HiBind® DNA Mini Column, instead of 50-100 µL indicated in the protocol.

16S rRNA gene was amplified by PCR using the universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). The PCR mixture (total volume of 10 µL) consisted in: 5 µL of Taq PCR Master Mix (Qiagen, Valencia, CA), 1 µL of primer 27F (2 µM), 1 µL of primer 1492R (2 µM) and 3 µL of DNA template. Negative controls were also included and consisted in replacing in the PCR mixture the DNA template by 3 µL of Nuclease-Free Water. The reaction started with an initial denaturation at 95 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 90 s, 72 °C for 90 s and a final extension at 72 °C for 10 min. PCR was performed in an Applied Biosystems ThermoCycler (Applied Biosystems Inc., Foster City, CA, USA). PCR products were separated on a 1.5% agarose gel containing SYBR Safe (ThermoFisher Scientific, USA), at 150 V for 30 min. Purification and sequencing of the amplified DNA 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 11.1.4. The resulting consensus sequences were used to establish taxonomic affiliation, by comparing these sequences with those of 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 (http://www.ezbioclound.net/) and the Sequence Match tool from the

Ribosomal Database Project (https://rdp.cme.msu.edu). For three potential new isolates, a phylogenetic tree was elaborated employing the Maximum likelihood method with 1000 bootstraps, based on the Tamura-Nei model. For the construction of the tree for each potential new isolate, the ten closest neighbour sequences were selected based on the BLAST results and aligned using the Geneious software. The tree was constructed using the Molecular Evolutionary Genetics Analysis program Version 7.0 (MEGA7) (Kumar et al., 2016).

2.3. Bioactivity Assays

2.3.1.Preparation of Crude Extracts

Crude extracts for the antimicrobial and anticancer assays were prepared by growing each actinobacterial isolate in 100 mL Erlenmeyer flasks containing 30 mL of the respective liquid culture medium (M1, M2 or SCN) (Fig. 3A), without the addition of antibiotics (cycloheximide, nalidixic acid and nystatin). The flasks were incubated at 28 ºC, 100 rpm, in the dark. After 3-5 days (depending on the growth rate of the microorganism), 0.5 g Amberlite® XAD16N resin (Sigma-Aldrich, St. Louis, Mo.) were added to the culture medium and the flasks were incubated for an additional 3 days period (Fig. 3B). The obtained biomass and resin were then harvested by centrifugation (4.600 g for 10 min), freeze-dried and preserved at-20 ºC. The freeze-dried samples were extracted twice, using 30 mL of acetone/methanol 1:1 (v/v) (Fig. 3C) and the organic layer was recovered and

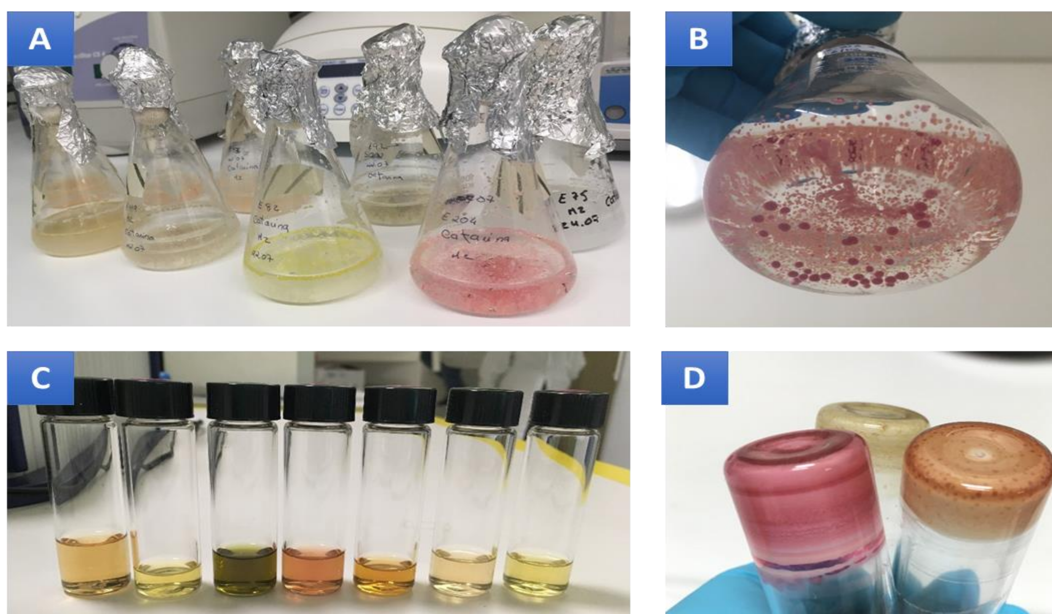

Figure 3. (A) Growth of some actinobacterial strains isolated from the sponge *H. perlevis* in liquid culture medium, (B) Bacterial growth in the presence of the Amberlite® XAD16N resin, (C) Crude extracts obtained from actinobacterial cultures dissolved in methanol, and (D) Dry crude extracts.

dried using a rotary evaporator. Part of the obtained crude extracts was dissolved in DMSO (≥ 99.9%, Sigma-Aldich, USA) for the preparation of stock solutions at the concentration of 10 mg mL^{-1}, 3 mg mL^{-1} and 1 mg mL^{-1} to be used in the bioactivity screenings. The dry crude extracts not dissolved in DMSO were conserved at -20 ºC (Fig. 3D).

2.3.2.Screening of Antimicrobial Activity

The antimicrobial activity of the obtained actinobacterial extracts was tested employing 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 in Muller-Hinton agar (MH; BioKar diagnostics) and the yeast, *Candida albicans*, in Sabouraud Dextrose Agar (SDA; BioKar diagnostics). After growth in solid medium, the test microorganisms were suspended in the corresponding liquid medium and the turbity was adjusted to 0.5 McFarland standard (OD_{625}=0.08-0.13). The suspensions were then used to inoculate agar plates (MH or SDA, according to the test microorganism), by evenly streaking the plates with a swab dipped in the suspension cultures. Blank paper disks (6 mm in diameter; Oxoid) were placed in the surface of the inoculated plates and loaded with 15 µl of crude extract at the concentration of 1 mg mL^{-1}. The positive control consisted in 15 µl of enrofloxacin (1 mg mL^{-1}) for the bacterial strains and 15 µl of nystatin for *C. Albicans,* and the negative control consisted in 15 µl of DMSO. The plates were incubated at 37 ºC for 24 h, and antimicrobial activity was determined by measuring the diameter of the inhibition zone formed around the disks. For extracts showing inhibition halos, minimal inhibitory concentration (MIC) was also determined (Wiegand et al., 2008). Inoculum suspensions of the reference microorganisms were prepared as described above. Stock solutions of extracts were prepared in the appropriate medium broth at the concentration of 2 000 µg mL^{-1}. Eleven two-fold dilutions were successively executed from the previous stock solutions, obtaining extracts solutions with concentrations ranging from 2 000 µg mL^{-1} to 1.95 µg mL^{-1}. The assay was performed in 96 well plates and each well included 50 µL of the microbial inoculum (diluted 1:100) and 50 µL of each extract dilution. All dilutions were tested in triplicate. The positive growth control consisted in 50 µL of microbial inoculum and 50 µL of medium broth and the negative growth control in 100 µL of medium broth. A DMSO control was also performed and consisted in testing several dilutions of this solvent in inoculum growth. DMSO was initially diluted five times and then, three two-fold dilutions were successively performed from this first solution. In each well, 50 µL of each DMSO dilution and 50 µL of microbial inoculum were added. The MIC was assessed by spectrophotometric analysis (570 nm) after 18 h at 37 ºC.

2.3.3. Screening of anticancer activity

The anticancer activity of actinobacterial extract was tested in two human cancer cell lines: human liver cancer (HepG2) and human colorectal cancer (HCT-116). The cell line HepG2 was grown in Dulbeco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts) supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 1% (v/v) penincillin/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 cell line HCT-116 was grown in McCoy's (Modified) medium in the same conditions of the cell line HepG2.

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, performed in triplicate. 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 extract solutions at a final concentration of 15 µg mL^{-1}. Solvent and positive controls consisted in 0.5% DMSO (Sigma-Aldrich, USA) and 20% DMSO, respectively. Cell viability was evaluated at 24 and 48 h by adding MTT at a final concentration of 0.2 mg mL^{-1} and incubating for 4 h at 37 °C. After this time, the medium was removed and 100 µL of DMSO were added per well, after which the absorbance was read at 570 nm (Synergy HT, Biotek, USA). Cellular viability was expressed as a percentage relative to the negative control.

2.4. Statistical analysis

Data from anticancer assays was tested for significant differences compared to the solvent control. The significance level was set for all tests at $p < 0.05$. The normality of data distribution was verified using the Kolmogorov Smirnov test. As all data followed normal distribution, one-Way ANOVA was applied followed by Dunnett's post hoc test.

3. Results

3.1. Phylogenetic identification of Actinobacteria associated with the marine sponge *Hymeniacidon perlevis*

Under the scope of a previous thesis, 184 bacterial strains were isolated from the marine sponge *H. perlevis*. Fig. 4 shows some examples of the morphological diversity of the isolates obtained. Many of these isolates exhibited morphologies typical of Actinobacteria, such as production of spores and hyphae, slow growth, production of pigments altering in some cases the colour of the culture medium, and rough colonies.

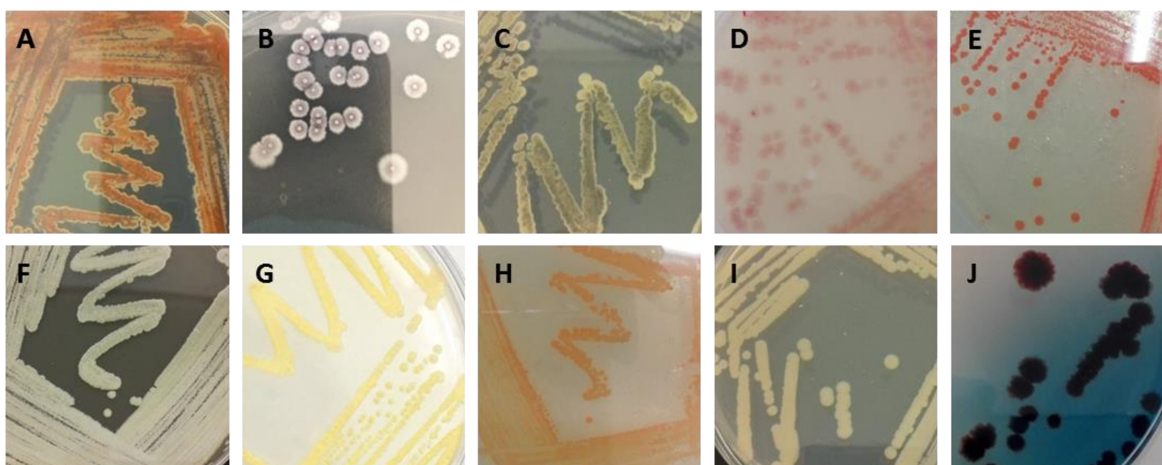

Figure 4. Examples of actinobacterial strains isolated from the marine sponge *H. perlevis*. (A) strain E226, (B) strain E102, (C) strain E5, (D) strain E204, (E) strain E196, (F) strain E18, (G) strain E215, (H) strain E66, (I) strain E37, (J) strain E230.

Of the 184 bacterial isolates obtained from *H. perlevis*, 119 strains were phylogenetically identified through 16S rRNA gene sequencing (Table 2). The results showed that 82 isolates were affiliated with the phylum Actinobacteria, 34 belonged to the phylum Firmicutes and 3 were associated with the phylum Proteobacteria (Fig. 5A). Sixty-five isolates were not possible to identify because PCR reactions or sequencing of amplified products failed.

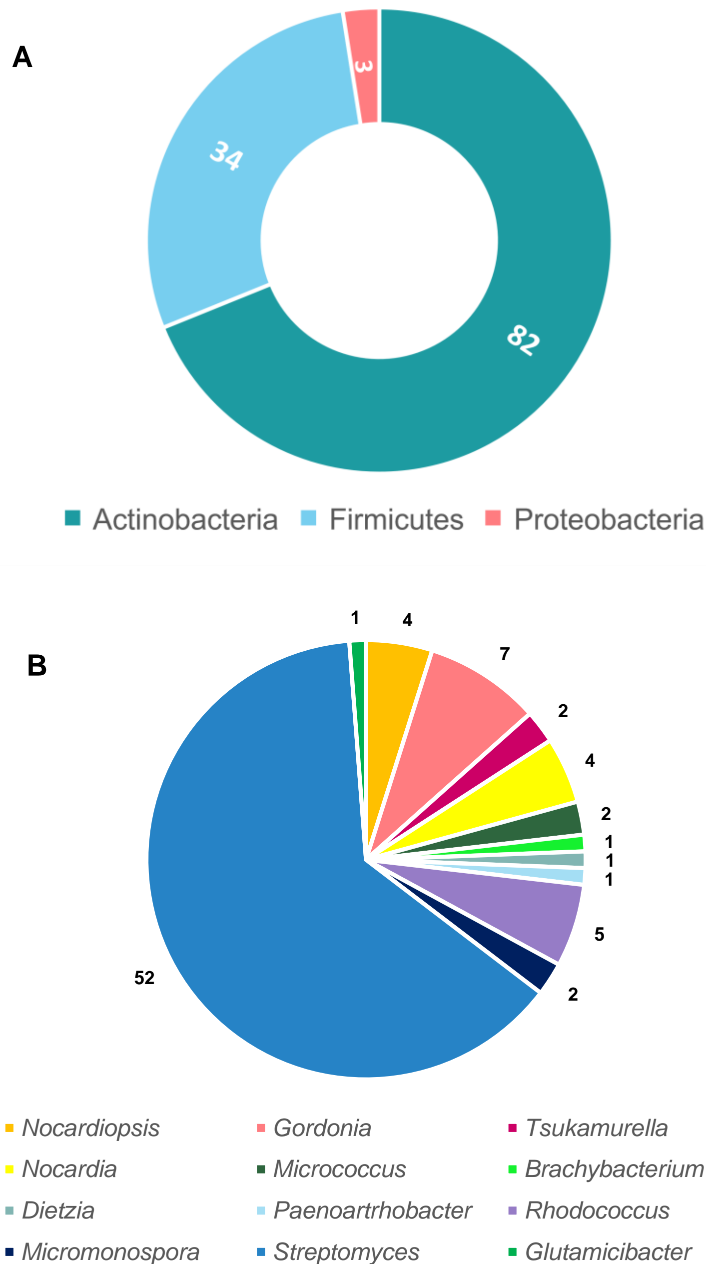

Figure 5. Number of strains isolated from *H. perlevis* affiliated with the shown phyla (A) and actinobacterial genera retrieved from *H. perlevis* and respective number of isolates (B).

The actinobacterial isolates identified were distributed by twelve genera: *Streptomyces*, *Gordonia*, *Rhodococcus*, *Nocardiopsis*, *Nocardia*, *Micrococcus*, *Tsukamurella*, *Paenoartrhobacter*, *Micromonospora*, *Brachybacterium, Glutamicibacter* and *Dietzia*, with the largest fraction of the actinobacterial isolates being affiliated with the genus *Streptomyces* (Fig. 5B). Regarding the isolates related with the phylum Firmicutes, 33 were affiliated with the genus *Bacillus* and 1 was affiliated with the genus *Staphylococcus*, while

the 3 isolates belonging to the phylum Proteobacteria were distributed by the genera *Loktanella*, *Paracoccus* and *Marinovum*.

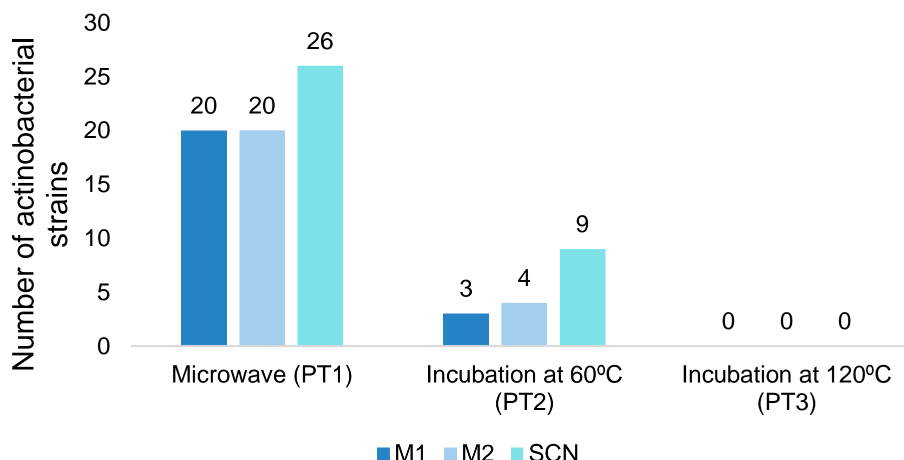

Figure 6. Distribution of the number of actinobacterial strains recovered from *H. perlevis*, according to the pretreatment applied and selective culture medium used.

Pretreatment 1 (microwave incubation) allowed the isolation of 66 actinobacterial strains (Fig.6), of which more than half (39) were affiliated with the genus *Streptomyces* (Fig. 7A). With the application of this pretreatment, 10 additional actinobacterial genera were also obtained (Fig. 7A).

Pretreatment 2 (incubation at 60 ºC) led to the isolation of 16 actinobacterial strains mainly belonging to the genus *Streptomyces*, but also to the genera *Gordonia* and *Micromonospora* (Figs. 6 and 7A). Pretreatment 3 (incubation at 120 ºC) proved to be too aggressive as no actinobacterial strains were recovered with this pretreatment (Fig. 6).

Regarding the three selective culture media used in the study, SCN led to a higher recovery of actinobacterial isolates, with 35 actinobacterial strains being isolated, while M1 and M2 media allowed the recovery of, respectively, 23 and 24 strains (Fig. 6). Isolates affiliated with the genera *Streptomyces* and *Nocardiopsis* were retrieved from the three selective media (Fig. 7B). Strains of the genera *Rhodococcus* and *Micrococcus* were only recovered from SCN and M1 media, while *Nocardia* strains were identified in M1 and M2 media. Actinobacterial strains of the genera *Tsukamurella*, *Brachybacterium*, *Dietzia*, *Micromonospora*, *Paenoarthrobacter* and *Glutamicibacter* were recovered from only one selective medium (Fig. 7B).

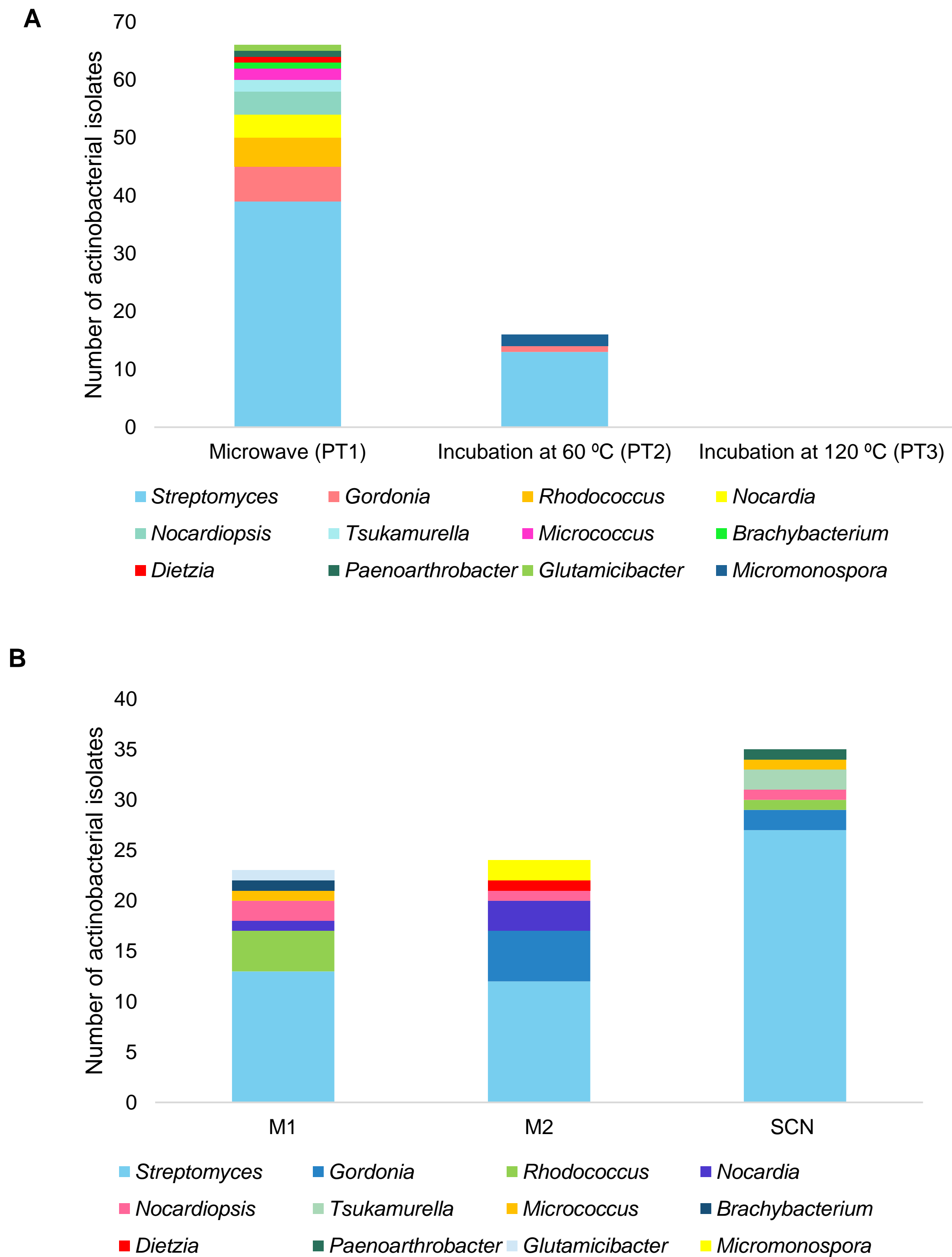

Figure 7. Distribution of actinobacterial genera recovered from *H. perlevis* according to (A) pretreatment and (B) selective culture medium used for isolation.

Phylogenetic analysis of the isolates revealed three potential new species (Table 2), according to the established similarity threshold for new species of 98.7% (Stackebrandt et al., 2006). From the analysis of the phylogenetic trees of strains E77, E194 and E228, it is possible to observe that these isolates always appear in a branch different from their closest neighbours, supporting the evidence that these strains may be new species (Fig. 8).

Table 2 – Phylogenetic identification of the isolates recovered from the marine sponge *H. perlevis*.

Isolate	Pretreatment	Culture Medium	Closest Identification	Phylum	*Similarity (%)
E2	1	M1	*Streptomyces gougerotii*	Actinobacteria	99.86
E3	1	M1	*Rhodococcus hoagii*	Actinobacteria	99.93
E5	1	M1	*Streptomyces anulatus*	Actinobacteria	99.93
E7	1	M1	*Nocardiopsis alba*	Actinobacteria	99.93
E9	1	M1	*Bacillus kochii*	Firmicutes	99.44
E10	1	M1	*Streptomyces olivaceus*	Actinobacteria	99.86
E12	1	M1	*Bacillus liqueniformis*	Firmicutes	99.30
E13	1	M1	*Streptomyces sp.*	Actinobacteria	99.93
E16	1	M1	*Streptomyces gougerotii*	Actinobacteria	99.86
E18	1	M1	*Streptomyces griseobrunneus*	Actinobacteria	99.78
E21	1	M1	*Streptomyces sampsonii*	Actinobacteria	99.93
E24	1	M1	*Terribacillus saccharophilus*	Firmicutes	99.76
E27	1	M1	*Solibacillus silvestris*	Firmicutes	99.51

E28	1	M1	*Gracilibacillus dipsosauri*	Firmicutes	99.79
E31	1	M1	*Bacillus kochii*	Firmicutes	99.58
E32	1	M1	*Ornithinibacillus scapharcae*	Firmicutes	98.51
E33	1	M1	*Bacillus licheniformis*	Firmicutes	98.10
E37	1	M1	*Glutamicibacter arilaitensis*	Actinobacteria	99.86
E38	1	M1	*Staphylococcus warneri*	Firmicutes	99.79
E41	1	M1	*Rhodococcus erythropolis*	Actinobacteria	99.64
E42	1	M1	*Nocardiopsis alba*	Actinobacteria	99.50
E43	1	M1	*Bacillus aerius*	Firmicutes	99.93
E45	1	M1	*Brachybacterium paraconglomeratum*	Actinobacteria	99.71
E49	1	M1	*Nocardia salmonicida*	Actinobacteria	99.42
E51	1	M1	*Lysinibacillus fusiformis*	Firmicutes	99.86
E52	1	M1	*Loktanella acticola*	Proteobacteria	99.26
E54	1	M1	*Micrococcus yunnanensis*	Actinobacteria	99.50
E59	1	M2	*Streptomyces janthinus*	Actinobacteria	99.88

E61	1	M2	*Streptomyces pratensis*	Actinobacteria	99.85
E65	1	M2	*Streptomyces pratensis*	Actinobacteria	100
E66	1	M2	*Gordonia sputi*	Actinobacteria	99.56
E66A	1	M2	*Streptomyces drozdowiczii*	Actinobacteria	99.56
E67	1	M2	*Nocardia salmonicida*	Actinobacteria	99.27
E67B	1	M2	*Nocardia salmonicida*	Actinobacteria	99.27
E74	1	M2	*Streptomyces parvus*	Actinobacteria	99.78
E75	1	M2	*Dietzia maris*	Actinobacteria	100
E77	1	M2	*Gordonia bronchialis*	Actinobacteria	98.39
E79	1	M2	*Nocardiopsis prasina*	Actinobacteria	99.85
E80	1	M2	*Streptomyces griseoaurantiacus*	Actinobacteria	99.57
E82	1	M2	*Streptomyces pratensis*	Actinobacteria	99.93
E83	1	M2	*Gordonia sputi*	Actinobacteria	99.86
E85	1	SCN	*Streptomyces sp*	Actinobacteria	99.71
E87	1	SCN	*Streptomyces sp*	Actinobacteria	99.70

E88	1	SCN	*Streptomyces diastaticus*	Actinobacteria	99.42
E90	1	SCN	*Streptomyces olivoverticillatus*	Actinobacteria	99.71
E92	1	SCN	*Streptomyces sp*	Actinobacteria	99.71
E94	1	SCN	*Streptomyces albogriseolus*	Actinobacteria	99.71
E96	1	SCN	*Streptomyces hygroscopicus*	Actinobacteria	99.78
E99	1	SCN	*Streptomyces sp*	Actinobacteria	99.85
E102	1	SCN	*Streptomyces sp*	Actinobacteria	99.93
E103	1	SCN	*Streptomyces sampsonii s*	Actinobacteria	99.71
E104	1	SCN	*Streptomyces sampsonii*	Actinobacteria	99.57
E106	1	SCN	*Streptomyces sampsonii*	Actinobacteria	99.93
E107	1	SCN	*Streptomyces fulvissimus*	Actinobacteria	100
E108	1	SCN	*Paracoccus mangrovi*	Proteobacteria	98.73
E109	1	SCN	*Gordonia terrae*	Actinobacteria	99.93
E113	1	SCN	*Streptomyces pratensis*	Actinobacteria	99.45
E114	1	SCN	*Nocardiopsis prasina*	Actinobacteria	99.79

E115	1	SCN	*Streptomyces olivaceus*	Actinobacteria	99.78
E116	1	SCN	*Paenarthrobacter nicotinovorans*	Actinobacteria	99.13
E117	1	SCN	*Tsukamurella ocularis*	Actinobacteria	99.71
E119	1	SCN	*Streptomyces sp.*	Actinobacteria	99.85
E121	1	SCN	*Streptomyces amphotericinicus*	Actinobacteria	99.14
E122	1	SCN	*Streptomyces camponoticapitis*	Actinobacteria	99.57
E123	1	SCN	*Streptomyces pratensis*	Actinobacteria	99.55
E126	2	M1	*Bacillus licheniformis*	Firmicutes	98.05
E128	2	M1	*Bacillus licheniformis*	Firmicutes	99.72
E129	2	M1	*Streptomyces sampsonii*	Actinobacteria	99.71
E130	2	M1	*Oceanobacillus polygoni*	Firmicutes	99.16
E131	2	M1	*Bacillus gibsonii*	Firmicutes	99.51
E135	2	M1	*Bacillus kochii*	Firmicutes	99.50
E138	2	M1	*Bacillus safensis*	Firmicutes	99.93
E139	2	M1	*Bacillus licheniformis*	Firmicutes	98.73

E146	2	M1	*Streptomyces sampsonii*	Actinobacteria	99.93
E147	2	M1	*Bacillus aerius*	Firmicutes	99.23
E150	2	M1	*Bacillus circulans*	Firmicutes	99.86
E152	2	M1	*Bacillus clausii*	Firmicutes	99.88
E154	2	M1	*Bacillus aerius*	Firmicutes	99.86
E157	2	M1	*Oceanobacillus aidingensis*	Firmicutes	98.92
E159	2	M1	*Bacillus plakortidis*	Firmicutes	99.79
E160	2	M1	*Bacillus sp.*	Firmicutes	99.51
E164	2	SCN	*Bacillus liqueniformis*	Firmicutes	99.78
E164A	2	SCN	*Bacillus licheniformis*	Firmicutes	100
E165	2	SCN	*Streptomyces matensis*	Actinobacteria	99.35
E167	2	SCN	*Streptomyces sampsonii*	Actinobacteria	99.78
E171	2	SCN	*Streptomyces sp*	Actinobacteria	99.93
E172	2	SCN	*Streptomyces cellulosae*	Actinobacteria	100
E173	2	SCN	*Streptomyces thermocarboxydus*	Actinobacteria	99.93

E176	2	M2	*Bacillus kochii*	Firmicutes	99.51
E178	2	M2	*Bacillus licheniformis*	Firmicutes	99.79
E184	2	M2	*Bacillus sp.*	Firmicutes	99.72
E185	2	M2	*Bacillus haynesii*	Firmicutes	99.51
E193	1	M1	*Marinovum algicola*	Proteobacteria	99.56
E194	1	M2	*Nocardia salmonicida*	Actinobacteria	98.62
E195	1	M2	*Gordonia sputi*	Actinobacteria	99.56
E196	1	M2	*Gordonia sputi*	Actinobacteria	99.93
E197	1	SCN	*Tsukamurella pseudospumae*	Actinobacteria	99.93
E198	2	SCN	*Bacillus licheniformis*	Firmicutes	97.39
E199	2	SCN	*Bacillus liqueniformis*	Firmicutes	99.72
E200	2	SCN	*Streptomyces sp.*	Actinobacteria	99.78
E201	2	SCN	*Gordonia sputi*	Actinobacteria	99.85
E202	2	SCN	*Streptomyces sampsonii*	Actinobacteria	100
E203	2	SCN	*Streptomyces sp.*	Actinobacteria	100

E204	1	M2	*Streptomyces diastaticus*	Actinobacteria	99.44
E206	1	M1	*Rhodococcus hoagii*	Actinobacteria	99.20
E209	1	SCN	*Streptomyces sp*	Actinobacteria	99.85
E210	1	M1	*Rhodococcus hoagii*	Actinobacteria	99.86
E215	2	M1	*Streptomyces griseorubens*	Actinobacteria	99.86
E221	1	SCN	*Rhodococcus sp*	Actinobacteria	98.92
E226	1	M1	*Streptomyces sp*	Actinobacteria	99.86
E227	1	M1	*Streptomyces sp*	Actinobacteria	99.71
E228	2	M2	*Streptomyces sampsonii*	Actinobacteria	98.34
E229	1	M2	*Streptomyces thermocarboxydus*	Actinobacteria	100
E230	1	M1	*Streptomyces sp*	Actinobacteria	100
E232	1	SCN	*Micrococcus yunnanensis*	Actinobacteria	99.71
E243	2	M2	*Micromonospora aurantiaca*	Actinobacteria	99.76
E244	2	M2	*Micromonospora echinofusca*	Actinobacteria	99.28
E247	2	M2	*Streptomyces sp*	Actinobacteria	99.93

(*according to 16s ribosomal RNA (Bacteria and Archea) data base from NCBI)

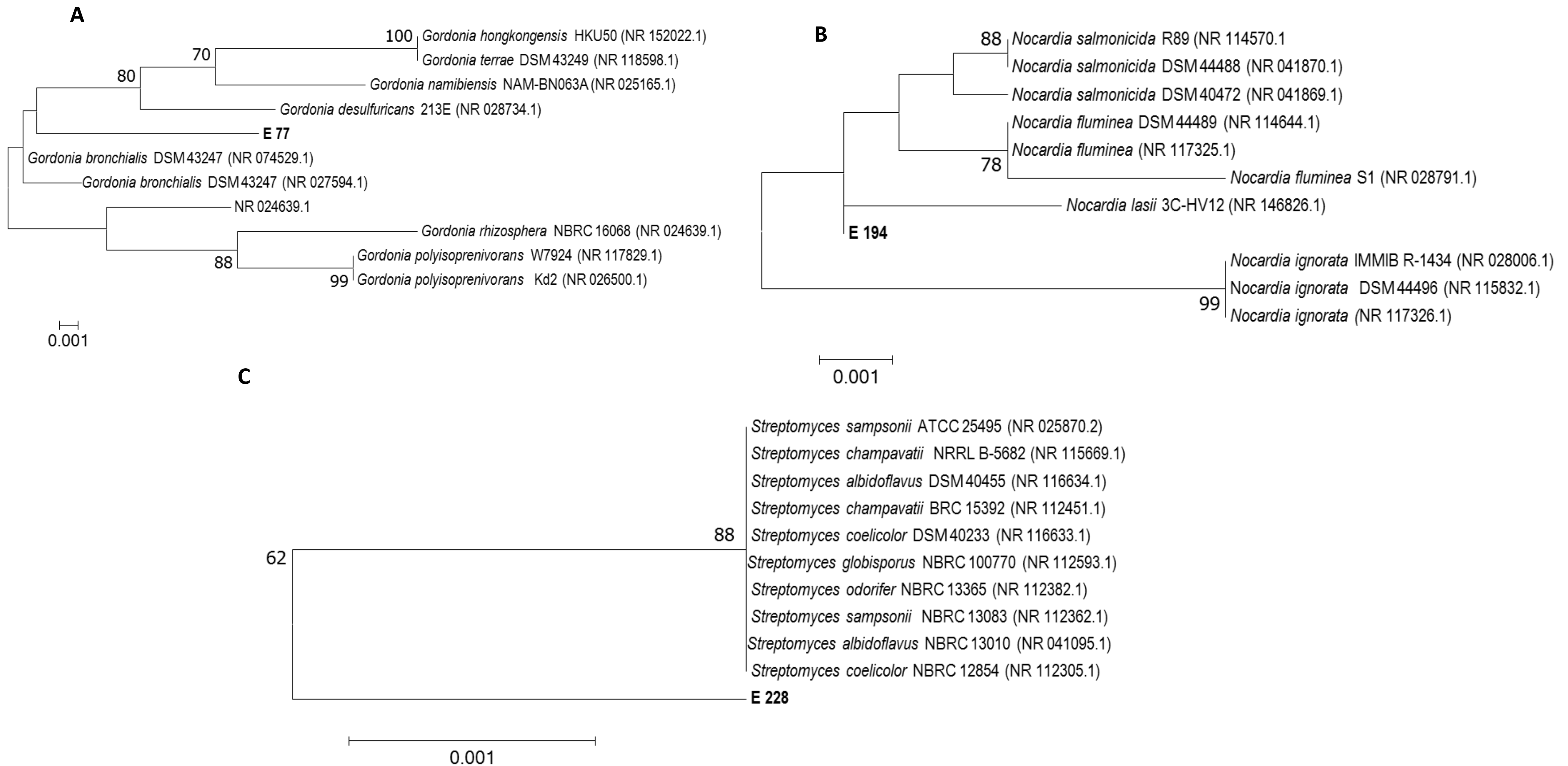

Fig. 8 - Phylogenetic relationship of the three potential new actinobacterial isolates recovered from *H. perlevis* based on 16S rRNA gene homology with their GenBank nearest neighbours. Numbers at nodes represent bootstrap values. Numbers in parenthesis correspond to GenBank accession numbers. (A) Strain E77, (B) Strain E194 and (C) Strain E228.

3.2. Screening of the bioactive potential of the isolated actinobacterial strains

The bioactive potential of the actinobacterial strains isolated from *H. perlevis* was tested by preparing crude extracts from liquid cultures of these strains and testing them for their antimicrobial and anticancer activities. During the timeframe of this thesis, only 57 actinobacterial crude extracts could be tested.

Antimicrobial assays allowed the identification of 7 actinobacterial extracts (of strains E10, E13, E74, E106, E115, E129 and E146, all *Streptomyces*) with ability to inhibit the growth of *Candida albicans* or *Bacillus subtilis* (Fig. 9 and Table 3).

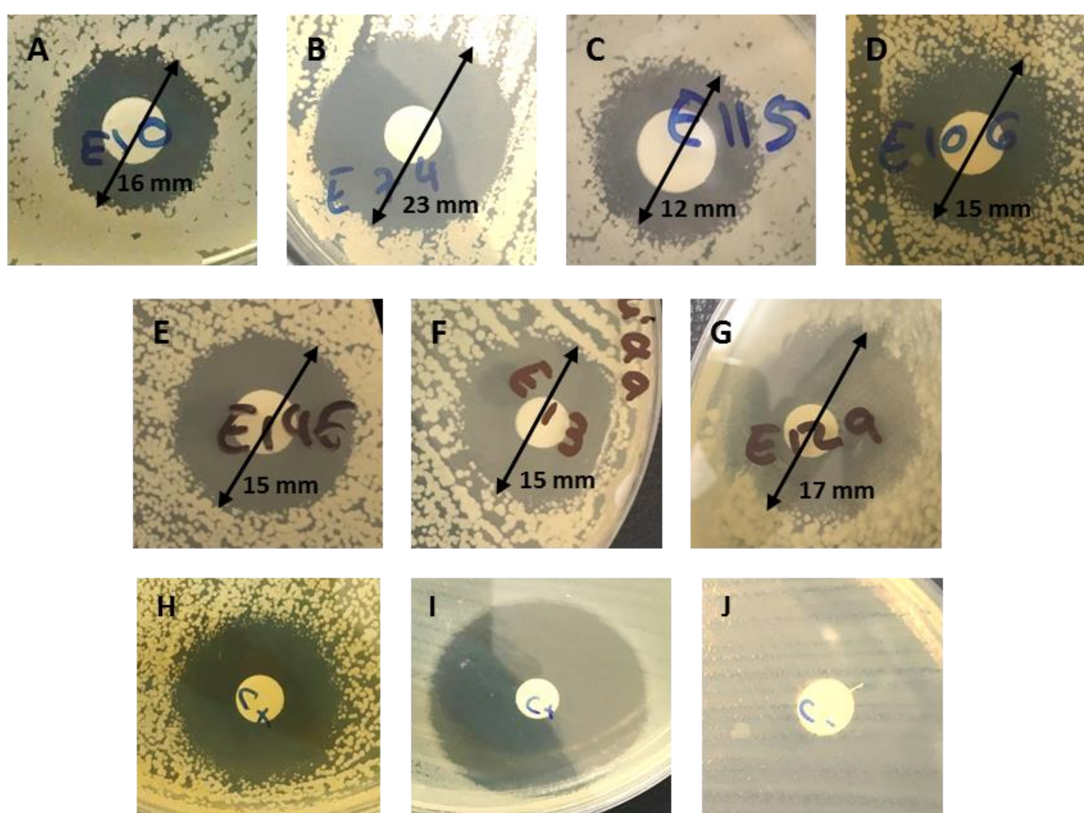

Figure 9. Inhibition halos obtained in the antimicrobial assays. A, B and C, inhibition halos against *Bacillus subtilis* caused by extracts from strains E10 (A), E74 (B) and E115 (C); D, E, F and G, inhibition halos against *Candida albicans* caused by extracts from strains E106 (D), E146 (E), E13 (F), E129 (G), Positive control (nystatin) (H), Positive control (enrofloxacin) (I) and Negative control (DMSO) (J).

Extracts of isolates E13, E106, E129 and E146 showed activity against *Candida albicans*, while extracts of isolates E10, E74 and E115 exhibited activity against *Bacillus subtilis*. For these crude extracts, MIC values were additionally determined, showing values ranging

from 15.62 to 125 µg mL^{-1} (Table 3). Crude extracts of strains E106 and E129 (both identified as *Streptomyces sampsonii*) showed the lowest MIC value against *Candida albicans* (31.25 µg mL^{-1}). The isolate E146, also belonging to the same species, presented a higher MIC value (125 µg mL^{-1}). The crude extract of strain E74 had the lowest MIC value against *Bacillus subtilis* (15.62 µg mL^{-1}).

Table 3 – Antimicrobial activitiy of crude extracts of actinobacterial strains isolated from the marine sponge *H. perlevis*. Diameter of inhibition halos and MIC values are shown for all strains with antimicrobial activity.

Isolates	Taxonomic Identification	Disk diffusion method			
		Diameter of Inhibition Halo (mm)		MIC (µg mL^{-1})	
		Candida albicans	***Bacillus subtilis***	***Candida albicans***	***Bacillus subtilis***
E10	*Streptomyces olivaceus*	0	16	ND	62.5
E13	*Streptomyces sp.*	15	0	125	ND
E74	*Streptomyces parvus.*	0	23	ND	15.62
E106	*Streptomyces sampsonii*	15	0	31.25	ND
E115	*Streptomyces olivaceus*	0	12	ND	62.5
E129	*Streptomyces sampsonii*	17	0	31.25	ND
E146	*Streptomyces sampsonii*	15	0	125	ND

*ND- Not determined.

The 57 actinobacterial crude extracts tested for antimicrobial activity were also investigated for their anticancer potential using the MTT assay. Results showed that 41 extracts had statistically significant activity against at least one of the tested human cancer cell lines (HepG2 or HCT-116) when compared to the solvent control. Twenty-one extracts decreased the viability of HepG2 cells at least 7,6%, while 33 extracts reduced the viability of HCT-116 cells in at least 15,16% (Fig. 10). Extracts of strains E194, E196, E197, E201, E221 and E228 (belonging to the genera *Nocardia*, *Gordonia*, *Tsukamurella*, *Gordonia*, *Rhodococcus* and *Streptomyces*, respectively) were able to reduce the viability of the cell line HepG2 in more than 80%, after 48 h of exposure. The first two extracts were also able to reduce the viability of the cell line HCT-116 in ca. 40% after 48 h. Strains E18 and E119

(*Streptomyces griseobrunneus* and *Streptomyces sp.*, respectively) reduced the viability in more than 60% of the cell line HCT-116, after 48 h of exposure (Fig. 10).

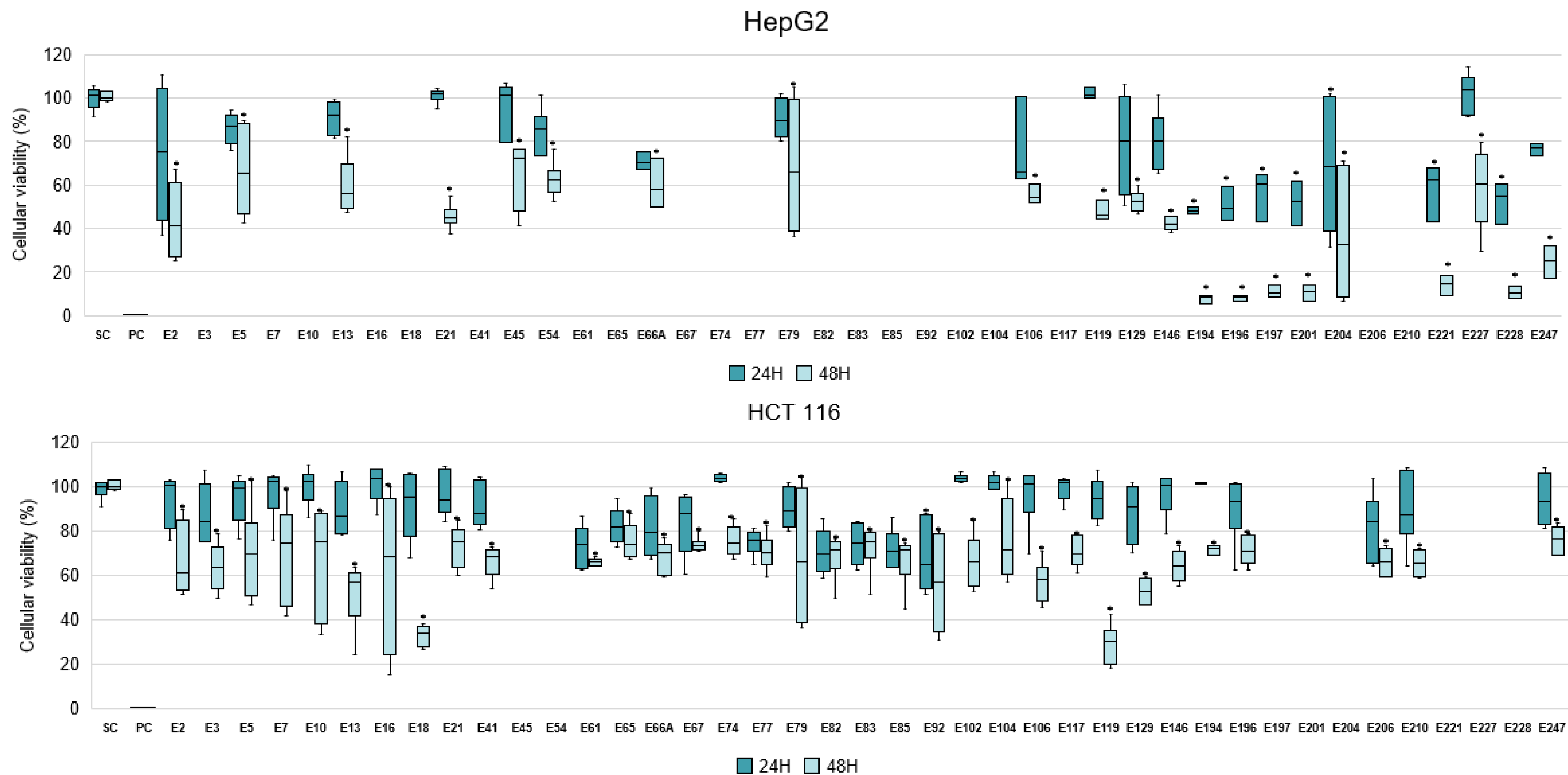

Figure 10. Percentage of cell viability of the cell lines Human Liver Cancer (HepG2) and Human Colon-Rectal Cancer (HCT-116), after 24 h and 48 h of exposure to crude extracts of actinobacterial strains isolated from *H. perlevis*. Only extracts showing statistically significant differences are shown in the figure. PC and SC indicate positive and solvent controls, respectively. *Statistically significant.

4. Discussion and Conclusions

4.1 Discussion

In recent years, many species of marine microorganisms have proved to be a promising source for the bioprospection of novel drugs (Santos et al. 2019). Sponges and their symbiotic microbiome have been described to be responsible for the production of many of these compounds (Parages et al., 2017). Actinobacteria are well represented in sponges microbiome and are considered high producers of bioactive molecules (Abdelfattah et al. 2019).

In this study, the cultivable community of Actinobacteria associated with the marine sponge commonly found in the Portuguese coast, *H. perlevis*, was investigated having as starting point a collection of 184 bacterial strains previously isolated. Phylogenetic analysis led so far to the identification of 82 actinobacterial strains affiliated with twelve genera, revealing that the studied sponge is rich in Actinobacteria, both in terms of abundance and phylogenetic diversity. More than half (52) of the identified strains were *Streptomyces*, which is an important result given the particular potential of members of this genus to produce bioactive substances (Sivalingam et al. 2019). Three potential new species associated with the genera *Streptomyces*, *Nocardia* and *Gordonia* were identified, based on the similarity threshold between 16S rRNA gene sequences of 98.7% (Stackebrandt and Ebers, 2006), opening good perspectives for the discovery of novel compounds. In addition to Actinobacteria, several isolates associated with the phylum Firmicutes, mostly *Bacillus* species, and three Proteobacteria strains were also identified. The fact that most of the retrieved Firmicutes strains produce endospores explain their survival to the pre-treatments and antibiotics used in this study for the selective isolation of Actinobacteria, however this does not apply to the identified Proteobacteria species. Since these latter species are Gram-negative and the antibiotic nalidixic acid was used in the isolation culture media with the purpose of inhibiting the growth of such microorganisms, one can conclude that the retrieved Proteobacteria strains are resistant to this antibiotic. Interestingly, these strains were reported in the literature to be isolated from marine related environments (Chen et al., 2017; Martens et al., 2006; Park et al., 2017).

In this study, different pretreatments and selective culture media were employed to optimise the isolation and growth of actinobacterial strains, a strategy followed by several researchers (Tiwari and Gupta 2016). The treatment of *H. perlevis* with microwave radiation (pretreatment 1) led to the isolation of a high number of actinobacterial strains affiliated with 11 genera (*Streptomyces*, *Gordonia*, *Rhodococcus*, *Nocardia*, *Nocardiopsis*, *Tsukamurella*,

Micrococcus, *Brachybacterium*, *Dietzia*, *Paenoarthrobacter* and *Glutamicibacter*). The success of this pretreatment in the isolation of Actinobacteria has been reported before. Bredholdt et al. (2008) submitted sediment from Trondheim fjord, in Norway, to microwave radiation for the isolation of Actinobacteria and the authors were able to isolate 2689 strains, most of them of the genera *Streptomyces*, *Micromonospora*, *Rhodococcus*, *Nocardia* and *Nocardiopsis*. Wang et al. (2013) also used microwave radiation to isolate Actinobacteria from soil samples from the Taibai Mountain, on the Shaanxi Province, in China, and similarly obtained a high number of actinobacterial isolates.

Pretreatment 2 used in the present study, consisting in incubating a sample of *H. perlevis* at 60 ºC for 30 min, allowed the isolation of 16 actinobacterial strains belonging to only 3 genera: *Streptomyces*, *Gordonia* and *Micromonospora*, showing to be more selective in the isolation of these microorganisms. This pretreatment has been reported in other studies to be effective in the isolation of slower-growing Actinobacteria, other than *Streptomyces*, however in this study this treatment did not seem to have the same effect. Öner et al., (2014) also applied a heat pretreatment to study the diversity of cultivable Actinobacteria associated with various species of marine sponges collected from the eastern Mediterranean Sea. The authors found that the incubation of the samples at 55 °C for 6 min led to the retrieval of a limited number of actinobacterial isolates, suggesting that actinobacterial strains associated with marine sponges may be particularly sensitive to heat.

Pretreatment 3 (incubation at 120 ºC for 60 min) revealed to be extremely selective, with no isolates being obtained. This pretreatment has been described in other studies to be efficient in the isolation of rare and slow-growing actinobacterial genera, but in this study it did not result (Hayakawa et al., 1991; Bredholt et al. 2008).

Three different selective culture media were used for the isolation of Actinobacteria from *H. perlevis*: M1, M2 and SCN, all made with seawater to simulate the sponge's natural environment. The majority of actinobacterial isolates were recovered from the culture medium SCN (66), followed by M2 (24) and M1 (23). The efficiency of the SCN medium in the isolation of actinobacterial strains has been reported in other studies. Girão et al., (2019) investigated the diversity of actinobacterial strains associated with the macroalgae *Laminaria ochroleuca* and found that, from the three selective media employed in the study (SCN, Raffinose-Histidine and Nutrient-Poor Sediment Extract agar), the one that allowed the isolation of the highest number of strains was SCN. Maldonado et al., (2005) also isolated a significant number of actinobacterial strains from marine sediment samples derived from the Japan Trench, Canary basin and selected fjords in Norway using the medium SCN.

The diversity of Actinobacteria associated with the sponge *H. perlevis* has been studied before with samples collected in the Yellow Sea and in the South China Sea, both in China (Sun et al., 2010; Zang et al., 2006). However, there is scientific controversy about the distribution of this species in China and some scientists believe that the sponge used in these studies was misidentified (Regueiras et al., 2019). One of the studies with this putative sponge collected in the Yellow Sea was performed by Zang et al., (2006). In that study, the authors used eight selective media (including M1 and M2) for the isolation of Actinobacteria and were able to recover 106 isolates. Similarly to the present study, the major fraction of the isolated actinobacterial strains was *Streptomyces* (74%), though the genera *Rhodococcus*, *Nocardiopsis*, *Nocardia*, *Actinoalloteichus*, *Pseudonocardia* and Micromonospora were also recovered. Sun et al. (2010) investigated the diversity of Actinobacteria associated with a presumed sample of *H. perlevis* collected in the Yongxing Island, in the South China Sea, and isolated eight genera: *Mycobacterium*, *Amycolatopsis*, *Arthrobacter*, *Brevibacterium*, *Microlunatus*, *Nocardioides*, *Pseudonocardia* and *Streptomyces.* The authors also employed in their study the selective media M1 and M2 and, while medium M1 did not led the isolation of colonies with specific characteristics of Actinobacteria, medium M2 allowed the isolation of microorganisms belonging to the genera *Nocardia*, *Rhodococcus*, *Gordonia* and *Streptomyces*. Comparing with the studies cited, the present study allowed the recovery of a greater diversity in terms of isolated genera belonging to the phylum Actinobacteria. In addition, the actinobacterial genera isolated in the present study from *H. perlevis* have been also associated with other marine sponges, like *Xestospongia* sp. (Montalvo et al., 2005; Thawai et al. 2017), *Cynachyra* sp. (Khan et al., 2012), *Haliclona sp.* (Jiang et al., 2007), *Halicondria panacea* (Schneemann et al., 2010) and *Spongia officinalis* (Vijayan et al., 2017). Similarly to this work, the genus Streptomyces has been reported as the dominant one.

In the present study, 57 actinobacterial strains were screened for their antimicrobial and anticancer potential. Antimicrobial assays revealed 7 actinobacterial crude extracts, all of them from *Streptomyces* strains, active against *Candida albicans* or *Bacillus subtilis*. The capacity of marine *Streptomyces* to produce antifungal compounds has been reported before. For example, *Streptomyces zhaozhouensis* is known to produce a polycyclic tetramic acid macrolactam (isoikarugamycin) active against *Candida albicans*, having a MIC value of 2-4 µg mL^{-1} (Lacret et al., 2015); a *Streptomyces* strain isolated from the marine sponge *Aplysina fistularis* produces the polyketide Saadamycin that also inhibits the growth of *Candida albicans*, with a MIC of 1–5 µg Ml^{-1}. When comparing these MIC values to the ones obtained in the presented study, one can see that the reported values are well above

the ones found here, however it is important to note that, unlike the reported studies, crude extracts and not pure compounds were tested.

Streptomyces strains isolated from several marine samples have also been reported to be active against *Bacillus subtilis*. Valli et al., (2012) evaluated the antimicrobial activity of actinobacterial strains isolated from marine sediment samples collected from Royapuram, Muttukadu and Mahabalipuram sea shores and Adyar estuary, in India, and, similarly to our study, some *Streptomyces* strains showed antimicrobial activity against *Bacillus subtilis*. Also, Manikandan et al. (2019) investigated the antimicrobial potential of *Streptomyces chumphonensis* BDK01 isolated from marine sediment collected from Palk Strait, Bay of Bengal, in India, and the strain showed activity against a *Bacillus* species.

Anticancer activity was also screened in this study for the same 57 actinobacterial strains, using the MTT assay. This assay is a convenient tool to assess the preliminary anticancer activity. It evaluates cellular viability through the measurement of mitochondrial dehydrogenase activity. In living cells, MTT is reduced by dehydrogenase to form a formazan with intense colour, which can be quantified by spectrometry (Grela et al., 2018; Mccauley, 201;).

Forty-one extracts presented anticancer activity against at least one the tested cancer cell lines. It is important to note that for a higher certainty of the results, anticancer activity of each actinobacterial extract should be tested at least twice and in independent bioassays, for each cancer cell line. However, due to time limitation, several actinobacterial extracts were tested only once in the cell line HepG2 (extracts from strains E65, E66, E66A, E75, E77, E82, E83, E85, E92, E96, E99, E 02, E104, E106, E109, E113, E114, E115, E116, E117, E119, E194, E196, E197, E201, E204, E228, E232 and E247) and, thus, for these extracts, anticancer activity needs to be re-evaluated in order to confirm the obtained results. Some of the tested extracts showed potent activities (though some of them need to be confirmed for the reasons indicated above), being able to reduce the viability of the cell line HepG2 in more than 80% (extracts from strains E194, E196, E197, E201, E221 and E228) and of the cell line HCT-116 in more than 60% (extracts from strains E18 and E119), after 48 h of exposure. Most of the strains exhibiting anticancer activity were *Streptomyces*, but strains affiliated with other genera were also identified.

Marine Actinobacteria have proven to be effective producers of compounds with anticancer activity. The genus *Streptomyces* is one of the most promising sources of these compounds. Some illustrative examples are Petrocidin A, produced by *Streptomyces* sp. isolated from the marine sponge *Petrosia ficiformis* collected in Milos, Greece, and exhibiting high activity against promyelocytic leukemia and colon adenocarcinoma cells; Mansouramycin A-D,

produced by a *Streptomyces* strain isolated from sediment of Jade Bay on the southern German North Sea Coast, and exhibiting significant activity on non-small-cell lung cancer, breast cancer, melanoma, and prostate cancer cells, with a high degree of selectivity; Macrolide tartrolon D produced by a *Streptomyces* species isolated from a 30 m deep marine sediment collected off the east coast of Madagascar, and exhibiting cytotoxic activity against lung (A549), colon (HT29) and breast (MDA-MB-231) cancer cell lines (Pérez et al. 2009) and Streptoanthraquinone A, isolated from a marine *Streptomyces* strain obtained from sediment samples from the east China sea, with activity against glioma cells (Cheng et al., 2017; Khalifa et al. 2019; Liang et al. 2016)

In addition to the genus *Streptomyces*, and similarly to the results obtained in the present study, other marine actinobacterial species have been described in the literature to exhibit anticancer activity. *Gordonia* species isolated from the marine sponge *Erylus discophorus* collected from the continental shelf at Berlengas, in Portugal, were reported to have anticancer activity against liver cancer cells (Santos et al., 2019). A strain of *Nocardia dassonvillei*, isolated from a marine sediment sample, was reported to produce the compound N-(2-hydroxyphenyl)-2-phenazinamine, exhibiting activity not only against the cancer cell lines tested in the present study, but also against lung adenocarcionoma epithelial cells and ovarian cancer cells (Gao et al. 2012). Ji et al. (2007) isolated a strain of *Nocardiopsis lucentensis* from marine sediment from Island of Little San Salvador, in the Bahamas, that produces Lucentamycins A and B with activity against colorectal cancer cells.

The results presented in this thesis, though interesting, are not yet complete. Due to the lack of time, several parts of the work were not concluded. In the future, it will be necessary to phylogenetically identify the strains isolated from *H. perlevis* whose identification failed and test the respective bioactive potential; anticancer assays will have to be repeated for the actinobacterial extracts that were tested only once in the cancer cell line HepG2; cytotoxicity of all extracts should be assessed in a non-cancer cell line; dereplication studies should be performed in the extracts showing bioactivity to investigate the presence of potential novel compounds and chemical studies should be conducted to unveil the identity of these putative novel molecules.

4.2 Conclusions

The objective of this study was to investigate the diversity of culturable Actinobacteria associated with the marine sponge *H. perlevis* and evaluate the potential of the isolated strains to produce compounds with antimicrobial and anticancer activities. Eighty-two strains of Actinobacteria were identified up to the moment from a collection of 184 isolates. The studied sponge revealed to host phylogenetically diverse *Actinobacteria,* with twelve genera identified. Antimicrobial and anticancer results indicate that many of these strains produce bioactive compounds, with some of these bioactivities being promising in terms of potency. Three potential new species were isolated, opening good perspectives for the discovery of novel bioactive molecules. In the future, it will be important to deeper investigate the more active crude extracts for the presence of novel compounds. This work contributed to increase the knowledge on the diversity and bioactive potential of Actinobacteria living in symbiosis with the marine sponge *H. perlevis*. To the best of our knowledge, no such studies have been conducted with this sponge species inhabiting Portuguese marine environments.

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