Bioprospection of deep-sea Actinobacteria for the discovery of novel natural compounds with pharmaceutical and industrial applications

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Francisca Branco

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Resumo

Inúmeros compostos bioativos, com as mais variadas aplicações, são produzidos naturalmente por microrganismos, destacando-se, as actinobactérias como uma das maiores fontes destes compostos. Devido ao seu elevado valor biotecnológico, os investigadores têm tido a sua atenção voltada para actinobactérias provenientes de ambientes extremos, como o mar profundo, uma vez que se acredita que devido às condições peculiares destes ambientes, as actinobactérias que neles habituam têm a capacidade de produzir metabolitos únicos e muito distintos dos encontrados até ao momento.

É neste contexto que se insere a presente tese, cujo objetivo principal foi rastrear uma coleção de culturas de 64 isolados de actinobactérias, previamente obtidos de esponjas, corais e sedimentos de mar profundo recolhidos no arquipélago da Madeira, para a descoberta de novos compostos naturais com propriedades anti-inflamatória, anti-obesidade e anti-incrustante. Os ensaios de atividade anti-inflamatória basearam-se na capacidade dos extratos actinobacterianos reduzirem inflamações induzidas pelo LPS nas células da linhagem celular RAW 264.7. Os ensaios de atividade anti-obesidade, consistiram na exposição de embriões de peixe-zebra aos extratos, com a finalidade de verificar se estes eram capazes de reduzir os lípidos presentes na sua região abdominal, sendo essa redução visualizada através da fluorescência do Nile Red. Os ensaios de atividade anti-incrustante consistiram na observação da capacidade dos extratos em inibirem o crescimento de bactérias marinhas incrustantes como *Cobetia marina, Roseobacter litoralis, Halomonas aquamarina, Vibrio harveyi* e *Pseudoalteromonas atlantica*.

Os resultados obtidos revelam 4 extratos, afiliados com o género *Brevibacterium*, que demonstraram atividade anti-inflamatória (2) ou anti-obesidade (2). Estes extratos foram desreplicacados na plataforma GNPS e foi construída uma rede molecular comparando estes extratos com outros de uma base de dados criada no laboratório. Esta rede molecular destacou que um dos extratos possuía um cluster único com dois nódulos

(m/z 545.08 e 543.04) e que não estava presente em nenhum outro extrato actinobacteriano. As massas correspondentes foram, posteriormente, pesquisadas em bases de dados (Dictionary of Natural Products e Natural Products Atlas), não se tendo encontrado nenhum resultado que correspondesse a essas massas, o que indica que apossibilidade de se tratar de moléculas novas.

Futuramente, será importante crescer em grande escala os isolados de actinobactérias cujos extratos se revelaram promissores, a fim de realizar uma análise química detalhada para o isolamento e caracterização desses potenciais novos metabolitos secundários.

Palavras-chave: Actinobacteria, Mar profundo, Compostos bioativos, Anti-inflamatório, Anti-obesidade, Anti-incrustante

Abstract

Inumerous bioactive compounds, with the most varied applications, are naturally produced by microorganisms, and, in this respect, actinobacteria are one of the largest producers of these compounds. Due to their enormous biotechnological value, researchers are looking for actinobacteria in extreme environments, such as the deep sea because it is believed that, given the environmental conditions in which they live, these marine actinobacteria can produce unique metabolites, very different from those found so far.

In this context, the objective of the present thesis was to screen a culture collection of 64 actinobacterial isolates, previously obtained from deep-sea sponges, corals and sediments collected in the Madeira archipelago, for the discovery of novel natural compounds with anti-inflammatory, anti-obesity and anti-fouling activities. Anti-inflammatory assays were based on the ability of actinobacterial extracts to reduce LPS-induced inflammation in RAW 264.7 cells. Anti-obesity assays were performed using the Nile Red fluoresce assay, where zebrafish embryos were exposed to actinobacterial extracts in order to see if they were able to reduce the neutral lipids present in their abdominal region. The anti-fouling assays consisted in observing whether the actinobacterial extracts were able of inhibiting the growth of five marine fouling bacteria: *Cobetia marina, Roseobacter litoralis, Halomonas aquamarina, Vibrio harveyi and Pseudoalteromonas atlantica*.

The results obtained revealed 4 extracts, associated to the *Brevibacterium* genus, that exhibited anti-inflammatory (2) or anti-obesity (2) activities. These extracts were dereplicated in GNPS platform and a molecular network was built after the comparison of these extracts with others from a database created in the laboratory. In this molecular network, it was found that one of the extracts had a single cluster with two nodules (m/z 545.08 and 543.04) that was not present in any other actinobacterial extract. The corresponding masses also showed no hits in the databases (Dictionary of Natural

Products and the Natural Products Atlas), indicating the potential presence of new molecules.

In the future, it will be important to grow on a large scale the actinobacterial isolates whose extracts have shown to be promising, in order to perform a detailed chemical analysis for the isolation and characterization of these potentially new secondary metabolites.

Keywords: Actinobacteria, Deep-sea, Bioactive compounds, Anti-inflammatory, Anti-obesity, Anti-fouling

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List of Abbreviations

BGC	BIOSYNTHETIC GENE CLUSTER
DMEM	DULBECCO'S MODIFIED EAGLE MEDIUM
DMSO	DIMETHYL SULFOXIDE
EIC	EXTRACTED ION CHROMATOGRAMS
GC	GUANINE AND CITOSINE
GNPS	GLOBAL NATURAL PRODUCTS SOCIAL MOLECULAR NETWORKING
IL	INTERLEUKIN
LPS	LIPOPOLYSACCHARIDE
MTT	3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE
NO	NITRIC OXIDE
NRPS	NONRIBOSOMAL PEPTIDE SYNTHASES
PBS	PHOSPHATE-BUFFER SALINE
PKS	POLYKETIDE SYNTHASE
PTU	1-PHENYL 2-THIOUREA
REV	RESVERATROL
TBT	TRIBUTYLTIN
TLR	TOLL-LIKE RECEPTOR
WHO	WORLD HEALTH ORGANIZATION

CHAPTER 1

INTRODUCTION

1. General introduction

Modern societies face today several serious health problems, of which anti-inflammatory diseases and obesity are at the forefront. In order to improve the quality of life for millions of people worldwide, finding new therapies for the treatment of these diseases is vital.

Inflammation processes are induced as a response against harmful exogenous agents. However, this process can trigger tissue damage and be associated with numerous chronic diseases, including autoimmune disorders like Grave's disease and rheumatoid arthritis (Nathan and Ding, 2010). Diseases associated with chronic inflammation and unrelated to autoimmune processes are generally linked to aging, these include type 2 diabetes, varied forms of cardiovascular diseases and most neurodegenerative diseases such as Alzheimer's disease (Tabas et al., 2013).

Another concerning disease is obesity. According to the World Health Organization (2022), it is estimated that currently more than one billion people in the world are obese, between 650 million adults, 340 million teenagers and 39 million children, and these numbers keep rising. One of the biggest problems with obesity is that it affects most of the body's systems, including the heart, liver, kidneys, joints, and reproductive system. This leads to several chronic diseases such as type 2 diabetes, cardiovascular disease, hypertension, stroke, various forms of cancer, as well as mental health problems (Jackson et al., 2015). Furthermore, people with morbid obesity are also 4.6 times more likely to be hospitalized due to COVID-19, another big threat of present times (Yu et al., 2021).

A large number of bioactive compounds is naturally produced by microorganisms, and actinobacteria are one of the largest producers of these compounds. Due to their enormous biotechnological value, terrestrial actinobacteria have been intensively explored. In the recent years, the results of these explorations have slowed down, forcing researchers to look for actinobacteria in alternative habitats like marine environments or even extreme environments, like the deep-sea.

It is believed that the peculiar environmental conditions of deep-sea can lead to the production of unique metabolites by deep-sea actinobacteria. The deep-sea environment

is very particular, presenting large variations in terms of nutrient availability, oxygen concentration, salinity concentration, lack of light, high pressure, and low temperature, which may trigger the development of unique biochemical and physiological properties in the organisms that inhabit it, consequently translating in the production of unique metabolites (Kamjam et al., 2017).

1.1. Phylum Actinobacteria

Actinobacteria, commonly called actinomycetes, are Gram-positive bacteria, generally free-living, typically with a high content of guanine and cytosine (GC) in their genome except for some freshwater-dwelling members that present relatively low GC content (Kavagutti et al., 2019).

The phylum Actinobacteria is one of the largest and most diverse phyla of bacteria. Microorganisms integrating this phylum can live as inhabitants of soil or aquatic environments (e.g., *Streptomyces*, *Micromonospora*, *Rhodococcus*, and *Salinispora* species), plant or animal pathogens (e.g., *Corynebacterium*, *Mycobacterium*,or *Nocardia* species), plant symbionts (e.g., *Frankia* spp.), or gastrointestinal commensals (e.g., *Bifidobacterium* spp.). This ubiquity causes these bacteria to present the most varied forms, from cocci to coccobacilli, different organizations of the mycelium, which can be branched or not, very different pigments, assuming practically all colors and, also, spore morphology and size very variable (Barka et al., 2016).

Recently, the phylum Actinobacteria was validly published with the name of Actinomycetota (Oren and Garrity, 2021). A new organization of this phylum was also proposed based on the phylogeny of the 16S rRNA gene. According to this rearrangement, this phylum now includes 6 classes (Actinomycetia, Acidimicrobiia, Coreobacteriia, Nitriliruptoria Rubrobacteria, and Thermoleophilia), 46 orders, 79 families, and 225 genera (Salam et al., 2020).

Actinobacteria have an enormous biotechnological potential due to their ability to produce unique bioactive compounds, such as antibiotics, antifungals, antitumor agents, immunosuppressants, anti-inflammatories, antivirals, enzymes, and enzyme inhibitors (Ettoumi et al., 2016).

The largest actinobacterial producers of bioactive compounds are affiliated with the

orders: Corynebacteriales, Glycomycetales, Jiangellales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Actinomycetales, Bifidobacteriales, Kineosporiales, Micrococcales Catenulisporales, Streptomycetales, Streptosporangiales and Frankiales (Ludwig et al., 2015). Of these, the order Streptomycetales stands out, categorized as the most important regarding the production of secondary bioactive metabolites (Dhakal et al., 2017).

1.2. Marine Actinobacteria

Terrestrial actinobacteria have been intensively explored over the last few decades. As a result, the discovery of new bioactive compounds isolated from these bacteria has slowed down in the recent years, leading researchers to look for unexplored habitats, such as marine environments and extreme environments, including high altitude environments, volcanic areas, regions of high salinity or temperature, and deep-sea environments, in order to find new phylogenies of actinobacteria and, consequently, new bioactive compounds (Dhakal et al., 2017).

Initially, actinobacteria were considered to be exclusively indigenous to terrestrial environments and it was believed that their presence in marine environments was due to the migration of resistant spores from terrestrial environments. However, currently, there are data that prove the existence of actinobacteria of marine origin (Jensen et al., 2005) and demonstrate their relevance in these ecosystems, being crucial for the mineralization of organic matter, immobilization of mineral nutrients, nitrogen fixation, improvement of physical parameters of water and environmental protection (Manivasagan et al., 2013).

Marine actinobacteria frequently live in association with a large variety of aquatic organisms (Fig. 1), including sponges, corals, echinoderms, and some vertebrates, and it is believed that this association can influence the production of unique secondary metabolites. These microorganisms can also exist in sediments and both in planktonic and biofilm niches (Jagannathan et al., 2021).

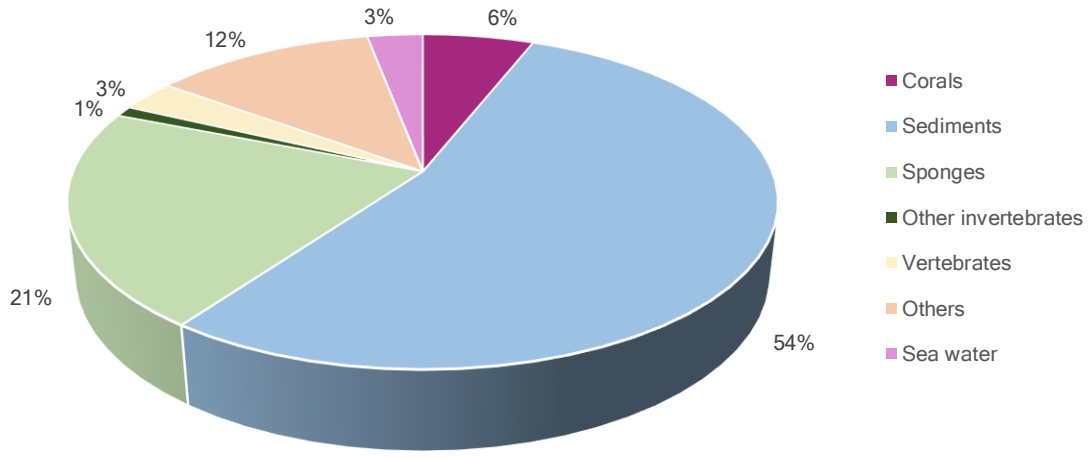

Figure 1 – Distribution of actinomycete-derived 16S rRNA gene sequences in the marine environment (adapted from Abdelmohsen et al., 2014).

One of the great challenges in the investigation of marine actinobacteria is the difficulty of cultivating them, when compared to terrestrial ones, due to their special growth requirements (Xu et al., 2018). Thus, to be more successful in their isolation, it is important to take into account the conditions of their natural environment in terms of pH, oxygen gradient, temperature, and nutritional composition (Dhakal et al., 2017). Stereotypically, actinobacteria are found at moderate pH levels, between 6 and 9, with a relatively small percentage being acidophilic and alkaliphilic (Barka et al., 2016). In terms of temperature, actinobacteria commonly prefer moderate temperature, with some members being thermophilic (Jagannathan et al., 2021).

1.2.1. Marine actinobacteria as a source of novel bioactive compounds

Marine actinobacteria have attracted increasing attention from researchers, as they have been shown to be a rich source of new bioactive molecules such as retamycin, cytotoxic and antibacterial compound produced by *Streptomyces lindensis*, fluostatins, produced by *Streptomyces* sp. and with antibacterial activity, taromycin, a compound with antibacterial activity produced by *Saccharomonospora* sp. (Barka et al., 2016), among others (Table 1).

Within marine actinobacteria, the largest producers of new bioactive molecules are affiliated with the genera *Streptomyces, Nocardiopsis, Salinospora, Micromonospora and Saccharomonospora*. In fact, *Streptomyces* species alone are responsible for the production of over than 7600 bioactive compounds (Tiwari and Grupta, 2016).

Table 1 – Examples of bioactive compounds produced by marine actinobacteria (adapted from Zotchev et al., 2012).

COMPOUND	SOURCE	ACTIVITY	REFERENCE
Abyssomycin C	*Verrucosispora maris*	Anti-bacterial	Bister et al. (2004)
2-Allyloxyphenol	*Streptomyces* sp.	Antimicrobial and antioxidant	Arumugam et al. (2010)
Albidopyrone	*Streptomyces* sp.	Cytotoxic	Hohmann et al. (2009)
Aureoverticillactam	*Streptomyces aureoverticillatus*	Cytotoxic	Mitchell at al. (2004)
Carboxamycin	*Streptomyces* sp.	Anti-bacterial and cytotoxic	Hohmann et al. (2009)
Cyclomarines	*Streptomyces* sp. *& Salinispora arenicola*	Anti-inflammatory	Schultz et al. (2008)
Dermacozines	*Dermacoccus* sp.	Cytotoxic and radical scavenging	Abdel-Mageed et al. (2010)
Diazepinomicin	*Micromonospora* sp.	Anticancer	Charan et al. (2004)
Lynamicins	*Marinispora* sp.	Anti-bacterial	Mc Arthur et al. (2008)
Mansouramycins	*Streptomyces* sp.	Cytotoxic	Hawas et al. (2009)
Marinopyrroles	*Streptomyces* sp.	Anti-bacterial and cytotoxic	Hughes et al. (2008)
ML-449	*Streptomyces* sp.	Cytotoxic	Jorgensen et al. (2010)
Proximicins	*Verrucosispora* sp.	Cytotoxic	Fiedler et al. (2008)
Salinamides	*Streptomyces* sp.	Anti-inflammatory	Moore et al. (1999)

Salinosporamide A	*Salinispora tropica*	Anticancer	Feling et al. (2003)
Thiocoraline	*Micromonospora* sp.	Anticancer	Perez Baz et al. (1997)
TP-1161	*Nocardiopsis* sp.	Anti-bacterial	Engelhardt et al. (2010)

The production of secondary metabolites by actinobacteria occurs during the stationary phase of microbial growth, that is, after primary metabolism, and is part of the adaptation of microorganisms to the surrounding environment. It can be favored by optimizing fermentation conditions, such as nutrient availability, pH, temperature, O_2 partial pressure, agitation and concentration of mineral salts and metal ions (Manivasagan et al., 2013).

In recent years, it was found that even closely related strains of actinobacteria own unique biosynthetic gene clusters (BGCs), locally clustered group of genes that encode a biosynthetic pathway to produce a secondary metabolite. This suggests that marine actinobacteria are very promising and valuable sources of natural products (Letzel et al., 2017).

Synthesis of secondary metabolites in actinobacteria is mainly driven by polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS). Numerous natural PKS and NRPS products have already been identified and characterized in actinobacteria and used on a large scale in antimicrobial and anti-tumor therapies (Wei et al., 2018).

PKS comprise several domains, such as β-ketoacyl synthases, acyl carrier proteins, and acyltransferases, and intervene in the production of polyketides over sequential decarboxylative condensations of acetate or other carboxylic acids (Du et al., 2010). NRPS are a class of multifunctional enzymes that synthesize peptides through various domains, such as condensation, adenylation and peptidyl carrier proteins (Du et al., 2010). The molecules assembled by PKS and NRPS are afterwards completed by tailoring enzymes, namely methyltransferases, glycosyltransferases, acyltransferases, transaminases, prenyltransferases, cyclases, ketoredutases, oxygenases, and halogenases (Li and Lou, 2013).

1.3. Deep-sea actinobacteria

1.3.1. Deep-sea environments

The deep-sea represents the greatest and least explored biome on the planet.

Deep-sea is demarcated into the bathyal zone, with depths that go from 200 to 2000 m, abyssal zone, whose depths lie between 2000 and 6000m, and the hadal zone, with depths below 6000 m (Harino et al., 2009).

Deep-sea organisms live under extreme conditions, with lack of light, variable salinity and oxygen concentrations, high pressures between 20 and 1100 atm, and very low temperatures (Skropeta, 2008).

These conditions impact microbial primary metabolic pathways and subsequently their secondary metabolites, making them capable of producing unique natural compounds found only in these environments (Jagannathan et al., 2021).

1.3.2. Deep-sea actinobacteria as a source of novel bioactive compounds

Actinobacteria can also be found in deep-sea environments, where they can inhabit deep-sea sediments or live in association with other marine dwellers, such as sponges (Abdelmohsen et al., 2010), corals (Valliappan et al., 2014) and sediments (Chen et al., 2016).

Between 2006 and 2016, the highest number of actinobacteria retrieved from deep-sea environments was obtained from samples collected from the abyssal region or deeper. The most frequent species being, in descending order, *Microbacterium, Dermacoccus, Streptomyces* and *Verrucosispora* (Kamjam et al., 2017).

In terms of bioactivity, deep-sea actinobacteria most commonly present antimicrobial and anticancer potential. Cytotoxic and anti-fouling activities have also been reported (Table 2) (Kamjam et al., 2017).

Table 2 – Examples of bioactive compounds obtained from deep-sea actinobacteria and respective locations from which they were obtained.

SPECIES	REGION AND DEPTH	COMPOUNDS	BIOACTIVITY	REFERENCE
Microbacterium sediminis	South-west Indian Ocean 2327 m	Microbacterins A and B	Anticancer	Liu D. et al., 2015
Pseudonocardia sp.	South China Sea 3258 m	Pseudonocardians A–C	Anticancer	Li et al., 2011
Serinicoccus profundi	Indian Ocean 5368 m	Indole alkaloid	Antibacterial	Yang et al., 2013
Streptomyces fungicidicus	Western Pacific 5000 m	Diketopiperazines	Anti-fouling	Li et al., 2006
Streptomyces lusitanus	South China Sea 3370 m	Grincamycins B–F	Anticancer	Huang et al., 2012
Streptomyces niveus	South China Sea 3536 m	Marfuraquinocins	Cytotoxic	Song et al., 2013
Streptomyces scopuliridis	South China Sea 3536 m	D sotamides B−D	Antibacterial	Song et al., 2014
Streptomyces xiamenensis	Pacific Ocean 2628 m	Xiamenmycin C and D	Anti-fibrotic	You et al., 2013
Streptomyces sp.	Pacific Ocean 5774 m	12-MTA	Anti-fouling	Xu et al., 2009
Streptomyces sp.	Bahamas 1618 m	Ammosamides A and B	Anticancer	Gaudêncio et al., 2008
Verrucosispora sp.	South China Sea 2733 m	Abyssomicins J–L	Antibacterial	Wang et al., 2013

1.4. Inflammatory diseases

Inflammation is an immune system response that can be activated by innumerous factors, including toxic compounds, damaged cells, irradiation, and pathogens, and aims to remove the harmful stimulus. This process can be induced in the kidney, intestinal

tract, pancreas, heart, among others, and can lead to tissue damage or disease (Chen et al., 2018).

Inflammation can be classified as acute or chronic.

Acute inflammation is characterized by a tissue damage caused by trauma, harmful compounds, or microbial invasion. Normally, this type of inflammation is triggered quickly and becomes severe in short period of time with the symptoms lasting a few hours or days (Pahwa et al., 2022).

In contrast, chronic inflammation is slow and can last for several months or even years, depending on the injury and the ability of the body to heal the damage. Roughly, chronic inflammation is caused by low-virulence microorganisms, exogenous substances that are difficult to eliminate, and self-antigens (autoimmune diseases). There are several conditions associated with this type of inflammation, namely, atherosclerosis, metabolic diseases (obesity, insulin resistance and type 2 diabetes), autoimmune diseases like rheumatoid arthritis and Grave's disease, and cancer (Nathan and Ding, 2010).

To treat inflammatory disorders, steroidal and nonsteroidal anti-inflammatory drugs are used. However, its long-term use is often associated with considerable side effects, including gastrointestinal discomfort, kidney and liver dysfunction, cardiovascular and endocrine systems damage, among others (Li et al., 2021). With that in mind, it is important to find new anti-inflammatory drugs that are effective and safe, especially in the case of chronic inflammatory diseases.

1.4.1. Actinobacteria as a source of anti-inflammatory compounds

Marine actinobacteria have shown to be very promising regarding anti-inflammatory compounds production.

It was already demonstrated that marine actinomycetes are an interesting source of anti-inflammatory compounds. For example, salinamides A and B, two topical anti-inflammatory drugs synthesized by *Streptomyces* sp.; 3-keto sterols bendigoles D–F, steroids produced by *Actinomadura* sp., whose anti-inflammatory action is based on NF-kB inhibition and glucocorticoid receptor–protein binding properties; and lobophorin B, isolated from *Streptomyces carnosus* (Chen et al., 2021).

1.4.2. Approach to anti-inflammatory assays

Comprehensively, anti-inflammatory assays are designed based on the induction of an inflammation in a given cell line and subsequent assessment of an extract ability to reduce or eliminate inflammation.

Lipopolysaccharide (LPS) is used as a stimulator of inflammation.

LPS is the major component of the outer cell wall of Gram-negative bacteria, and it induces the release of interleukin 8 (IL-8), interleukin 6 (IL-6), interleukin 1β (IL-1β), and other inflammatory cytokines in numerous cell lines, causing acute inflammation in the presence of pathogens (Ngkelo et al., 2012). This signaling occurs via Toll-like receptor
4 (TLR4) localized in the cell membrane that activates NF-κB, a downstream transcriptional factor, which in turn leads to the transcription and translation of pro-inflammatory factors like IL-6 and TNF-α (Li et al., 2020).

RAW 264.7 cell line is used as target cells for the anti-inflammatory assays.

These cells are monocyte/macrophage-like cells that were established from a tumor in a male mouse induced with the Abelson murine leukemia virus. Their viable shape can vary from cuboidal or spindle-shaped to rounded (Fig. 2). They are adherent cells, but when viable, they are detachable and able to float, especially when there is a confluent culture.

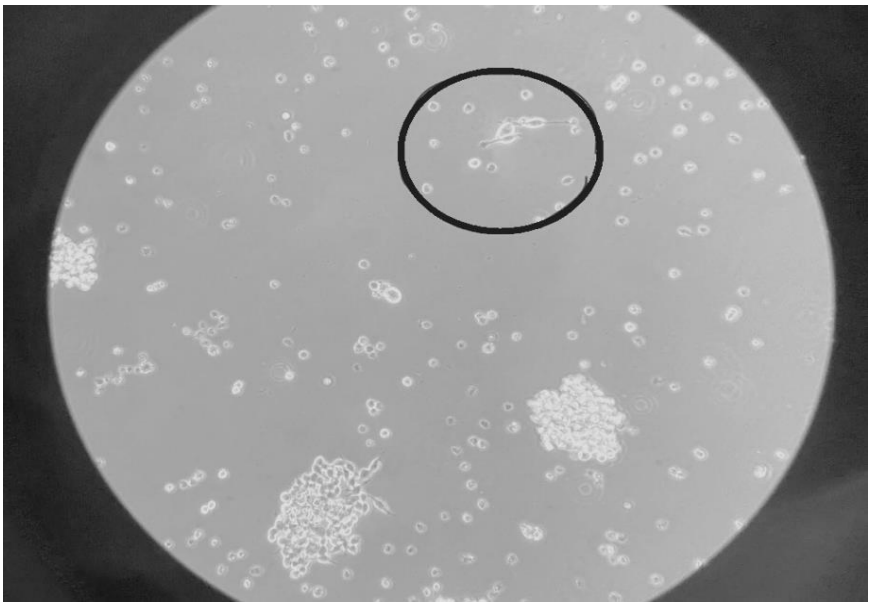

Figure 2 – RAW 264.7 cell line morphology. The black circle highlights spindle-shaped cells that is the most common viable shape of this cell line.

When stimulated by LPS, RAW 264,7 cells boost nitric oxide (NO) production and lead to phagocytosis. Therefore, NO is considered a pro-inflammatory mediator that can induce inflammation when overproduced in anomalous situations (Taciak et al., 2018).

1.5. Obesity

Obesity is an abnormal fat accumulation that translates into a bodymass index over 30 kg/m^2 (Yu et al., 2021).

This condition can lead to the increase in insulin resistance (diabetes), hypertension, dyslipidemia, abnormal left ventricular geometry, endothelial dysfunction, increased systemic inflammation, prothrombotic state, systolic and diastolic dysfunction, heart failure, coronary heart disease, atrial fibrillation, obstructive sleep apnea, albuminuria, osteoarthritis and several types of cancer (Lavie et al., 2009).

According to the World Health Organization (WHO), from 1975 to 2016, worldwide obesity has nearly tripled with more than 1.9 billion adults being overweight and of these over 650 million being obese (World Health Organization, 2021).

In Portugal, according to the INE, in 2019 more than 53% of the population over 18 years old were overweight or obese along with 41.6% of children (Instituto Nacional de Estatística, 2020).

These numbers are very alarming, and it is crucial to take measures to reduce them. These measures involve raising the awareness of the population to reduce their daily intake, namely of sugars and fats, and to the importance of physical exercise, but also of the food industry, which should reduce the amount of fat, salt, and sugar in its products and also make healthy eating accessible to everyone.

1.5.1. Current treatments for obesity

Currently, there are several pharmacological therapies to help obese patients, with the most common medicines being: Orlistat, that permanently inhibits pancreatic lipases

avoiding the absorption of more than 32% of ingested fats; Liraglutide, which slows gastrointestinal transit, alters glucose homeostasis and also contributes to appetite suppression; and Naltrexone/Bupropion, two substances that, when conjugated, have a synergistic action on inhibiting hunger centers located in the hypothalamus (Ruban et al., 2019).

These drugs can have, however, numerous side effects such as fecal urgency, incontinence, gastrointestinal disorders, acute pancreatitis, nausea, constipation, and headaches. In addition to these side effects, which in themselves are worrisome, these drugs also have the limitation that their continuous use is not recommended, so they only present a solution for the first few months of weight loss (Ruban et al., 2019).

Therefore, science can play a key role in the development of innovative treatments to help fighting obesity, particularly through the use of natural products.

1.5.2. Anti-obesity compounds produced by marine actinobacteria

Several natural compounds isolated from plants have been shown to be effective in metabolic regulation, one of the keys in the treatment of obesity.

Among these compounds we can highlight polyphenols, such as resveratrol, quercetin, catechins, procyanidins, anthocyanins, epigallocatechins gallate and procyanidins, whose interest in its anti-obesity properties has been increasing and that have already shown results in assays carried out with animals and cell models. Its functioning is related to the inhibition of pancreatic lipases, α-amylases, and α-glucosidases, as well as the reduction of appetite, in line with the action of the current treatments (Bhardwaj et al., 2021). In addition to these compounds, we can also emphasize the flavonoids which generally inhibit weight gain, reducing food intake and providing a feeling of satiety (Nieuwenhuizen et al., 2006).

Many of these compounds can also be obtained from actinobacteria (Hozzein et al., 2021). Therefore, it seems relevant to explore these microorganisms as a source of anti-obesity compounds, especially since they are practically unexplored for this bioactivity. To date, no actinobacterial compound has been identified as having undoubtedly anti-obesity properties.

1.5.3. Approach to anti-obesity assays

Anti-obesity assays are based on the evaluation of the capacity of organic extracts to reduce the neutral lipids present in the abdominal region of zebrafish (*Danio rerio*) through fluorescence quantification.

In these assays, two key compounds stand out: Nile Red (9-diethylamino-5H-benzo[a]phenoxazine-5-one), that is a lipophilic stain used for the detection of neutral lipids through fluorescence microscopy or cytofluorometry (Greenspan et al., 1985), and resveratrol (REV), used as positive control due to its ability of inhibit adipogenesis and prevent triglyceride accumulation, increasing energy spending by stimulating intracellular mitochondrial functions (Zhao et al., 2017).

During the assays, the various stages of zebrafish development (Fig. 3) are always considered to ensure that all evaluated fish are at the same stage and have, consequently, equivalent lipid accumulation.

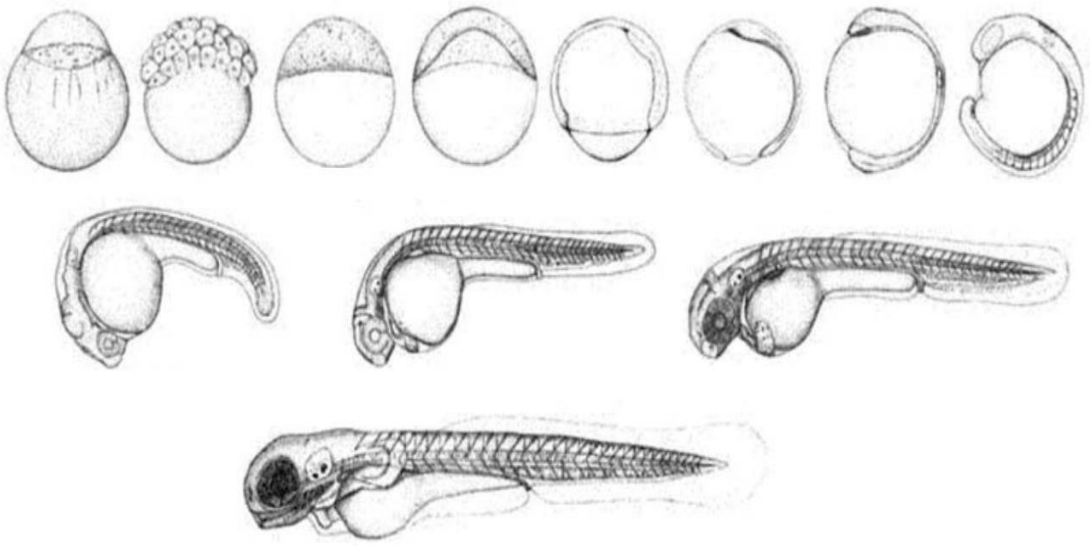

Figure 3 – Schematic representation of characteristic stages during zebrafish development (adapted from Kimmel at al., 1995).

1.6. Marine biofouling

In non-sterile seawater, any natural and synthetic substrata rapidly become colonized by living organisms. The undesired colonization and buildup of micro and macroorganisms and their by-products on underwater surfaces is called biofouling (Pereira et al., 2020).

Biofouling occurs as a dynamic multistage process that includes bacteria, algae, larvae, and adults of many phyla, and involves i) adsorption of dissolved organic molecules in the marine substratum, ii) primary colonization by bacteria, iii) formation of microbial biofilm and establishment of microscopic eukaryotes, such as fungi, diatoms and heterotrophic eukaryotes, iv) settlement of macroorganisms through the growth of algal spores and invertebrate larvae, and v) association of large marine invertebrates (Fig. 4) (Dobretsov et al., 2006).

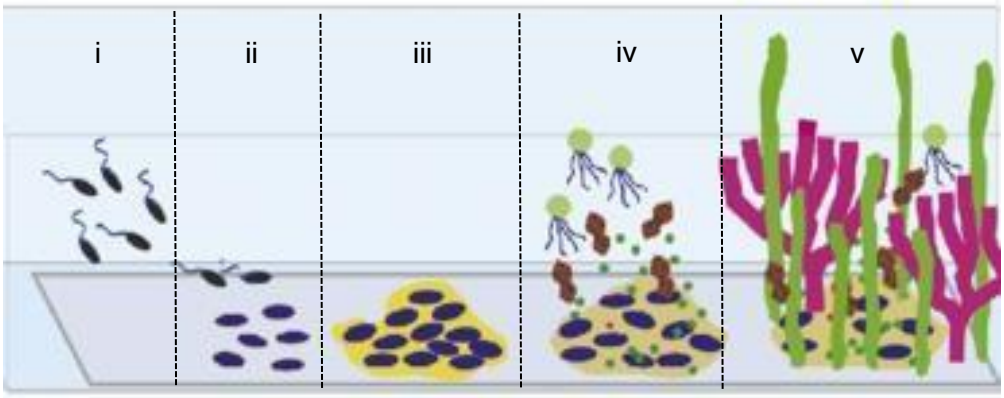

Figure 4 – Illustration portraying the stages (i, ii, iii, iv and v) of marine biofouling (adapted from Mohammed, 2015).

Marine biofouling poses substantial issues to many industries, such as sea and land-based aquaculture, naval industry through damage caused to maritime transports and increase of fuel consumption, the oil industry through the destruction of oil platforms, among others (Armstrong et al., 2000). Furthermore, from an ecological point of view, biofouling constitutes a major vehicle for the spread of invasive organisms (Costas et al., 2013).

1.6.1. Solutions for preventing marine biofouling

Historically, very early in time, the Phoenicians and Carthaginians utilized pitch on the bottom of their ships to prevent biofouling. Other solutions such as wax and asphalt were also used. Meanwhile, the Greeks and Romans both presented lead sheathing and this material was used until the 16th century by the British and Spanish. During the 17th and 18th centuries, the use of wooded sheathing painted with mixtures of tar and grease began to be frequent. In the meantime, the Portuguese charred deeply the outer surface of the bottom of the ships. However, since the 17th century copper remained the most used solution for biofouling. At the end of the 18th century, bigger ships with iron hulls appeared, which made copper no longer a plausible solution due to the corrosive action of copper on iron. This ultimately led to the development of the modern paint methods, which have substituted copper sheathing (Laidlaw, 1952).

In the early 1970s, tributyltin (TBT) was introduced as an anti-fouling biocide (Sonak et al., 2009). However, TBT turned out to be a very toxic compound for the marine environment, affecting aquatic life, even in small concentrations of 1 ng/L. In fact, TBT was found to work as an endocrine disrupter in some molluscs, mainly in *Nucella* species, leading females to develop male sex organs (Armstrong et al., 2000). For that reason, in January 2008, TBT was completely banned by the International Maritime Organization (Kotrikla, 2009).

The restriction on the utilization of TBT led to a new approach to the use of copper-based paints incorporating high concentration of copper and other metals that are also harmful for the environment, as well as the use of other toxic compounds such as dichlofluanid, chlorothalonil and diuron (Thomas and Brooks, 2010).

With this in mind, the discovery of new alternative anti-fouling compounds not harmful to marine life is urgent and, in this respect, compounds produced naturally by marine microorganisms have shown to be very promising (Satheesh et al., 2016).

1.6.2. Anti-fouling compounds produced by marine actinobacteria

In the past years, numerous anti-fouling compounds have been isolated from marine organisms, namely sea weeds, tunicates, sponges, soft corals (Armstrong et al., 2000),

but also microorganisms including microalgae, cyanobacteria and actinobacteria (Satheesh et al., 2016). However, to date very few compounds reached the requirements of being effective towards a wide range of fouling organism and at the same time environmentally safe. In addition, compounds isolated from natural sources have been shown to be more effective against barnacles and algae and not against fouling bacteria that are at the origin of biofouling colonization succession, as it is bacteria that will induce the settlement of other micro and macroorganisms, which establish themselves in the biofilms through chemical signals (Landini et al., 2010).

Nevertheless, compounds produced by bacteria seem to be more promising in terms of biofilm reduction and, consequently, as a solution against the initial stages of biofouling.

Despite not being a much-explored source, several actinobacterial products have shown to be efficient anti-fouling compounds. In 2014, a team of Indian researchers concluded that *Streptomyces* sp. produced antibacterial compounds which, in combination with zirconia oxide nanoparticles, had a high anti-fouling potential against *Bacillus thuringiensis* and *Pseudomonas aeruginosa* (Agarwal et al., 2014). Later, in 2019, extracts obtained from *Glycomyces sediminimaris* greatly affected the biofilm formation of *Kocuria* sp. and *Mesorhizobium* sp., two dominant species in the marine environments of Iran (Heidarian et al., 2019). More recently, Portuguese researchers have demonstrated the anti-fouling activity of a napyradiomycin, isolated from *Streptomyces aculeolatus* and found to reduce the growth of five fouling bacteria by 80% and to prevent the establishment of *Mytilus galloprovincialis* larvae (Pereira et al., 2020).

1.6.3. Approach to anti-fouling assays

Broadly, an anti-fouling assay consists in testing microbial extracts against fouling bacteria. The main purpose is therefore to inhibit growth of these bacteria, which would demonstrate the ability of an extract to prevent biofouling.

1.7. Aim and outline of this thesis

The objective of this thesis was to explore the bioactive potential of an actinobacterial collection obtained previously from deep-sea samples collected in the Madeira archipelago. This collection was created under the scope of a project financed by Fundação para a Ciência e a Tecnologia (FCT) — the ActinoDeepSea project (reference: (PTDC/BIA-MIC/31045/2017) — and comprises ca. 300 actinobacterial strains isolated from various deep-sea samples of corals, sponges, and sediments.

In this work, part of the actinobacterial collection obtained from the Madeira archipelago was explored for the bioprospection of novel compounds with anti-inflammatory, anti-obesity and anti-fouling activities that can be of relevance for human health and industrial applications.

This thesis is organized in 5 chapters. Chapter 1 consists in a general introduction where several aspects associated with actinobacteria are described, mainly focusing on marine and deep-sea actinobacteria, their secondary metabolism and examples of bioactive produced by these microorganisms. Also in this chapter, a reference is made to inflammatory diseases, obesity and marine biofouling, addressing the main problems associated with them and how actinobacteria can be part of the solution to help tackling these issues. Chapter 2 presents the methodology used in terms of bioactivity assays, namely anti-inflammatory, anti-obesity and anti-fouling assays, and in terms of dereplication of the most promising extracts. Chapter 3 consists in the presentation of the obtained results and in their discussion. Chapter 4 includes a general discussion and main conclusions of the performed work. Finally, chapter 5 list the references used in this thesis.

CHAPTER 2

MATERIAL AND METHODS

2.1. Actinobacterial strains selected for this study

The actinobacterial strains used for this study were isolated from deep-sea samples previously collected from the regions of Ribeira Brava and Garajau in the Madeira archipelago at depths between 463 and 865 m, using the manned submersible Lula1000.

The taxonomic affiliation of the isolates used in this study, together with information about the culture medium from which they were isolated in listed in Table 3.

In the moment this study began, each isolate was stored at -80 ºC in 30% (v/v) of glycerol. Each isolate was revived by culturing it on plates with the respective isolation medium.

Table 3 — List of the deep-sea actinobacterial strains used in this study, isolated from deep-sea samples from the Madeira archipelago. The similarity was analyzed based on the 16S rRNA database from NCBI BLAST tool.

	ISOLATE CODE	SELECTIVE ISOLATION MEDIUM	ENVIRONMENTAL SOURCE	CLOSEST SPECIES	SIMILARITY (%)
1	S_020_2	M1 [1]	Sponge	*Tsukamurella tyrosinosolvens*	99.93
2	S_020_3	M1	Sponge	*Brevibacterium aureum*	99.93
3	S_020_4	M1	Sponge	*Tsukamurella tyrosinosolvens*	99.86
4	S_020_5	M1	Sponge	*Brevibacterium sediminis*	99.86
5	S_020_6	NPS [2]	Sponge	*Tsukamurella tyrosinosolvens*	99.93
6	S_020_8	NPS	Sponge	*Tsukamurella tyrosinosolvens*	99.93

[1] M1: 10 g of casamino acids, 5 g of neopeptone, 1 g of yeast extract, 5 g of NaCl, 50 mL of Tris/HCl buffer (pH 7.8) and1 L of distilled H_2O (Moynet, 1976).

[2] NPS: 100 mL of sand extract (900 ml of sand washed with 500 mL of seawater), 17 g of agar and 1 L of seawater (Jensen et al., 2005).

7	S_020_9	M1	Sponge	*Tsukamurella strandjordii*	99.14
8	S_020_10	M1	Sponge	*Tsukamurella tyrosinosolvens*	99.78
9	S_020_13	NPS	Sponge	*Brevibacterium aureum*	99.79
10	S_021_1	M4 [3]	Sponge	*Tsukamurella tyrosinosolvens*	99.93
11	S_021_2	NPS	Sponge	*Brevibacterium aureum*	99.79
12	S_021_3	NPS	Sponge	*Brevibacterium sediminis*	99.35
13	S_023_3	NPS	Sponge	*Tsukamurella tyrosinosolvens*	99.86
14	S_023_4	NPS	Sponge	*Microbacterium aerolatum*	99.64
15	S_001_1	M1	Sponge	*Brevibacterium sediminis*	99.86
16	S_001_2	M1	Sponge	*Brevibacterium siliguriense*	98.99
17	S_001_4	M1	Sponge	*Microbacterium aerolatum*	100
18	S_001_5	M1	Sponge	*Tsukamurella tyrosinosolvens*	99.78
19	S_001_10	M1	Sponge	*Microbacterium ginsengiterrae*	98.85
20	S_001_12	NPS	Sponge	*Tsukamurella tyrosinosolvens*	99.78
21	S_026_1	M1	Sponge	*Brevibacterium sediminis*	99.71
22	S_026_2	M1	Sponge	*Microbacterium aerolatum*	99.71
23	S_026_3	M1	Sponge	*Microbacterium oxydans*	99.78

[3] M4: 2 g of chitin, 18 g of agar and 1 L of seawater (Mincer et al., 2002).

24	S_026_4	M1	Sponge	*Brevibacterium sediminis*	99.86
25	S_026_5	M1	Sponge	*Microbacterium aerolatum*	99.93
26	S_026_6	M1	Sponge	*Microbacterium aerolatum*	99.78
27	S_026_7	M1	Sponge	*Brevibacterium sediminis*	99.85
28	S_026_8	NPS	Sponge	*Brevibacterium sediminis*	99.57
29	S_026_9	NPS	Sponge	*Brevibacterium sediminis*	99.93
30	S_026_10	M4	Sponge	*Brevibacterium aureum*	100
31	S_026_13	M4	Sponge	*Brevibacterium aerolatum*	99.71
32	S_026_15	NPS	Sponge	*Brevibacterium sediminis*	99.86
33	S_026_16	NPS	Sponge	*Microbacterium aerolatum*	99.78
34	S_026_17	NPS	Sponge	*Microbacterium ginsengiterrae*	98.84
35	S_026_19	M4	Sponge	*Brevibacterium sediminis*	99.43
36	C_014_1	M1	Coral	*Brevibacterium sediminis*	99.86
37	C_014_6	M4	Coral	*Microbacterium ginsengiterrae*	98.71
38	C_014_7	NPS	Coral	*Rhodococcus qingshengii*	99.93
39	C_014_9	NPS	Coral	*Brachybacterium rhamnosum*	99.06
40	C_014_10	NPS	Coral	*Brevibacterium siliguriense*	99

41	C_014_11	NPS	Coral	*Microbacterium aerolatum*	99.64
42	C_014_12	NPS	Coral	*Tsukamurella tyrosinosolvens*	100
43	C_014_13	NPS	Coral	*Tsukamurella tyrosinosolvens*	99.86
44	C_014_16	M4	Coral	*Rhodococcus erythropolis*	99.71
45	C_017_1	M1	Coral	*Microbacterium amylolyticum*	97.90
46	C_017_2	M1	Coral	*Brevibacterium sediminis*	99.64
47	C_017_3	NPS	Coral	*Brevibacterium sediminis*	99.86
48	C_017_5	NPS	Coral	*Microbacterium amylolyticum*	98.05
49	C_017_6	M1	Coral	*Microbacterium aerolatum*	99.78
50	C_017_7	M4	Coral	*Brevibacterium aureum*	100
51	C_025_1	M1	Coral	*Leucobacter komagatae*	99.71
52	C_025_2	M1	Coral	*Micrococcus luteus*	99.43
53	C_025_3	NPS	Coral	*Tsukamurella tyrosinosolvens*	100
54	C_025_4	M1	Coral	*Microbacterium aerolatum*	99.50
55	C_025_6	NPS	Coral	*Brevibacterium aureum*	99.64
56	C_025_7	NPS	Coral	*Microbacterium aerolatum*	99.42
57	C_025_9A	M4	Coral	*Microbacterium aerolatum*	99.86

58	C_025_11	NPS	Coral	*Brevibacterium aureum*	99.93
59	Sed_004_2	M1	Sediment	*Microbacterium ginsengiterrae*	98.92
60	Sed_004_12	NPS	Sediment	*Micrococcus luteus*	100
61	Sed_004_13	NPS	Sediment	*Brachybacterium paraconglomeratum*	99.35
62	Sed_004_22	M1	Sediment	*Brevibacterium sediminis*	99.72
63	Sed_004_27	NPS	Sediment	*Microbacterium aerolatum*	99.78
64	Sed_004_28	M4	Sediment	*Tsukamurella tyrosinosolvens*	100

2.2. Preparation of crude extracts

For the preparation of the crude extracts from the actinobacterial isolates listed from table 3, each isolate was grown in 100 mL Erlenmeyer flasks containing 30 mL of the corresponding isolation medium. Cultures were incubated in the dark for 1 week at 25 °C with 100 rpm shaking, and after that period, 0.5 g of Amberlite® XAD16N resin (Sigma-Aldrich, U.S.A.) was added to each flask and allowed to incubate for an additional period of 72 hours.

After this period, the cultures were centrifuged at 4600 g for 10 minutes, and the obtained biomass and resin were lyophilized. Cultures were extracted twice with a mixture of methanol and acetone 1:1 (v/v) followed by evaporation in a rotary evaporator. The obtained crude extracts were dissolved in 100% dimethyl sulfoxide (DMSO) in order to obtain solutions with final concentrations of 10 and 3 mg/mL.

2.3. Stock libraries preparation

To make the bioactivity assays less time-consuming, two libraries were built in 96-well plates, one for anti-inflammatory and anti-obesity assays and the other for anti-fouling assays.

For the first library, regarding anti-inflammatory and anti-obesity assays, 180 µL of phosphate-buffered saline (PBS) and 20 µL of each extract (at a concentration of 3 mg/mL) were added to each well of the library, performing a 1:10 dilution to obtain a final concentration of 0.3 mg/mL. A DMSO control was also included in the library, by adding to the 180 µL of PBS 20 µL of 100% DMSO.

For the second library, corresponding to the anti-fouling assay, the procedure was similar, but instead of adding PBS, 10% of DMSO was used to obtain the same concentration.

2.4. Anti-inflammatory assays

The anti-inflammatory assays were performed as described by Regueiras et al (2022).

Cells of RAW 264.7 cell line were previously seeded in Dulbecco's Modified Eagle Medium (DMEM, Biowest, France) supplemented with 10% fetal bovine serum (100 IU/mL), 1% penicillin/streptomycin (10 mg/mL) and 0.1% amphotericin.

Every 3 days, a passage was performed to maintain the viability of the cells. This passage consisted of aspirating all the culture medium from the flask, once the cells adhered to the bottom of the flask, washing with PBS and aspirating again. Then, 10 mL of supplemented DMEM medium was added and scraping was performed so that the cells resuspended. Then, the entire content of the flask was pipetted into a 15 mL falcon which was centrifuged for 5 min at 1600 rpm (Centrifuge 5430, Eppendorf, Germany). After the centrifugation, the supernatant was aspirated, and 1 mL of fresh culture medium was added to resuspend the cells. From this final cell solution, 200 µL were withdrawn into two new flasks and a further 10 mL of supplemented DMEM medium was added to each flask.

On the first day of each assay, cell viability was analyzed, ensuring a minimum viability of 85%. This viability was measured using an automatic counter (Countess, Invitrogen, U.S.A.) with the help of Trypan Blue to stain the cells. Considering the number of viable cells, a dilution in DMEM medium was performed to obtain a final concentration of 3.5x105 cells/mL. Each assay was performed in triplicate in 96-well plates to which 100 µL of cell suspension was added to each well. The 96-well plates were allowed to incubate in a CO_2 incubator (Thermo Fisher Scientific, U.S.A.) for 24 hours at 37 °C.

After the incubation time, the medium was removed, leaving the cells adhered to the bottom of each well. The following tests were after carried out: a pro-inflammatory assay, to test if the extracts could cause inflammation; and an anti-inflammatory assay, to determine the anti-inflammatory potential of each extract. In the case of the first test, 95 µL of fresh DMEM medium and 5 µL of each extract present in the library were added to each well seeded with the RAW 264.7 cell line (no LPS was added). For the second test, in addition to adding the medium and the extract, 5 µL of the inflammation agent — LPS – was added at a concentration of 1 µg/mL.

For this assay, four controls were used: one blank with cells and 100 µL of DMEM medium only, one negative control consisting of cells in 95 µL of DMEM medium plus 5 µL of DMSO, one positive control consisting of the cells in 95 µL of DMEM medium plus 5 µL of LPS, and a final control with cells in 90 µL of DMEM medium plus 5 µL of DMSO and 5 µL of LPS to make sure DMSO does not compromise the inflammation caused by LPS.

After 24 hours of incubation, viability of the cells by MTT and inflammation by nitric oxide (NO) were analyzed.

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the medium was removed from each well of the plates exposed to the compounds and 100 µL of MTT at a concentration of 0.5 mg/mL was added to each well and left to incubate for 45 min at 37 °C. After this period, the supernatant was totally removed and 100 µL of 100% DMSO was added. Absorbance was after measured at 570 nm in a microplate plate reader (Cytation 5, Biotek, U.S.A.).

For the NO assay, 75 µL of supernatant from each well of the plates exposed to the compounds was transferred to a new 96-well plate to which 75 µL of Griess Reagent (20 mL of 2% phosphoric acid plus 200 mg of 1% sulphanilamide and 20 mg of 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride) was added and left to incubate on a plate-shaker for 15 min.

Finally, the absorbance was measured at 562 nm in a microplate reader (Cytation 5, Biotek, U.S.A.).

The assays were performed at least in two independent assays and the results of all the replicates (n=6) were pooled and statistically analyzed with GraphPad Prism 9 software. In this analysis, outliers were first removed, and the cleaned data was used for normality and lognormality tests, namely Shapiro-Wilk test, and Kolmogorov-Smirnov test. When the results did not have a normal distribution, a Kruskal-Wallis nonparametric test was chosen. All results were compared to the controls containing only DMSO, in the case of the pro-inflammatory assays, and DMSO and LPS, in the case of anti-inflammatory assays.

2.5. Anti-obesity assays

For the anti-obesity assays, zebrafish (*Danio rerio*) males and females were gathered in the late afternoon of the day before of the assay in order to be possible to collect the eggs in the next morning.

After harvesting, the eggs were cleaned with egg water by passing the eggs in the sieve and the live and dead eggs were separated. After hatching, the larvae were transferred to petri dishes (approximately 50 larvae at the same stage of development per petri dish)containing 20 mL of E3 medium[4] with methylene blue.

The next day, the larvae were observed under a microscope and the embryos with malformations were removed.

After 48 hours, the fishes were transferred to 96-well plates (4 larvae per well) to start the exposure. For the exposure 10 µL of each of the extracts from the library was addedto the wells containing 200 µL of E3 medium with 1-phenyl 2-thiourea (PTU). To ensurethe accuracy of the results, a negative control composed by 10 µL of DMSO at a final concentration of 0.1% plus 200 µL of E3 medium with PTU and a positive control consisting of 10 µL Resveratrol (REV) at a final concentration of 50 µM plus 200 µL of E3 medium with PTU were used.

Results were observed 48 hours after the start of exposure. For this, 24 hours after the exposure, Nile Red was added to a final concentration of 10 ng/mL to stain the neutral

[4] E3: 34.8 g of NaCl, 1.6 g of KCl, 5.8 g of $CaCl_2 \cdot 2H_2O$ and 9.78 g of $MgCl_2 \cdot 6H_2O$.

lipids present in fish. On the next day, larvae were anesthetized by tricaine (0.03% MS-222) and the fluorescence was measured in the imaging reader Cytation 5 (BioTek, U.S.A.) and compared with the controls.

Fluorescence intensity was quantified in individual zebrafish larvae by ImageJ.

The results of the quantifications were statistically analyzed with GraphPad Prism 9 software. In this analysis, outliers were first removed, and the cleaned data was used for normality and lognormality tests, namely Anderson-Darling test, D'Agostino & Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. When the results did not have a normal distribution, a Kruskal-Wallis nonparametric test was chosen where all results were compared to the negative control.

2.6. Anti-fouling assays using biofilm-forming marine bacteria

The anti-fouling assays were performed as described by Neves et al (2021).

Five strains of marine fouling bacteria were used for the anti-fouling assays: *Cobetia marina, Roseobacter litoralis, Halomonas aquamarina, Vibrio harveyi* and *Pseudoalteromonas atlantica.*

In each assay, each of the bacteria mentioned was inoculated separately in 200 µL of Marine Broth (Carl Roth GmbH, Germany) in 96-well flat-bottom microtiter plates at an initial density of 0.1 (OD_{600}) and 4 replicates were performed.

After a 24-hour incubation period at 26 °C, the extracts were added to the wells at a final concentration of 30 µg/mL.

As a negative control, 0.1% DMSO was used and, as a positive control a penicillin-streptomycin-neomycin stabilized solution (2%) was included. The bacteria were left to incubate for 24 hours at 20 °C, and, after this period, the absorbance was read at 600 nm in a microplate reader (Cytation 5, Biotek, U.S.A.).

The bacterial growth inhibition was calculated based on the growth of the negative control and, since growth inhibition was never greater than 30%, no statistical analysis was performed.

2.7. Dereplication of the bioactive extracts

Extracts showing bioactivity in the performed assays were selected for metabolic analysis, including MS-based dereplication and molecular networking analyses. The bioactive extracts were resuspended in methanol at a final concentration of 2 mg/mL and analyzed by liquid chromatography-high resolution electrospray ionization tandem mass spectrometry (LC-HRESIMS/MS) to identify extracts with potentially new bioactive compounds. This analysis was carried out on a Dionex Ultimate 3000 HPLC coupled to a qExactive focus mass spectrometer managed by XCalibur 4.1 software (Thermo Fisher Scientific, U.S.A.). The chromatographic step was performed in an ACE UltraCore 2.5 Super C18 column (75 mm x 2.1 mm, Advanced Chromatography Technologies, U.K.).

For this, 5 µL of each extract was injected and separated starting with 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, for 9.5 min and then held for 6 min before returning to initial conditions. The UV absorbance of the eluate was measured at 254, 300, 455 and 600 nm and full MS scans, with a resolution of 70,000 full width at half maximum (FWHM) (range 150–2000 m/z) were performed. The flow of the mobile phase was 0.35 mL/min.

The raw data resulting from MS-based dereplication was converted to the mzML format and submitted to the Global Natural Products Social Molecular Networking (GNPS) platform dereplication workflow to analyze if the extracts showed relevant hits that could explain the activities obtained.

For this purpose, three dereplication analyses were used: Insilico Peptidic Natural Product Dereplicator, Dereplicator VarQuest and Dereplicator+ with default parameters,excluding ion mass precursor tolerance (0.005 Da) and fragment ion mass tolerance (0.005 Da).

In this analysis, only compounds with a score greater than 10 were taken into account, in order to increase the confidence of the results, and all compounds not associated with bacteria were excluded from the search.

In addition, a molecular network was designed using the GNPS data analysis workflow applying default parameters (except precursor ion mass tolerance and fragment ion mass tolerance which were set to 0.02 Da). The resulting molecular network was loaded into Cytoscape v3.8.2 to visualize the sets of spectra of related molecules and the

distribution of m/z clusters between the actinobacterial extracts used in this work and the actinobacterial database from the laboratory.

Only the unique m/z clusters of a single actinobacterial strain were selected and manually checked in the corresponding LC-HRESIMS data to obtain the mass of each target metabolite.

These presumed masses were searched in two databases, the Dictionary of Natural Products (version 31.1, CRC Press, U.K.) and the Natural Products Atlas, to determine whether they may be related to compounds produced by Actinobacteria or whether theycould be new compounds.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Anti-inflammatory assays

The anti-inflammatory activity of 64 actinobacterial strains previously isolated from deep-sea samples collected in the Madeira archipelago was investigated by screening crude extracts obtained from these isolates in terms of their capacity to reduce the inflammation of the RAW 264.7 cell line promoted by LPS.

To rule out any extract that by itself could cause inflammation, pro-inflammatory assays were performed without the addition of LPS (Fig. 5 and 6). In this approach, the MTT assay evaluated the viability of the cells, and the NO assessed the pro-inflammatory potential.

In Fig. 5, it is possible to observe that in the presence of the extracts, RAW 264.7 cells maintained their viability. Moreover, none of the 64 extracts tested caused inflammation in the RAW 264.7 cell line (Fig. 6), as no statistically significant values of percentage of inflammation after 24h of exposure were obtained when compared to the control with only DMSO.

However, for the extracts E19, E20 and E45, slightly higher inflammation percentages, accompanied by a considerable standard deviation, were observed, which were however not significantly different.

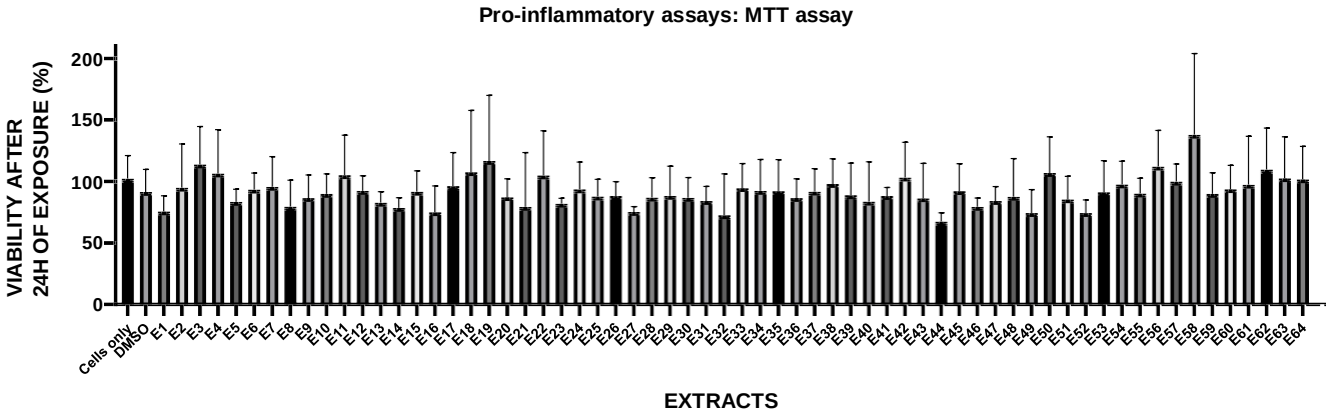

Figure 5 — Viability of RAW 264.7 cells evaluated through the MTT method in the pro-inflammatory assays. For this analysis, the percentage of viability after 24 h of exposure to extracts was compared to the control containing only DMSO. No statistically significant values were obtained, indicating a linear cell viability, thus validating the assay.

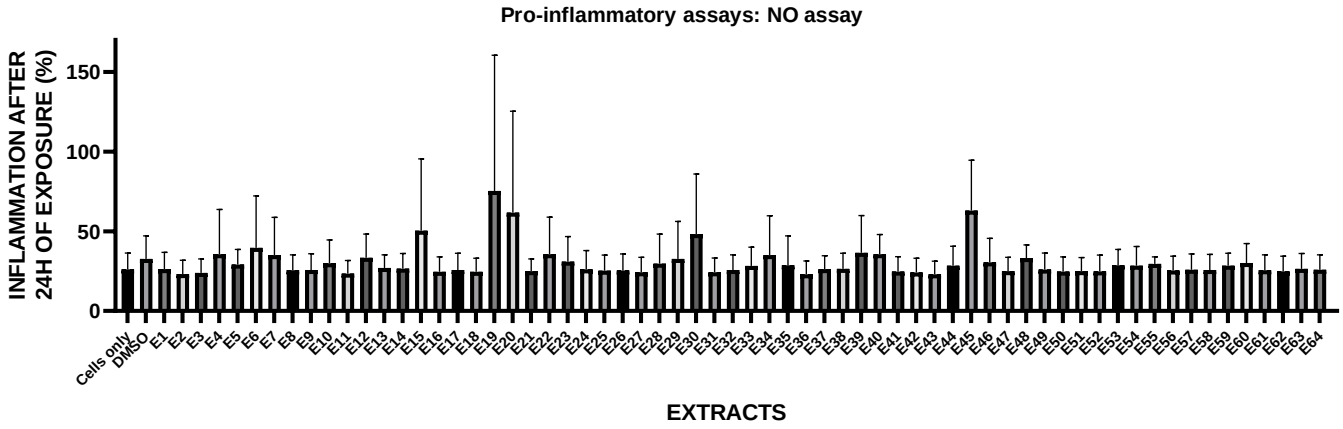

Figure 6 — Results obtained in the pro-inflammatory assays, in which extracts were analyzed for their capacity to induce the production of nitric oxide (NO). For this analysis, the percentage of inflammation after 24 h of exposure to extracts was compared to the control containing only DMSO. No statistically significant values were obtained, indicating no pro-inflammatory activity of the extracts.

A bibliographic review was carried out, for those 3 strains with slightly increased pro-inflammatory activity. The *Tsukamurella tyrosinosolvens* strain (E20), may have pro-inflammatory activity, since this genus has already been associated several times with bacteremia, cutaneous infections, lung infections, conjunctivitis and brain abscesses (Chen et al., 2016). Still, this species in particular is very rare when it comes to human infections with only 6 cases being reported until 2009 (Ménard et al., 2009). The extracts E19 and 45, from *Microbacterium ginsengiterrae* and *Microbacterium amylolyticum*, respectively did not have bibliographic records of causing inflammation.

Concerning the results obtained in the anti-inflammatory assays, as shown in Fig. 7, cellular viability was not compromised by the presence of the extracts, thus validating the anti-inflammatory assay performed.

When the RAW 264.7 cells were stimulated with LPS, two extracts, namely E11 and E21, showed statistically significant anti-inflammatory activity with RAW 264.7 cells presenting a percentage of inflammation after 24 h of only 52.73% and 46.88%, respectively, in thepresence of these extracts (Fig. 8).

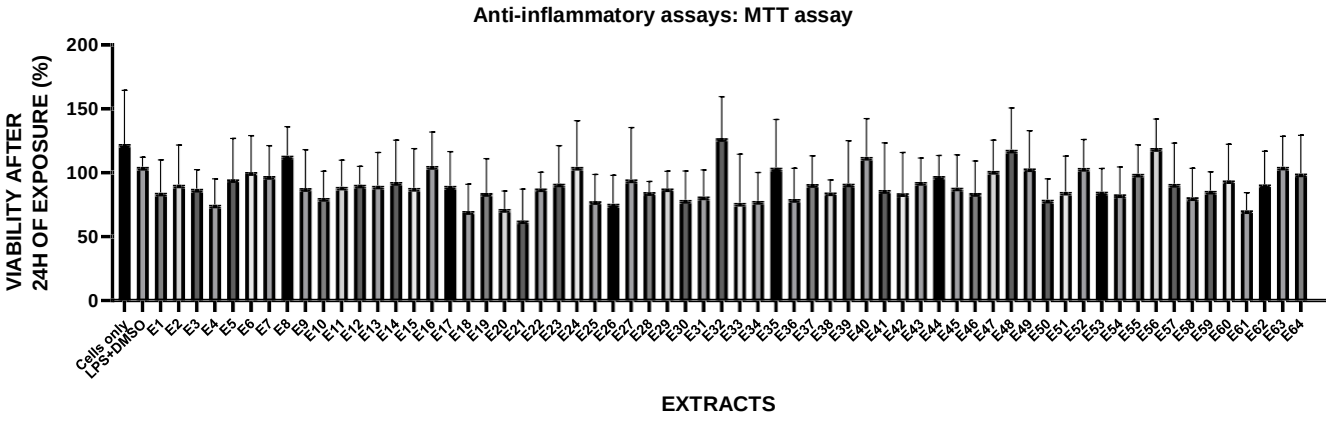

Figure 7 — Viability of RAW 264.7 cells evaluated through the MTT method in the anti-inflammatory assays. For this analysis, the percentage of viability after 24 h of exposure to extracts was compared to the control containing LPS and DMSO. No statistically significant values were obtained, indicating a linear cell viability, thus validating the assay.

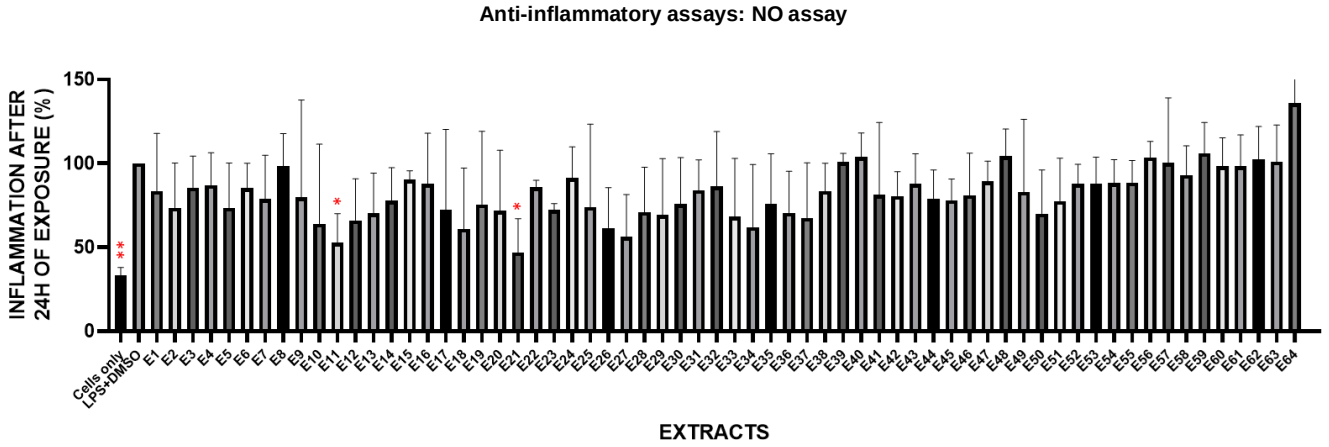

Figure 8 — Results obtained in anti-inflammatory assays, in which extracts were analyzed for their capacity to reduce the production of nitric oxide (NO). For this analysis, the percentage of inflammation after 24 h of exposure to extracts was compared to the control containing LPS and DMSO. Statistical significance is indicated by asterisks, * = $p < 0.05$, ** = $p < 0.01$.

Both extracts were derived from strains affiliated with the genus *Brevibacterium*, namely *Brevibacterium aureum* (E11) and *Brevibacterium sediminis* (E21). In fact, this genus has already been associated with antibacterial and anti-inflammatory activities (Srilekha et al., 2017). In a study conducted by Srilekha, in 2017, an isolated of marine *Brevibacterium* sp., demonstrated anti-inflammatory activity in a *in vivo* assay realized in

Wistar Albino rats after a paw edema was induced with carrageenan. In this assay, it was verified a reduction of the size of paw edema. Nevertheless, there are very few studies that prove this anti-inflammatory activity, and this genus is still practically unexplored in that field.

The results obtained may be quite promising, as the two extracts showing anti-inflammatory activity may contain new natural anti-inflammatory compounds. For this reason, these extracts were selected for dereplication analysis.

3.2. Anti-obesity assays

In the anti-obesity assays, the capacity of the actinobacterial extracts to reduce neutral lipids present in its abdomen of zebrafish was evaluated by exposing these extracts to zebrafish larvae.

The reduction was analyzed by quantifying the Nile red fluorescence by utilizing Image J software. The results were then statistically treated in GraphPad Prism 9 where the percentage of fluorescence intensity was compared to the solvent control.

A solvent control consisting in zebrafish exposed to DMSO (Fig. 9) and a positive control consisting in zebrafish exposed to REV (Fig. 10) were used.

Observing the images of the solvent and the positive controls, the differences are evident, with a clear effect of REV in the reduction of lipids in the abdominal region of zebrafish (clear reduction of fluorescence intensity when compared to the DMSO control), as expected.

During the assays, two of the extracts, namely E5 and E48, were not analyzed since they alone showed fluorescence, which did not allow the quantification of the fluorescence ofthe abdominal region of zebrafish (Fig. 11).

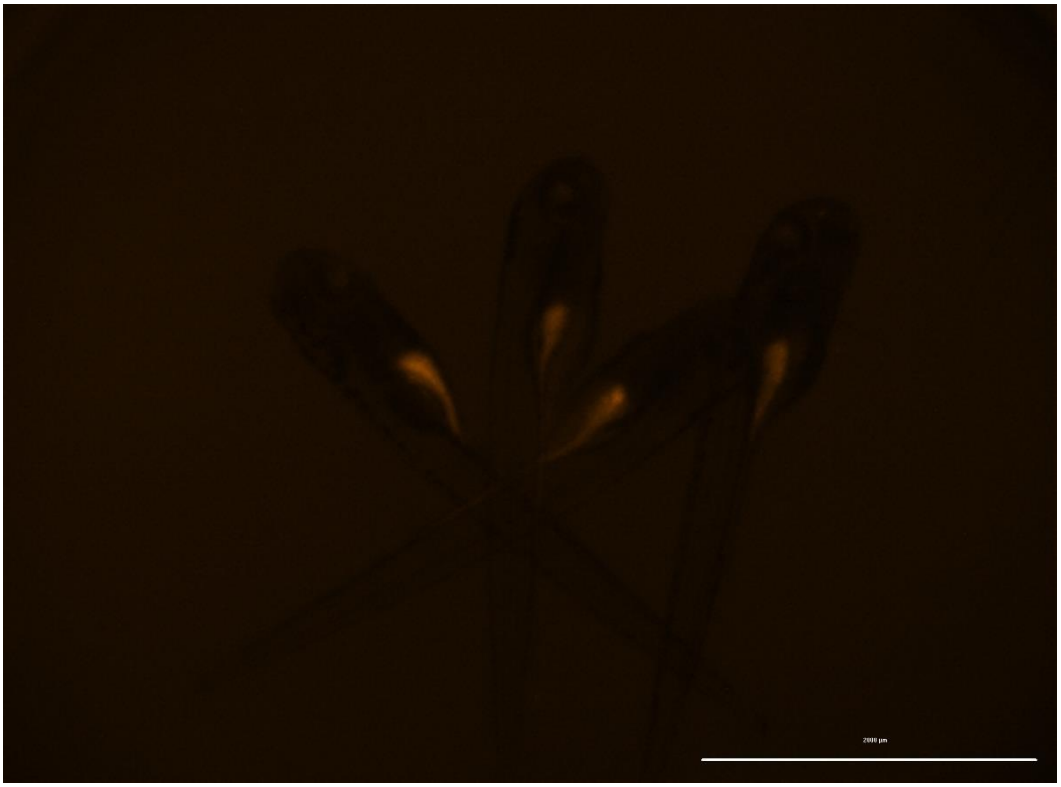

Figure 9 – Representative image of the solvent control containing DMSO in zebrafish Nile red assay obtained in Image J software.

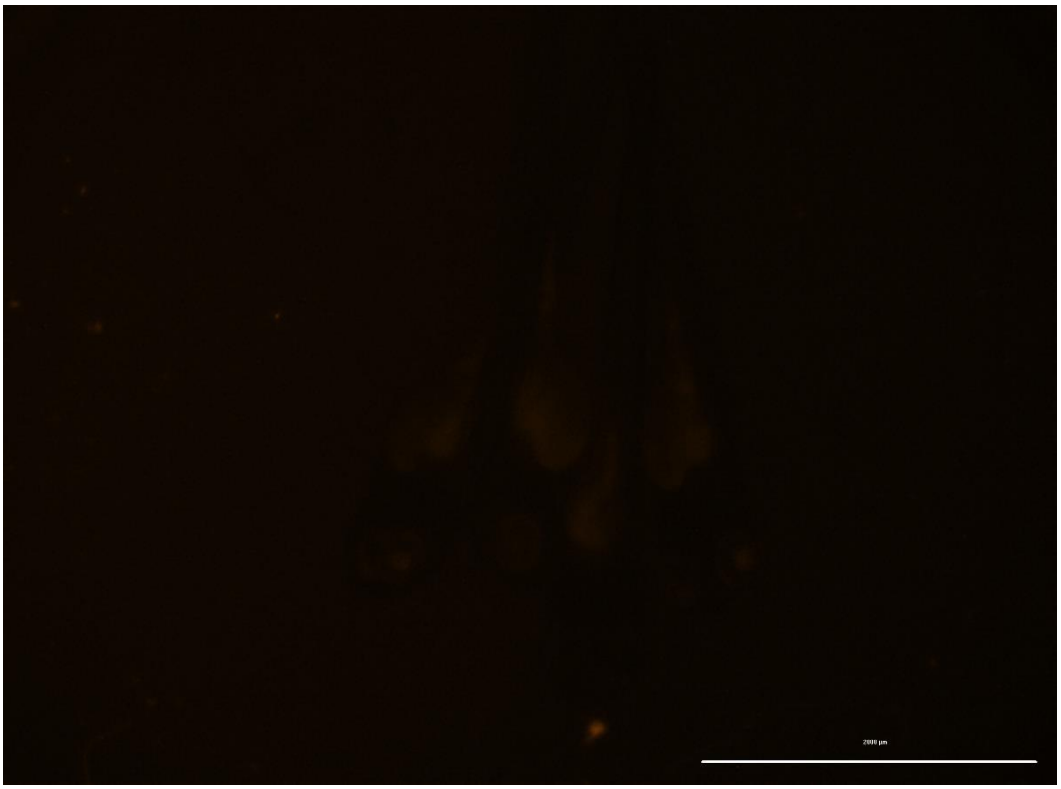

Figure 10 – Representative image of the positive control containing resveratrol (REV) in zebrafish Nile red assay obtained in Image J software.

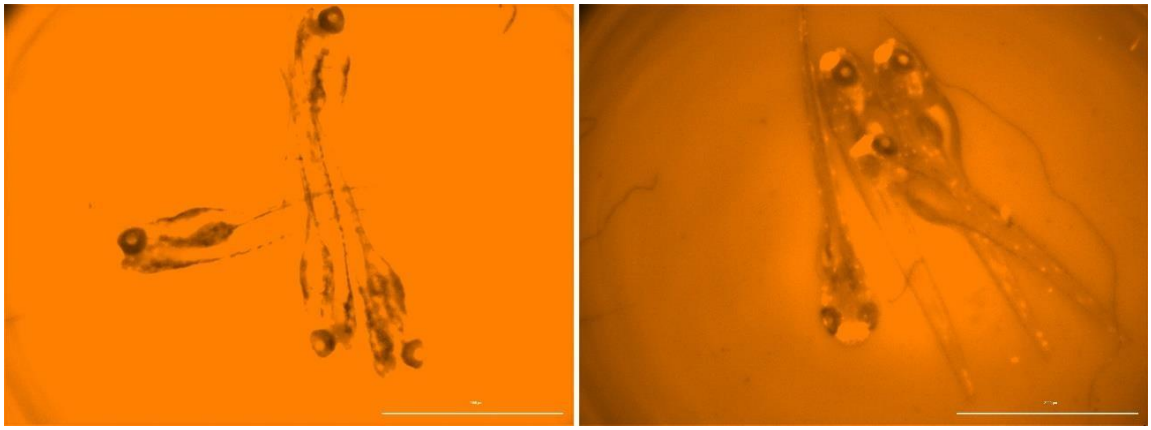

Figure 11 — Zebrafish embryos in the presence of the extracts E5 (left) and E48 (right) that showed fluorescence bythemselves.

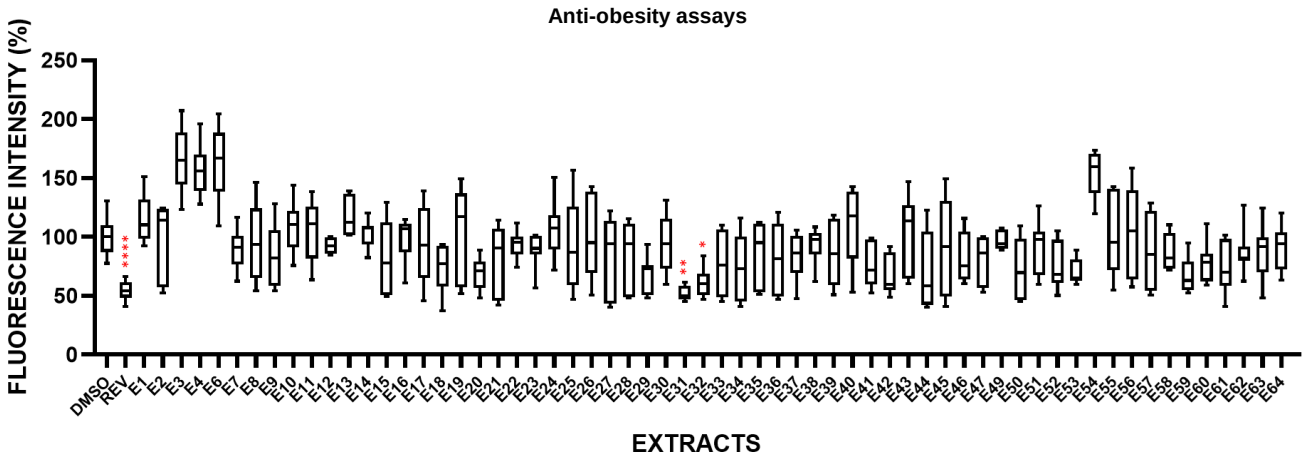

Figure 12 — Results of the anti-obesity screening for the 64 extracts derived from actinobacterial strains isolated from deep-sea samples collected in the Madeira archipelago. For this analysis, the percentage of florescence intensity of zebrafish exposed to the extracts was compared to the solvent control containing DMSO. Statistical significance is indicated by asterisks, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

The results of anti-obesity screening for the 64 actinobacterial extracts, revealed that two extracts, namely E31 and E32, significantly reduced the lipids present in zebrafish abdomen (Fig. 12). The mean values of fluorescence intensity in these extracts were of 53.61% and 59.66%, respectively, which corresponds to a lipid reduction of 47% and 40%.

This anti-obesity potential was also visualized in the images obtained referring to extracts E31 (Fig. 13) and E32 (Fig. 14) where a clear difference in the fluorescence intensity is visible compared to the solvent control (Fig. 9).

As in the anti-inflammatory assays, both extracts were obtained from species affiliated with the *Brevibacterium* genus, specifically *Brevibacterium aerolatum* and *Brevibacterium sediminis*.

Since actinobacteria is still largely unexplored in terms of the production of anti-obesity compounds, it is not possible to find information in the literature linking this activity to this group of microorganisms. However, there are already studies that prove that actinobacteria can have anti-obesity activity (Santos et al., 2019).

However, in terms of scientific progression in this particular field, these results show that actinobacteria can be promising for the discovery of new anti-obesity molecules.

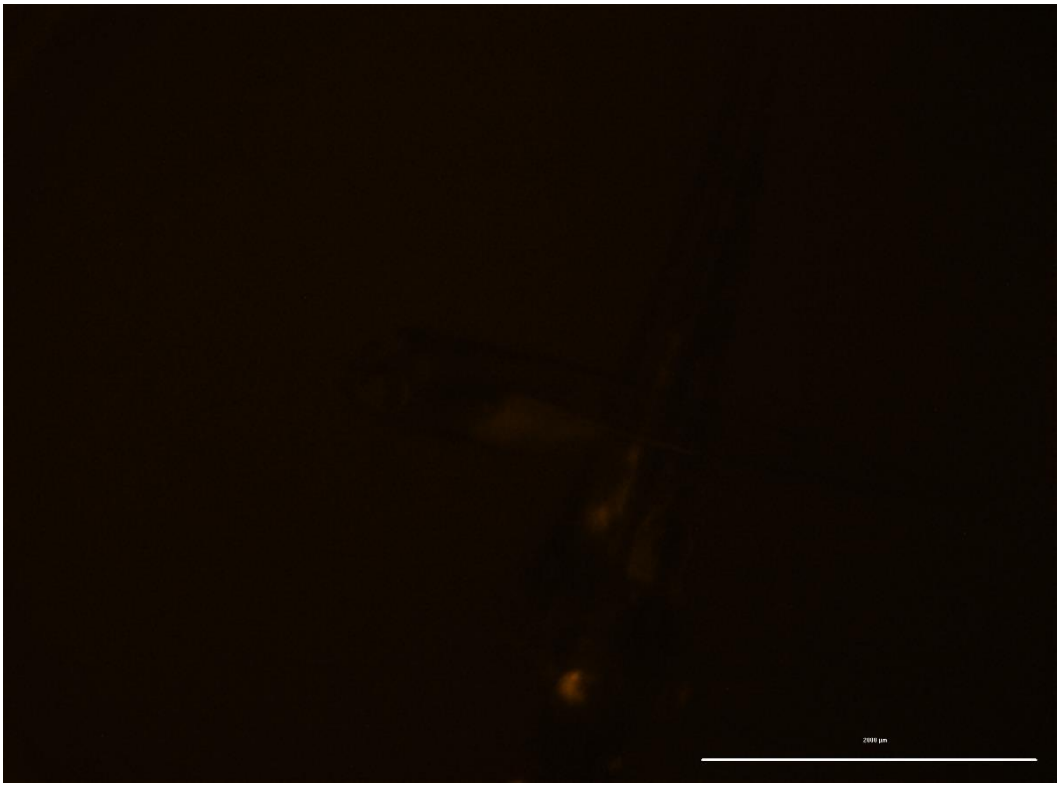

Figure 13 – Image obtained in Image J software of zebrafish in the presence of extract E31.

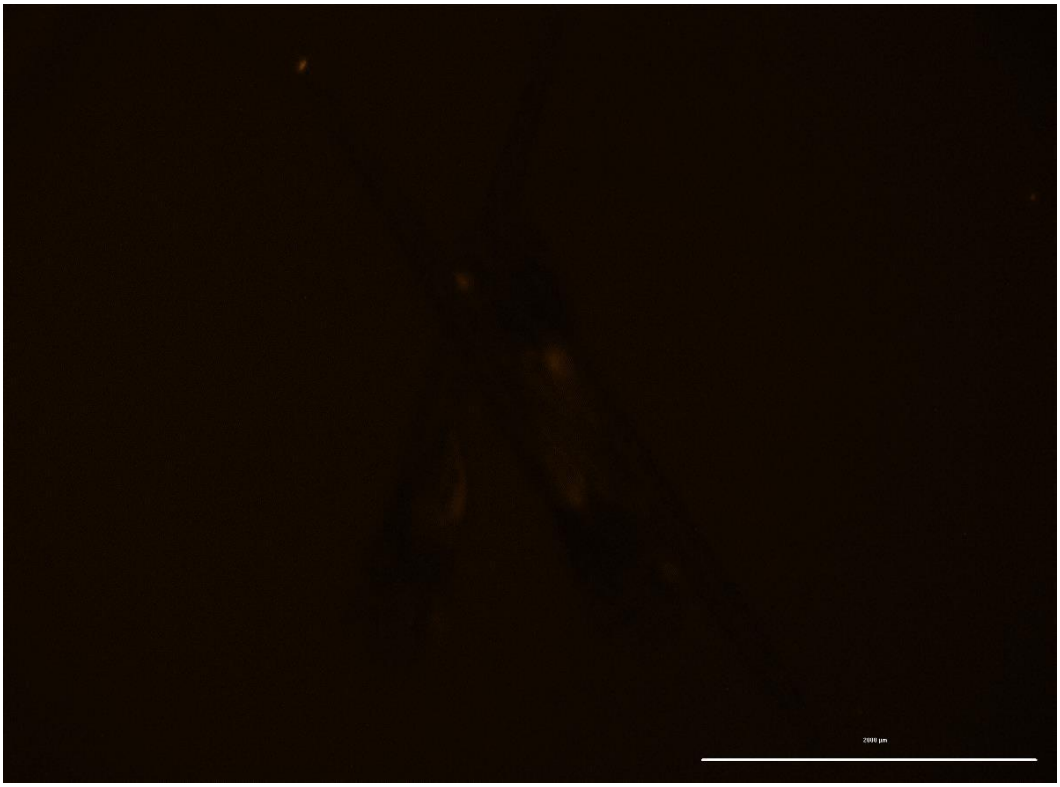

Figure 14 – Image obtained in Image J software of zebrafish in the presence of extract E32.

Although not directly related to the observed activity, during the assays it was found that some of the extracts, namely E3, E4 and E6, caused Nile Red to mark a structure different from that usually stained (Fig. 15).

In order to understand what this structure was, the anatomy of the zebrafish was studied, and it was concluded that it could be cells of the olfactory epithelium, also called olfactory rosette (Fig. 16).

However, it was inconclusive what type of cells were actually being stain as no bibliographic reference to the marking of this structure by Nile Red was found. It will be interesting in the future to follow-up this strong staining, trying to evaluate if some biotechnological application may be developed from this.

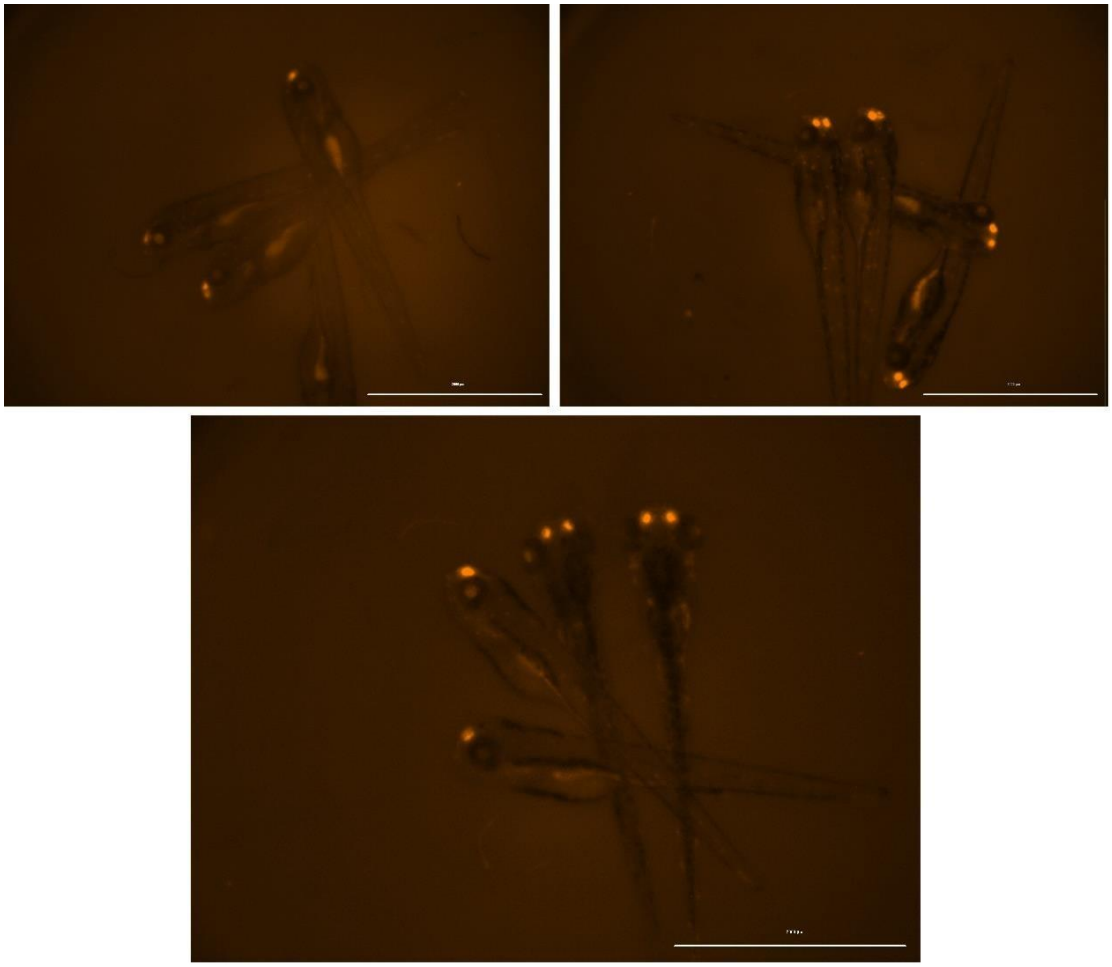

Figure 15 — Image obtained in Image J software of zebrafish in the presence of the extracts E3 (top left), E4 (top right) and E6 (bottom), where a structure different from those usually marked by this stain appeared.

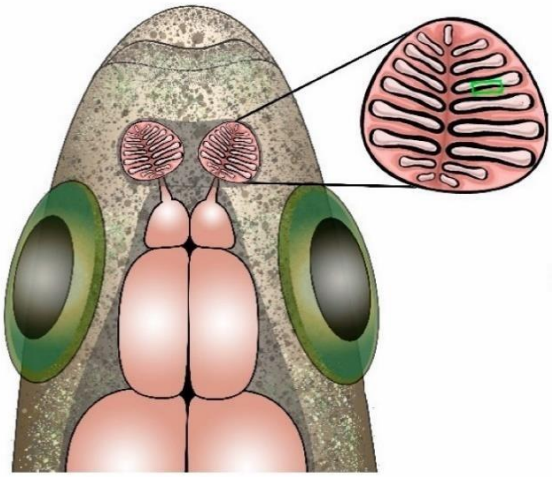

Figure 16 – Olfactory epithelium (rosette) possibly marked by Nile Red during the anti-obesity assays (adapted from Calvo-Ochoa and Byrd-Jacobs, 2019).

3.3. Anti-fouling assays using biofilm-forming marine bacteria

The anti-fouling activity of the actinobacterial strains previously isolated from deep-sea samples collected in the Madeira archipelago was studied by screening crude extracts obtained from these isolates in terms of their capacity to inhibit the growth of five fouling bacteria: *Cobetia marina, Roseobacter litoralis, Halomonas aquamarina, Vibrio harveyi* and *Pseudoalteromonas atlantica.*

The inhibition of bacterial growth was calculated based on the growth of the negative control, containing only the fouling bacteria and DMSO.

Anti-fouling activity was considered significant when the growth of fouling bacteria was inhibited by at least 30%. However, as shown in Figs 17 to 21, none of the extracts significantly inhibited the growth of fouling bacteria.

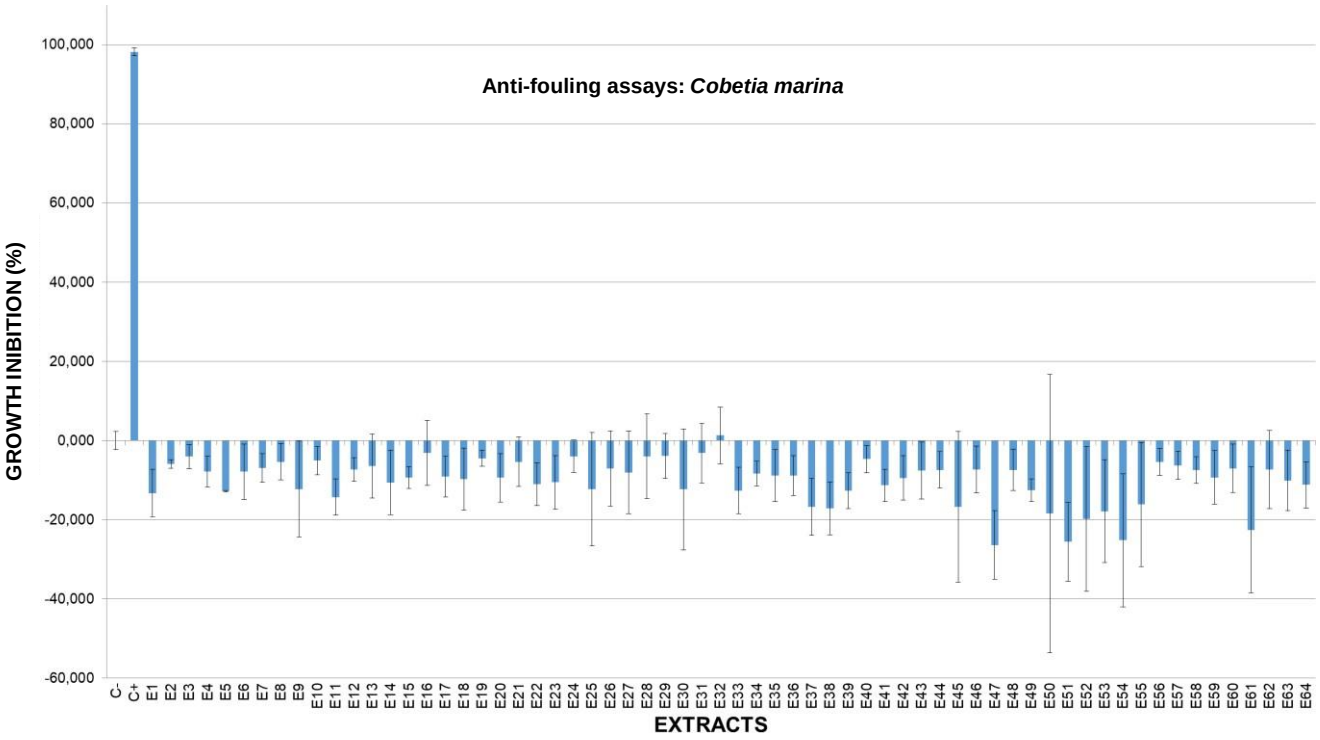

Figure 17 – Results of the anti-fouling assays where *Cobetia marina* was exposed to the actinobacterial extracts. The results were represented based on the percentage of growth inhibition, comparing to the negative control.

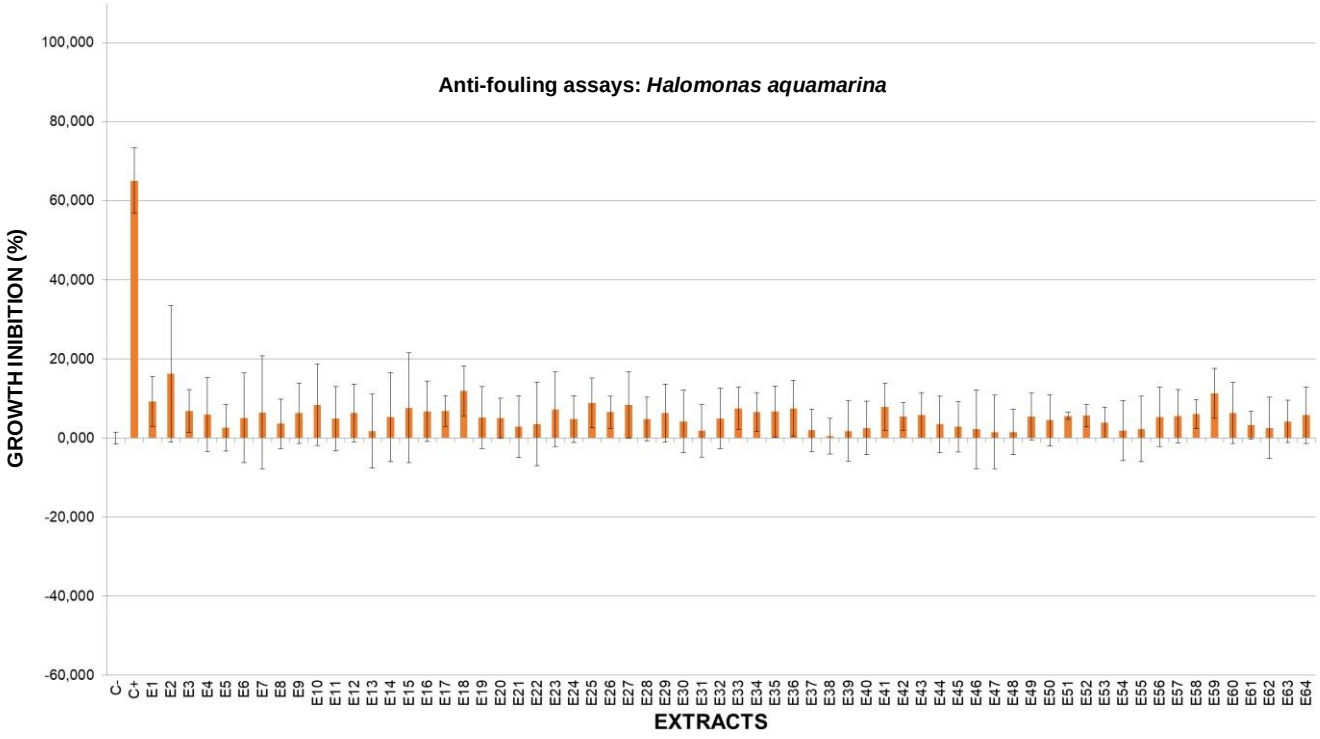

Figure 18 — Results of the anti-fouling assays where *Halomonas aquamarina* was exposed to the actinobacterial extracts. The results were represented based on the percentage of growth inhibition, comparing to the negative control.

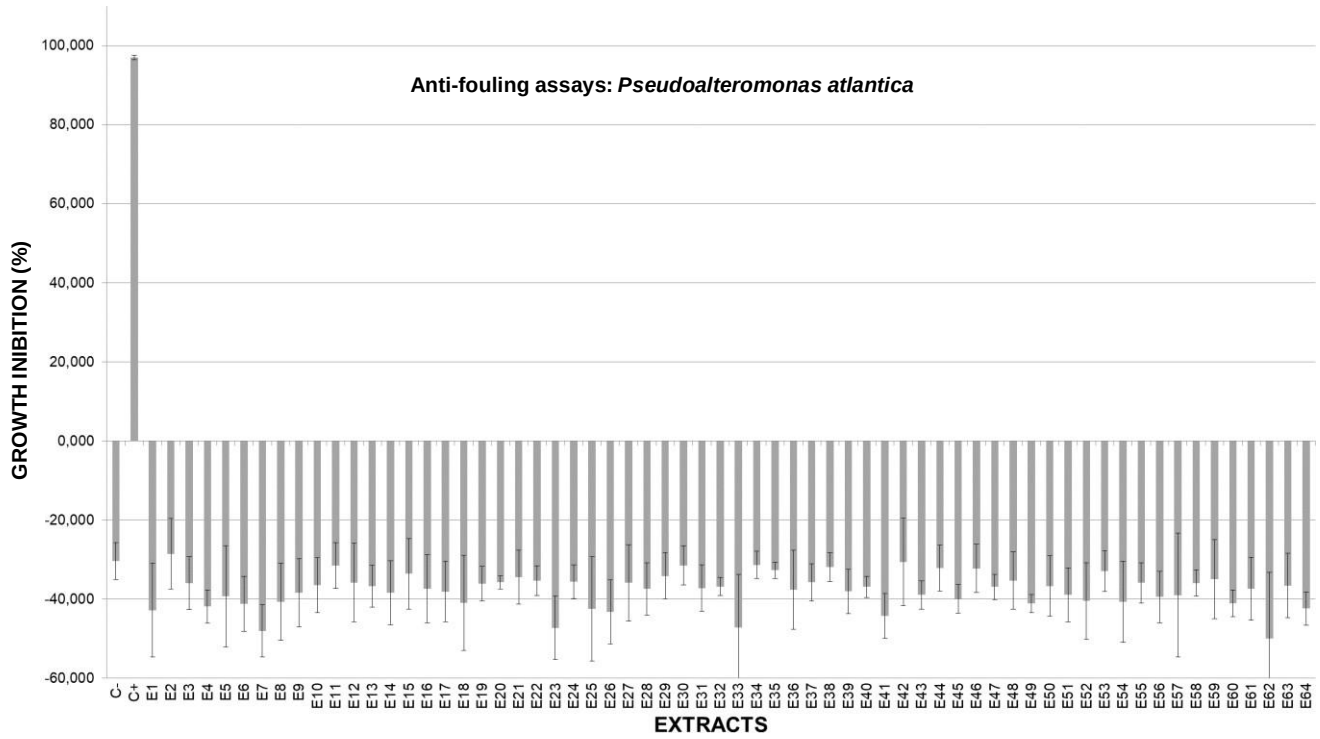

Figure 19 — Results of the anti-fouling assays where *Pseudoalteromonas atlantica* was exposed to the actinobacterial extracts. The results were represented based on the percentage of growth inhibition, comparing to the negative control.

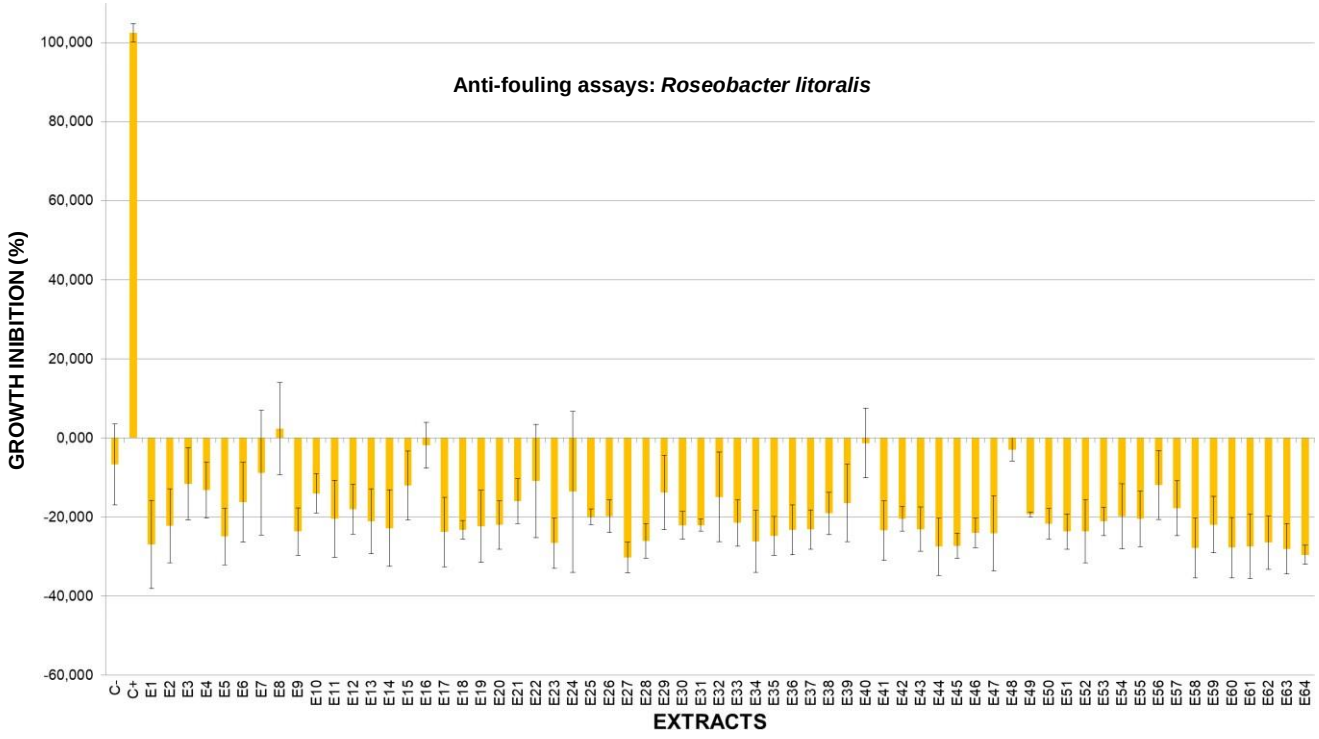

Figure 20 — Results of the anti-fouling assays where *Roseobacter litoralis* was exposed to the actinobacterial extracts. The results were represented based on the percentage of growth inhibition, comparing to the negative control.

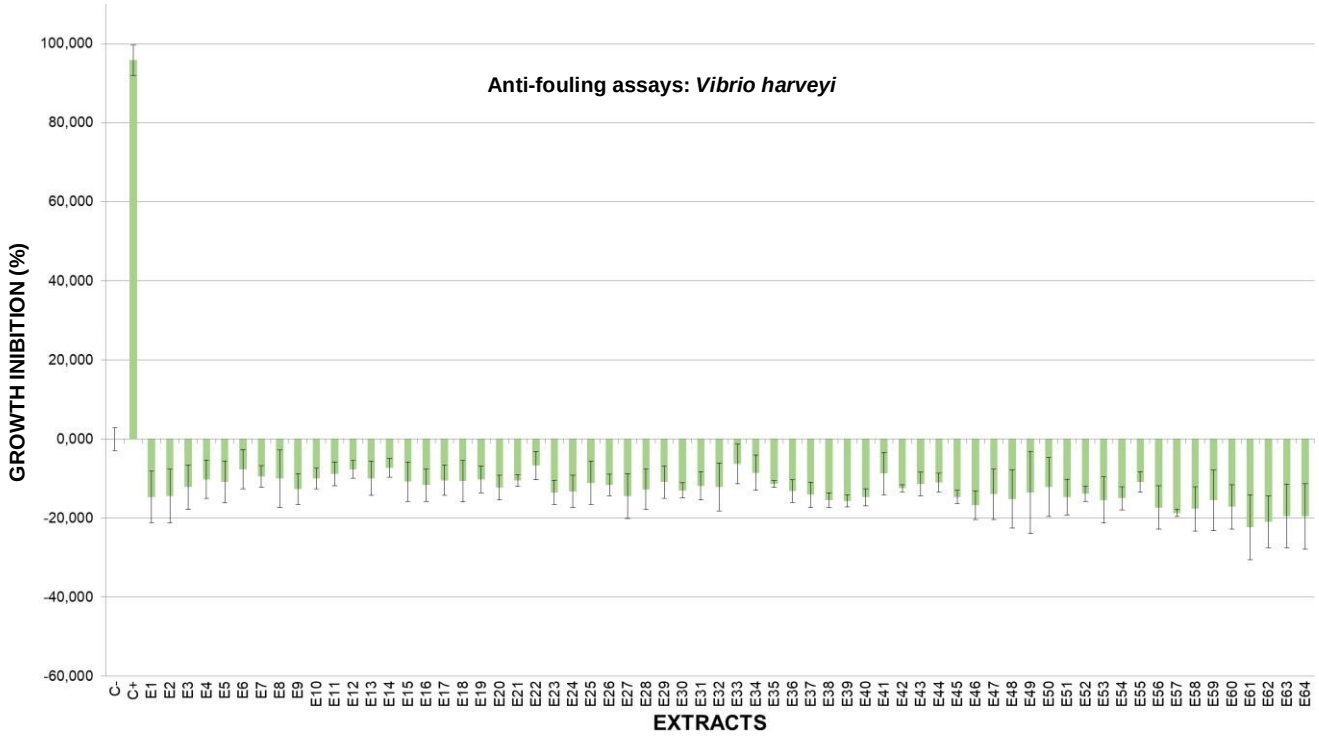

Figure 21 – Results of the anti-fouling assays where *Vibrio harveyi* was exposed to the actinobacterial extracts. The results were represented based on the percentage of growth inhibition, comparing to the negative control.

The results obtained in the anti-fouling assays with *Cobetia marina* (Fig. 17), *Pseudoalteromonas atlantica* (Fig. 19), *Roseobacter litoralis* (Fig. 20), and *Vibrio harveyi* (Fig. 21), clearly indicate that none of the actinobacterial extracts was able to significantly inhibit the growth of these bacteria.

In terms of the results of *Halomonas aquamarina* (Fig. 18), it is possible to observe that some of the actinobacterial extracts slightly inhibited its growth though not significantly, reaching not even 20% of growth inhibition. However, for the actinobacterial extract E2, a considerable standard deviation in growth inhibition values was obtained, with some replicates presenting an inhibition greater than 30%. Thus, particularly for this extract, it would be important to test it again in the future against the species *Halomonas aquamarine*, as well as against other fouling bacteria.

3.4. Dereplication of the bioactive extracts and molecular network analysis

3.4.1. Dereplication of the bioactive extracts

To analyze whether the detected bioactivity could be related with already known secondary metabolites, or, on the contrary, could suggest the presence of new natural compounds, a dereplication analysis of the four extracts that showed anti-inflammatory (E11 and E21) and anti-obesity (E31 and E32) activities was performed.

The dereplication results revealed that the actinobacterial extracts E21 and E32 associated to *Brevibacterium sediminis* both contained in its composition the antibiotic A201A ($C_{37}H_{50}N_6O_{14}$) and skyllamycin B ($C_{74}H_{92}N_{12}O_{20}$). The first compound is associated with antibacterial activity and is typically produced by a biosynthetic gene cluster (*ata*) of *Saccharothrix mutabilis*, a soil actinobacteria affiliated with the family Pseudonocardiaceae (Saugar et al., 2017), but it was also discovered to be produced by *Marinactinospora thermotolerans*, a deep-sea actinobacteria isolated from a sediment of the South China Sea at a depth of 3865 m (Zu et al., 2012). The skyllamycin B is a cyclodepsipeptide produced by *Streptomyces* sp. and contains a high content of α- and β-hydroxylated amino acids. These amino acids are commonly found on antibiotics like vancomycin (Pohle et al., 2011), which was also identified in the composition of both

extracts. In the actinobacterial extract E21 was also found nocardamine ($C_{27}H_{48}N_6O_9$), also known as deferrioxamine E. This compound is an antibiotic found to be produced by *Streptomyces wadayamensis* and *Streptomyces leeuwenhoekii*, isolated from a marine sponge (Lee et al., 2005).

In addition to the fact that no records have been found indicating the production of the antibiotic A201A, skyllamycin B and nocardamine by any *Brevibacterium* species, there is also no studies linking these compounds to anti-inflammatory (E21) or anti-obesity (E32) activity. Thus, it is inconclusive whether the bioactivities presented by these two extracts are due to the presence of the mentioned compounds.

The actinobacterial extract E11, derived from a *Brevibacterium aureum* strain, contained, in addition to antibiotic A201A, daptomycin ($C_{72}H_{101}N_{17}O_{26}$), neoviridogrisein III (C45H64N8O11) and actinomycin Y8 (C61H82N12O18). Daptomycin is an antibiotic derived from the actinobacteria *Streptomyces roseosporus* and is used to treat complex skin and tissue infections (Kelesidis, 2014). Neoviridogrisein III is a depsipetide antibiotic found in *Streptomyces griseoviridus* (Okumura et al., 1979). Actinomycin Y8 is an antibiotic with cytotoxic activity produced by various species of *Streptomyces*.

These compounds have not been previously found in *Brevibacterium* species and, apparently, do not explain the anti-inflammatory activity exhibited by the extract.

As for the actinobacterial extract E31, also from a *Brevibacterium* strain (*Brevibacterium aerolatum*), it was found that this actinobacterial extract contains monensin M2, a polyether antibiotic previously isolated from *Streptomyces cinnamonensis* (Královcová et al., 1984).

However, this compound, has not been identified before in *Brevibacterium* species and should not explain the anti-obesity activity of the extract E31.

3.4.2. Molecular network analysis

Additionally, a molecular network of the 4 actinobacterial extracts exhibiting anti-inflammatory or anti-obesity activities was build using GNPS data analysis workflow.

The resulting molecular network allowed to detect sets of spectra from related molecules and visualize the distribution of m/z clusters between the actinobacterial extracts.

From this, it was possible to obtain an individual cluster with two nodules (m/z 545.08 and 543.04) that was only present in extract 31 (Fig. 22).

For this reason, two more databases were consulted, namely the Natural Products Atlas and the Dictionary of Natural Products, where no hits were found for the masses obtained. This fact makes this extract highly promising, as it may present new metabolites.

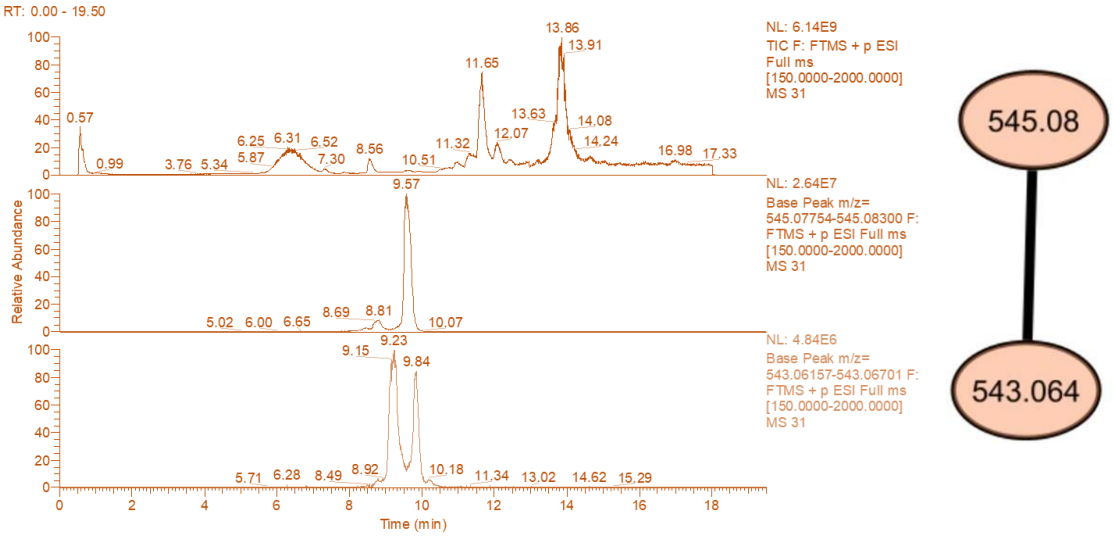

Figure 22 — Analysis of the GNPS-generated molecular network LC-HRESI MS/MS of the actinobacterial crude extract E31 that showed an exclusive cluster represented by connected nodules and marked with the corresponding m/z value for the parental ion that did not show hits in the databases used for dereplication. For the cluster it is shown the extracted ion chromatograms (EICs).

Thus, in the future, the next step will be to perform a scale-up of the corresponding actinobacterial culture in order to isolate and characterize the compounds relative to these masses and determine if they are new molecules.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

There is no doubt that actinobacteria have an enormous biotechnological value due to their ability to produce unique bioactive compounds with various biotechnological applications.

In this study, it was aimed to explore the bioactive potential of an actinobacterial collection of 64 isolates obtained previously from deep-sea samples (sponges, corals and sediments) collected in the Madeira archipelago. The crude extracts obtained from these isolates were bioprospected for anti-inflammatory, anti-obesity and anti-fouling activities.

Anti-inflammatory assays revealed two actinobacterial extracts, namely E11 and E21, capable of reducing the inflammation induced by LPS in cells of the RAW 264.7 cell line. These extracts were obtained from strains of *Brevibacterium aureum* and *Brevibacterium sediminis*, respectively. When dereplication was performed, it was revealed that extract E11 contained the antibiotic A201A, daptomycin, neoviridogrisein III and actinomycin Y8, while extract E21 presented the antibiotic A201A, skyllamycin B, vancomycin and nocardamine. Nevertheless, none of these compounds was able of explaining the observed anti-inflammatory activity.

In the anti-obesity assays, there were two actinobacterial extracts, specifically E31 and E32, showed the ability of reduce the neutral lipids present on the abdominal region of zebrafish. These extracts were both also affiliated to the *Brevibacterium* genus (Brevibacterium aerolatum and Brevibacterium sediminis, respectively). Like extract E21, extract E32 also contained the antibiotic A201A, skyllamycin B and vancomycin, whereas in extract E31, only the compound monensin M2 was identified. None of the detected compounds can, however, properly justify the observed anti-obesity activity.

None of the actinobacterial extracts exhibited anti-fouling activity, as they were not able to significantly inhibit the growth of the fouling bacteria *Cobetia marina, Roseobacter litoralis, Halomonas aquamarina, Vibrio harveyi* and *Pseudoalteromonas atlantica*.

After constructing the molecular network using the GNPS data analysis workflow, it was found that the actinobacterial extract E31 had a cluster with two nodules (m/z 545.08 and 543.04) not shown in any other extract in the laboratory database. These masses were not identified when consulting the Natural Products Atlas and the Dictionary of Natural Products platforms, suggesting that these masses might correspond to new molecules.

In conclusion, the 4 extracts demonstrating anti-inflammatory or anti-obesity activities are very promising since the dereplication did not identify compounds associated with these activities. Among these actinobacterial extracts, the E31 extract stands out since it presents a unique cluster that may indicate the presence of new molecules. Overall, these results indicate that deep-sea actinobacteria can be an important source of new compounds with relevant bioactivities. Nonetheless, as anti-obesity, anti-inflammatory and antifouling activities are scarcely explored in actinobacteria, it would be relevant to extend their screening to actinobacteria with origins other than the deep-sea.

As future work, it would be essential to further study extract E31 in terms of its chemical profile. To achieve this objective, it would be necessary to (i) grow in a larger scale the corresponding actinobacterial strain (about 20-30 L of liquid culture), (ii) prepare the respective crude extract, (iii) isolate the unidentified masses and (iv) characterize these masses in order to conclude if they could be new molecules. Furthermore, it would be relevant to test again the actinobacterial extract E2 to confirm its activity against the fouling bacterium *Halomonas aquamarina* and, also, to extend the assays to other marine fouling bacteria.

CHAPTER 5

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