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Analysis of secondary metabolism in marine Actinobacteria: searching for novel compounds.

José Diogo Neves dos Santos

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José Diogo Neves dos Santos

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto, Instituto de
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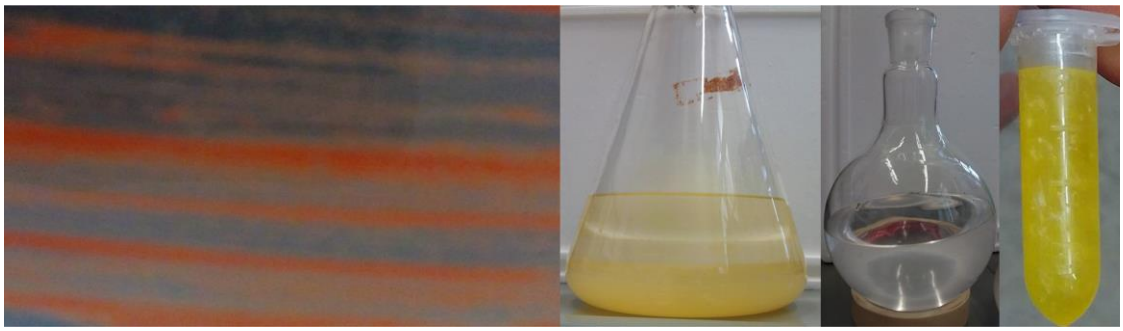

Analysis of secondary metabolism in marine Actinobacteria: searching for novel compounds.

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Ano 2016/2017

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

“Sometimes science is more art than science, Morty. A lot of people don’t get that”

Rick Sanchez, “Rick and Morty”

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FCT Fundação para a Ciência e a Tecnologia
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Resumo

Os antibióticos, mais do que qualquer outro fármaco, são responsáveis por melhorar a expectativa de vida do Homem. No entanto, com o aumento do número de estirpes multirresistentes aos antibióticos e a sua disseminação pelo planeta, a capacidade de tratamento de infeções bacterianas está afetada. Esta é uma preocupação mundial em matéria de saúde pública. A obesidade e as doenças metabólicas também são uma ameaça global à saúde, com 600 milhões de adultos e 100 milhões de crianças diagnosticadas como obesas. A obesidade aumenta a probabilidade de outras doenças ocorrerem, como doenças cardiovasculares, diabetes tipo 2, apneia do sono, cancro, osteoartrite e depressão. Infelizmente, ainda não existe uma abordagem farmacológica segura e eficiente para tratar da obesidade. Por isso, a luta contra estes dois problemas exige o reforço da procura de novos compostos.

Novas moléculas bioativas são, frequentemente, encontradas em ambientes sub-explorados, como habitats marinhos. Muitos animais marinhos já provaram ser reservatórios para tais moléculas. Certas evidencias apontam para que a origem das moléculas bioativas esteja nas comunidades microbianas que colonizam e interagem com esses animais. Em face ao exposto, o principal objetivo deste trabalho foi confirmar a capacidade bioativa de bactérias marinhas, otimizar a produção das suas moléculas bioativas, isolar e caracterizar as mesmas e elucidar os seus modos de ação.

No decorrer deste trabalho, várias bactérias pertencentes aos filos Actinobacteria, Gammaproteobacteria e Firmicutes, previamente isoladas de esponjas marinhas provenientes da costa marinha de Portugal, foram avaliadas quanto à capacidade de produção de moléculas com propriedades antimicrobianas. Ensaios preliminares já tinham mostrado o potencial bioativo dessas bactérias, o qual foi confirmado. No entanto, ao testar as bactérias em vários ensaios, nenhuma bioatividade antimicrobiana foi detetada. Modificações feitas aos métodos de incubação, de extração e ensaios antimicrobianos não resultaram em bioatividade consistente. Quando o volume total de cultura extraído foi aumentado, bioatividade consistente foi obtida. Duas estirpes de Actinobacteria, *Rhodococcus equi* (# 91-36.1) e *Microbacterium foliorum* (# 91-36.2) demonstraram bioatividade contra *Bacillus subtilis* e *Micrococcus luteus*, e *M. foliorum* demostrou ainda atividade contra *Chlamydia trachomatis*. Nos ensaios de anti-obesidade, uma Gammaproteobacteria, *Pseudovibrio sp.* (B02-61), e uma Actinobacteria, *Microbacterium sp.* (B02-79), induziram a redução da acumulação de lipídios em larvas de peixes-zebra (*Danio rerio*). Extratos de *R. equi* e *M. foliorum* foram

submetidos a cromatografia de permeação em gel com o intuito de isolar compostos ativos. Contudo nenhuma das frações recolhidas revelou bioatividade.

Apesar de termos conseguido verificar que várias bactérias produziam moléculas bioativas, apesar de nem sempre de forma consistente, não foi possível, infelizmente, no tempo de realização deste trabalho, conseguir o isolamento dos princípios ativos.

Este trabalho demonstrou o potencial bioativo de bactérias marinha e é o ponto de partida para explorar as bioatividades encontradas, em particular o potencial anti-obesidade.

Palavras-chave

Bioquímica; Microbiologia; Actinobacteria; Gammaproteobacteria; Firmicutes; Antibióticos; Resistência a antibióticos; Agentes Patogénicos; Metabolismo secundário; Obesidade; Extração de moléculas bioativas;

Abstract

Antibiotics, more than any other drug, are responsible for the improvement of Human life expectancy. Nonetheless, as multi-drug resistance increases and spreads throughout bacterial populations our ability to treat bacterial infections becomes greatly weakened. This is a worldwide public health concern. Obesity and metabolic diseases are also a global health threat, with 600 million adults and 100 million children diagnosed as obese. Obesity also increases the likelihood of various diseases and conditions like cardiovascular diseases, type 2 diabetes, sleep apnea, cancer, osteoarthritis and depression. Unfortunately, a lack of an efficient and safe pharmacological approach to treat obesity still exists. As such, novel compounds must be found.

Novel bioactive molecules are frequently found in underexplored environments, like marine habitats. Many marine animals have already proven to be reservoirs for such molecules. Evidences points to a microbial origin of these bioactive molecules present in the communities that colonize such animals. Based on the above referred, the main goal of this work was to confirm the bioactive capacity of marine bacteria, optimize the production of their bioactive molecules, isolate and characterize them and clarify their modes of action.

Over the course of this work, several Actinobacteria, Gammaproteobacteria and Firmicutes bacteria, previously isolated from marine sponges off the coast of Portugal, were screened for the capacity to produce molecules with antimicrobial properties.

Preliminary assays proved the bioactive potential of these bacteria, which was confirmed in this study. However, when testing bacteria in several assays, no antimicrobial bioactivity was detected. Modifications made to the incubation, extraction and test methods resulted in no evidence of consistent bioactivity. When the total culture volume for extraction was increased, consistent bioactivity was obtained. Two Actinobacteria strains, *Rhodococcus equi* (#91-36.1) and *Microbacterium foliorum* (#91-36.2) were found to be bioactive against *Bacillus subtilis* and *Micrococcus luteus*. Additionally, *M. foliorum* showed activity against *Chlamydia trachomatis*. In the anti-obesity assays, one Gammaproteobacteria, *Pseudovibrio sp.* (B02-61), and one Actinobacteria, *Microbacterium sp.* (B02 79), induce the reduction of lipid accumulation in zebrafish larvae (*Danio rerio*). Gel permeation chromatography was attempted in extracts from *R. equi* and *M. foliorum* with the goal to isolate bioactive compounds. However, none of the fractions collected proved to be bioactive.

Despite various bacteria having produced bioactive molecules, although not always consistently, it was not possible to achieve the isolation of the active principles, in the limited timeframe of this thesis.

This work demonstrated the bioactive potential of some marine bacteria and is the starting point for exploring the bioactivities found and in particular, the anti-obesity potential.

Keywords

Biochemistry; Microbiology; Actinobacteria; Gammaproteobacteria; Firmicutes; Antibiotics; Antibiotic resistance; Pathogens; Secondary Metabolism; Obesity; Bioactive molecule extraction;

List of Figures and Tables

Tables

Table 5 – Levels of inhibition produced by the 33 bacterial extracts against the 3 targets.

Figures

List of Abbreviations

% - Percentage sign

® - Registered trademark symbol

3T3-L1 adipocytes – Mouse cell line derived from 3T3 adipose cells

A+T – Adenine and Thymine ratio

ACP - Acyl Carrier Protein

B. subtilis - *Bacillus subtilis*

BMI - body mass index

C. albicans - *Candida albicans*

C. trachomatis - Chlamydia trachomatis

CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental da Universidade do Porto, Porto, Portugal

CO_2 - Carbon dioxide

D. rerio - *Danio rerio*

DAPI- 4',6-diamidino-2-phenylindole

DCM – dichloromethane

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

E. coli - *Escherichia coli*

e.g. - *exempli gratia* (for example)

Fig/ Figs –Figure/ Figures

g - Grams

G+C – Guanine and Cytosine ratio

GPC - Gel permeation chromatography

$Gram^+$/ $Gram^-$ - Gram positive bacteria/ Gram negative bacteria

h - Hour/ hours

HEp-2 - Cell line derived from the eukaryotic Human Epidermoid carcinoma

IP_3 - inositol 1,4,5-triphosphate

L- Liters

LEMUP - Laboratory of Microbial Ecophysiology from University of Porto

LPS - lipopolysaccharide

M. luteus - *Micrococcus luteus*

MeOH - methanol

mg - Milligrams

mL - Milliliters

MoA- Mode of action

MRSA - Methicillin-Resistant *Staphylococcus aureus*

NA/NB - Nutrient Agar/Broth medium

NaCl - sodium chloride

Nm - Nanometers

NRPS - Nonribossomal peptide synthetases

º C – Degrees Celsius

OD_{600nm} - Optical density at 600 nanometers

PABA - para-aminobenzoic acid

PBPs - penicillin-binding proteins

PCP - Peptidyl Carrier Protein

PKSs/ PKS-I - Polyketide synthases/ Type I Polyketide synthases

PTU – Phenylthiocarbamide

R.p.m. - Rotations per minute

RNA - Ribonucleic acid

S – Svedberg

SIRT1 - silent mating type information regulation 2 homolog 1 protein

^{TM} – Trademark symbol

WHO- World Health Organization

X-Gal- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

µg – Micrograms

µL- Microliters

Table of contents

Introduction

Antibiotics are a type of antimicrobial pharmaceutical drugs used in the treatment and prevention of bacterial infections, either by killing (bactericidal) or by inhibiting (bacteriostatic) the growth of bacteria and can range from broad spectrum, meaning that they can act on both $Gram^+$ and $Gram^-$ bacteria, to narrow spectrum, drugs with limited activity primarily only useful against particular species of microorganisms (Taber & Thomas, 1997). Although exists evidence that some traditional medicines used mixtures with antimicrobial properties for the treatment of infections, with poultices using specially selected molds and plant material extracts (Forrest, 1982), it was only in the late nineteenth and early twentieth centuries that laboratorial observations of antagonistic interactions between microorganisms led to the discovery of natural antibacterial compounds produced by microorganisms and the appearance of the concept of antibiosis, to describe the action of the metabolites produced in by microorganisms (Foster & Raoult, 1974).

The history of antibiotics

In the late 1880s in Germany, Paul Ehrlich noted that certain dyes would color human, animal, or bacterial cells, whereas others did not, and proposed the idea that it might be possible to create chemicals that would act as a selective drug that would bind to and kill bacteria without harming the human host. After screening hundreds of dyes against various organisms, in 1907, he discovered a medicinally useful drug, the first synthetic antibacterial arsphenamine, used in the treatment of syphilis (Schwalbe *et al.*, 2007). This was the beginning of synthetic antibiotic chemotherapy, which delivered other useful drugs such as the first sulfonamides, Prontosil was developed by Bayer in 1935 from coal-tar dyes (Hager, 2006). Nevertheless, it was only with the discovery of penicillin in 1928 by Alexander Fleming that antibiotics started to revolutionize humanity in the 20th century. Penicillin displayed potent antibacterial activity against a wide range of bacteria, had low toxicity in humans, was not inhibited by biological constituents such as pus and could be mass-produced in World War II, helping with casualties (Florey, 1945; Sykes, 2001). The discovery of such a powerful antibiotic was unprecedented, and, in conjunction with other antibiotics and vaccination, near eradication of some diseases was achieved. Nonetheless, almost all compounds with antimicrobial activity were discovered between the 1920s and the 1960s in the golden age of antibiotics. Afterwards, innovation came mainly though derivatives of the previously discovered compounds (Lewis, 2013).

How do antibiotics work

Antibiotics work by disrupting key biological processes in the bacterial cell, ranging from inhibiting enzymes or interfering with other cellular components through the disruption of essential cellular processes like the synthesis of cell wall, nucleic acid and proteins. The specific biochemical interaction between the antibacterial compound and its biological target to produce the disruption of the biological process are known as the mechanisms of action (Spratto & Woods, 2012).

As such, antibiotics can be either divided into two categories, broad and narrow-spectrum action, or into more specific groups, that specify the target of the antibacterial compounds action. Broad-spectrum antibiotics, act against a wide range of bacteria normally both $Gram^+$ and $Gram^-$ bacteria (Taber & Thomas, 1997). An example of antibiotics in this category is ampicillin. Ampicillin is part of the β-lactam family of antibiotics, like penicillin G. Ampicillin, unlike this, can penetrate $Gram^+$ and some $Gram^-$ bacteria, as it has an amino group that helps the drug in the penetration of the outer membrane. This allows the normal action of the β-lactam antibiotics, stopping the enzyme that catalyzes the transpeptidation that crosslinks the peptide side chains of peptidoglycan strands. This action prevents normal cell wall regeneration and leads to cell lysis (Goodman *et al.*, 2006). Narrow-spectrum antibiotics, as the name implies, are active against a selected group of bacterial types and as such, are only typically used for specific infections when the bacterium is known. This selectivity also means that resistance is less likely to emerge. An example is fusidic acid, a steroid antibiotic active mainly against $Gram^+$ bacteria, that works by inhibiting bacterial protein synthesis (Rang *et al.*, 2015).

Nonetheless, this nomenclature provides almost no information about the underlying mechanisms behind their function and, as such, antibiotics are primarily divided into four main groups: inhibitors of cell wall synthesis, inhibitors of DNA synthesis, inhibitors of RNA synthesis and inhibitors of protein synthesis (Kaufman, 2011; Rang *et al.*, 2015). Many of these are part of the Class III and Class II biochemical reactions.

Unlike eukaryotes, prokaryotes possess a cell wall with peptidoglycan, and this makes the bacterial cell wall an ideal target for antibiotic compounds to act, since these should only affect prokaryote cell and not eukaryotes. Therefore, inhibitors of cell wall synthesis will prevent components of the bacterial machinery responsible for the cell wall regeneration from working appropriately. For example, penicillin and other β-lactams interrupt peptidoglycan synthesis by binding to the proteins from the penicillin-binding proteins (PBPs) group. These have been shown to catalyze several reactions involved

in the process of synthesizing cross-linked peptidoglycan from lipid intermediates and mediating the removal of D-alanine from the precursor of peptidoglycan (Basu *et al.*, 1992). This results in the formation of a weak or deformed cell wall, which swells and bursts (Rang *et al.*, 2015).

DNA replication and cell division are fundamental in maintaining and produce new bacterial cells. As such, some antibiotics can work by inhibiting replication of DNA. Interference with nucleic acid synthesis is possible in four different ways, by disruption of the DNA chains, inhibition of the synthesis of nucleotides, altering the base-pairing properties of the DNA template or inhibiting DNA gyrase, which uncoils supercoiled DNA to allow transcription (Kaufman, 2011). Disruption of DNA can be difficult to achieve, as it could affect eukaryotic cells besides prokaryotic ones. For this reason, drugs that act by disruption of DNA only work in very specific conditions. This is the case of metronidazole, which though reduction of its nitro group leads to the production of the short-lived cytotoxic intermediates that then interact with DNA, leading to cell death (Muller, 1983). Inhibition of the synthesis of the nucleotides can be accomplished by an effect on the metabolic pathways that generate nucleotide precursors. The folate biosynthetic pathway, that is only found in bacteria but not in humans, is an example. Folate is required for DNA synthesis in both bacteria and in humans, but humans must obtain it from the diet and concentrate it in cells by specific uptake mechanisms, while most species of bacteria lack these essential transport mechanisms and need to therefore synthesize it *de novo.* Sulfonamides are structural analogs of para-aminobenzoic acid (PABA), an essential component in the synthesis of folate, and compete with PABA for the enzyme dihydropteroate synthetase, and thus inhibit bacterial growth without impairing mammalian cell function (Rang *et al.*, 2015). Alteration of the base-pairing properties of the template agents act by intercalating in the DNA and causing frameshift mutations or mispairing. Examples of these agents include acridines, that double the distance between adjacent base pairs causing frameshift mutations, while inhibitors of DNA gyrase like fluoroquinolones act by selectively inhibiting the topoisomerase II ligase domain, which leads to fragmentation of DNA and cell death (Suto *et al.*, 1992).

Besides the three ways described above, DNA polymerase III inhibitors like anilinouracils are being developed. These are analogs of uracil that cannot form the 5'-3' links between nucleotides, halting DNA elongation and also sequestering DNA polymerase III with high affinity ternary complexes, eventually leading to cell lysis (Svenstrup *et al.*, 2008; Tarantino *et al.*, 1999).

Inhibition of RNA synthesis has a catastrophic effect on prokaryotic nucleic acid metabolism and is a potent means for inducing bacterial cell death. Specific inhibitors of bacterial RNA polymerase like rifampicin act by binding to RNA polymerase during the formation of the first two phosphodiester bonds and blocking the translocation step that would follow (McClure & Cech, 1978).

Both eukaryotes and prokaryotes require *de novo* protein synthesis as to maintain normal cellular functions, which in both happens in the cytosol, when mRNA is translated into an aminoacidic chain, on the ribosomes in a three-step process (Initiation, Elongation and Termination). Ribosomes also have three binding sites for tRNA, that transfer amino acids to ribosome, labelled the A, P and E sites. However, eukaryotic and prokaryotic ribosomes are structurally different which provides the basis for selective antimicrobial action of some antibiotics (Rang *et al.*, 2015). The bacterial ribosome consists of a 50S subunit and a 30S subunit, which combine into a 70S when mRNA is attached to the 30S subunit, unlike the eukaryote subunits are 60S and 40S (Ben-Shem *et al.*, 2011). Inhibition of mRNA translation can be achieved by inhibitors of 50S subunit or inhibitors of 30S subunit. 50S ribosome inhibitors include the macrolides, lincosamides, streptogramins, amphenicols and oxazolidinones and in general terms work by blocking either initiation of protein translation or translocation of tRNA, inhibiting the peptidyltransferase reaction (Kohanski *et al.*, 2010). However, 30S ribosome inhibitors like the tetracyclines and aminoglycosides, can disrupt the 30S ribosome in either of two ways. Tetracyclines compete with tRNA by blocking access of aminoacyl-tRNAs to the ribosome or by interacting with the 16S component of the 30S subunit that leads to instability of the connection between tRNA and ribosomes. Aminoglycosides cause an alteration in the conformation of the complex formed between an mRNA codon and its cognate charged aminoacyl-tRNA at the ribosome and promote tRNA mismatching (Kohanski *et al.*, 2010; Rang *et al.*, 2015).

Molecular mechanisms of antibiotic resistance

As most natural antibiotic compounds are secondary metabolites that bacteria use to outcompete others for resources, resistance to these compounds has evolved in specific species and strains, which means that intrinsic resistance precedes medical use of antibiotics (D'Costa *et al.*, 2011). Widespread use and misuse of antibiotics after World War II are due to wider global availability, uncontrolled sale, their use in livestock feed at low doses for animal growth promotion (Ferber, 2002) and release of large quantities of antibiotics into the environment during pharmaceutical manufacturing through inadequate wastewater treatment have caused evolutionary pressure on bacteria

(Ventola, 2015), resulting in a selective pressure of bacteria that lead to a spreading of resistance mechanisms throughout populations.

Essentially there are three key mechanisms by which bacteria become resistant to antibiotics: drug inactivation or modification, reduced drug accumulation and alteration of target site (Hawkey, 1998). In inactivation or modification of the antibiotic compound, the resistant bacteria retain the same sensitive target as antibiotic sensitive strains, but the antibiotic is prevented from reaching it. An example is the resistance to β-lactams, in which β-lactamases enzymatically cleave the four membered β lactam ring, rendering them inactive (Livermore, 1995). Reducing drug accumulation in the cell is another method bacteria have developed to prevent cell death. This can be achieved either thought an efflux pump, which pumps the antibiotic out of the cell, or inactivation of the original transporter responsible for the influx of the antibiotic (Hawkey, 1998). A case of this kind of resistance, is the lack of effect of β-lactams in *Pseudomonas aeruginosa* which has been found to lack a specific porin in the cell membrane, impeding β-lactams from entering the cell (Livermore, 1984). *Pseudomonas aeruginosa* has also been found to have a very efficient active efflux for tetracyclines, chloramphenicol and norfloxacin, which also explains this organism's general resilience (Li *et al.*, 1994). Bacteria, due to their fast division cycle and lack of many DNA repair mechanisms, tend to accumulate mutations fairly quickly (Martinez & Baquero, 2000), and can even integrate foreign DNA into their chromosome (Pozzi & Guild, 1985). These characteristics can be beneficial in achieving resistance to an antibiotic compound, as it may alter a target site in such a way that the antibiotic becomes unable to produce its effect. Most strains of *Streptococcus pneumoniae* are susceptible to β-lactams, but can acquire DNA from other bacteria with penicillin-binding proteins (PBPs) that have less affinity for penicillin (Zighelboim & Tomasz, 1980). Hence the altered PBPs still synthesize peptidoglycan for the cell wall, but are no longer susceptible to inhibition by β-lactams (Hawkey, 1998).

Consequences for the future

Since 2014, the World Health Organization (WHO) releases yearly reports on the global rise of resistance in pathogenic bacteria and a list of priorities on the need of new research. The Global Antimicrobial Resistance Surveillance report, released in April 2014, foresees an alarming future for mankind, since, for example, in patients with third-generation cephalosporin resistant *Escherichia coli* infections there was a two-fold increase in mortality attributable to bacterial infection and in patients with Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections there was also a significant increase in bacterial attributable mortality (WHO, 2014). These bacteria are some of the most common among humans. *S. aureus* colonizes the anterior nares (external portion

of the nostrils) while *E. coli* forms part of the gut microbiome (Eckburg *et al.*, 2005) and are a cause of the most common bacterial infection, such as urinary tract infections, food poisoning and bacteremia or blood poisoning (Brown *et al.*, 2014; Vogt & Dippold, 2005). And the latest pathogen priority list, released in the 27th of February 2017, that does not include *Mycobacteria*, which has its own report, places various Gram- bacteria, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and various Enterobacteriaceae (all of them resistant to carbapenems) as the top priorities for research and development of new drugs. Most antibiotics discovered in the past century were done so by screening soil microorganisms, but this limited resource of cultivable bacteria has already been over-explored, and synthetic approaches to produce antibiotics have not been able to replace them (Lewis, 2013). Unfortunately, these and other obstacles, like economic and regulatory ones, have made big pharmaceutical companies heavily reduce the investment in the field, making academia the main drive for the development of new compounds (Bartlett *et al.*, 2013; Gould & Bal, 2013; Ventola, 2015). This means that discovery and development of new antibiotics, specifically active against multidrug Gram- bacteria, and in particularly members of the Enterobacteriaceae family, such as *Klebsiella pneumonia* and *E. coli*, have been severely hampered. All these factors combined led to the production of several scientific review papers that showed haunting predictions for the future. In one of these it is expected that by the year 2050, mortality due to resistant bacterial infections would surpass deaths by cancer, the todays “in vogue” disease and worldwide costs in the trillions of dollars of gross domestic product (The Review on Antimicrobial Resistance, 2014).

New sources of potential antimicrobials.

Despite this seemingly apocalyptical prevision for the future, we still hold two major reserves of untapped producers of interesting bioactive molecules. New techniques, like the now famous iChip (Nichols *et al.*, 2010), have allowed previously unculturable soil bacteria to be screened for production of antimetabolites of interest (Piddock, 2015). These previously unculturable bacteria are assumed to account for 99% of the soil microbiome and they are now expected to provide interesting new leads (Pham & Kim, 2012).

Besides uncultured soil bacteria, marine organisms, and in particular sponges, have also been providing interesting compounds. Already several drugs have been described, namely Xestospongin C, an inhibitor of the inositol 1,4,5-triphosphate (IP_3) receptor (Miyamoto *et al.*, 2000) and Manoalides, a set of terpenoid compounds that has shown potential as biofilm modulators (Stowe *et al.*, 2011). Sponges show a great diversity in their microbiological communities, often showing both the presence of Archaea, Fungi,

microalgae and a great diversity of bacterial phyla (Webster *et al.*, 2001), with evidences showing that these interactions started over 600 million years ago (Wilkinson, 1984). Unfortunately, sponges may only contain minute quantities of these compounds in their body. For example, in *Lissodendorix sp.*, halichondrin B only exist in a concentration of 400 µg/kg (Sipkema *et al.*, 2005). This makes it impractical for clinical trials due to the need to collect vast amounts of marine organisms to isolate the amount of compound necessary to conduct these trials well as for commercialization. Interestingly, several studies point to the symbiotic bacteria as the true origin of these compounds. This opens the possibility of isolating and cultivating producing symbionts, thus insuring a sufficient and constant supply of the bioactive molecules and this relationship has already revealed interesting compounds (Yoo Kyung *et al.*, 2001). One such example is Alteramide A, which was isolated from an *Alteromonas sp.* symbiont of a sponge specimen of *Halichondria okadai* off the coast of Nagai, Japan and is a macrocyclic lactam with powerful cytotoxic effect on murine leukemia P388 cells, murine lymphoma L1210 cells and human epidermoid carcinoma KB cells (Shigemori *et al.*, 1992). Besides sponges, other marine animals have also provided bacteria with valuable compounds. An example of this is Tauramamide, a new lipopeptide with antimicrobial characteristics that was extracted from the isolate *Brevibacillus laterosporus* PNG276 obtained from an unidentified tubeworm collected off the coast of Loloata Island in Papua New Guinea and showed a broad-spectrum antibiotic activity, inhibiting both MRSA and Vancomycin-resistant *Enterococcus* (Desjardine *et al.*, 2007).

Actinobacteria: complex with interesting secondary metabolism

The *Actinobacteria* phylum is one of the largest taxonomic units among the 18 major lineages currently recognized within the domain *Bacteria*, including 5 classes and 14 orders. Actinobacteria are $Gram^+$ bacteria, meaning that they stain violet with Gram staining and have a thick peptidoglycan layer. Additionally, Actinobacteria possess a high Guanine/Cytosine ratio (G+C ratio) in their DNA, which can range from 51 to 70% (Lechevalier & Lechevalier, 1967). Morphologically, Actinobacteria can exhibit a wide range of forms, from coccoid or rod-coccoid to fragmenting hyphal forms or permanent and highly differentiated branched mycelium (Atlas, 1997). Ecologically, they are present in all ecosystems, be they terrestrial or aquatic and can act both in the carbon cycle and nitrogen cycle, either by helping decomposing organic matter such as the *Actinomycetales* or fixing nitrogen in the symbiotic relationship with plant roots like *Frankia* (Goodfellow & Williams, 1983). Additionally, Actinobacteria can be both symbionts, such as *Bifidobacterium* or pathogens, like *Mycobacterium* and *Nocardia* (Schell *et al.*, 2002; Wilson, 2012).

Besides all these characteristics, Actinobacteria are also prolific producers of bioactive metabolites, with emphasis on species and strains belonging to the *Actinomycetales,* most notably the *Streptomyces* genus. There is a total of fifteen antibacterial pharmaceutical agents still in use that were obtained from *Streptomyces*. Besides the staggering number of antimicrobials, several anti-fungal, anti-parasitic, herbicides and pesticides have their origin in this genus (Mahajan & Balachandran, 2012). This unusual density of useful compounds is attributed to two characteristics of Actinobacteria: their big genome and high G+C content ratio. A high G+C ratio is indicative of a higher number of coding DNA, since A+T are biased towards stopping codons. An example is *S. coelicolor* that possesses 7825 protein coding genes which includes over twenty gene clusters for the synthesis of known or predicted natural products (Bentley *et al.*, 2002). Already four antimicrobial compounds were isolated from this bacterium, including undecylprodigiosin and perimycin (Brian *et al.*, 1996; Lee & Schaffner, 1969).

Polyketide synthases and non-ribosomal peptide synthetases

Most of the biosynthesized compounds of interest have their roots in two groups of enzymes, the polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS). So, the presence of these genes in newly discovered organisms or strains can indicate the possibility of discovering a new interesting bioactive molecule (Donadio *et al.*, 2007).

PKSs are a family of enzyme complexes that produce polyketides, a common class of secondary metabolites, by catalyzing, almost as a molecular LEGO set, linear carbon extensions in simple two, three and four carbon building blocks and keto-group processing reactions, that share a great similarity with fatty acid biosynthesis (Dutta *et al.*, 2014). They are classified in three different sets. Type I PKS are large, highly modular proteins, that further subdivide in to two other categories. Iterative PKS are composed of several monofunctional or bifunctional proteins and Modular PKS contain a sequence of separated modules with unique domains of 100 to 400 amino acids in size, responsible for one cycle of polyketide chain extension and functional group modification (Khosla *et al.*, 1999). While Type I PKS are predominantly found in Actinobacteria, Myxobacteria, Pseudomonades Cyanobacteria and more recently in Planctomycetes (Bode & Muller, 2005; Graca *et al.*, 2016), Iterative PKS are typically found in Fungi (Cox & Simpson, 2009). Type II PKS are multienzyme complexes of discrete proteins only found in bacteria (Shen, 2000). Type III PKS are found in bacteria, fungi and plants and their main characteristic is that Type III PKS do not use the Acyl Carrier Protein (ACP) (Jenke-Kodama *et al.*, 2005; Yu *et al.*, 2012), an important carrier of acyl intermediates in both fatty acid and polyketide biosynthesis that exhibit characteristic structural features of

natively unfolded proteins *in vitro* with a thioester-linked acyl group attached to a 4'-phosphopantetheine (Byers & Gong, 2007).

PKSs genes in bacteria, are usually organized in a single operon with modules featuring common domains such as the ACP, already discussed above, the Acyltransferase, and Keto-synthase domains. Additionally, other domains such as Dehydratase, Enoyl-reductase, Keto-reductase, Methyltransferase, and Sulf-hydrolase and Thio-esterase can also be part of the biosynthetic pathway (Jenke-Kodama *et al.*, 2005).

Non-Ribosomal Peptide Synthetases (NRPS) are a cluster of modular enzymes that catalyze, through complex regiospecific and stereospecific reactions, important peptide products. NRPS synthesis, similarly to ribosomal protein synthesis, is divided into three steps, Initiation, Elongation and Termination. Despite these similarities, NRPSs are only limited by the length of the peptide chain, which is between two to forty-eight residues and can use a variety of standard and non-proteinogenic amino acids (amino acids that are not naturally encoded or found in the genetic code of any organism) (Strieker *et al.*, 2010). As with PKSs, NRPSs in bacteria are also organized in a single operon with different modules featuring common domains. There are four ubiquitous domains to all NRPSs, Adenylation domains, Peptidyl Carrier Protein (PCP) domains, Condensation domains and the Thioesterase domain. The Thioesterase domain is only found at the termination module and is responsible for the release of the peptide by hydrolysis or macrocyclization (Kopp & Marahiel, 2007). Other domains include Formylation, Cyclization, Oxidation, Reduction, Epimerization and *N*-methylation (Miller & Gulick, 2016). In the Initiation step, adenylation of the amino acids though ATP hydrolysis occurs, forming an unstable intermediate, that is subsequently transferred to another site of the enzyme where it is bound as a thioester to the cysteamine group of an enzyme-bound 4′-phosphopantetheinyl that forms the backbone of the PCP (Marahiel *et al.*, 1997; Miller & Gulick, 2016). In the next step, Elongation, the condensation domain catalyzes the peptide bond between the thioester group of the growing peptide chain downstream from the PCP to another PCP-bound amino acid upstream. It is also during this step that another reaction catalyzed by the non-essential domains may occur. Lastly, the Termination stage finishes biosynthesis by releasing the completed polypeptide from the PCP domain from the last module (Kopp & Marahiel, 2007).

Combating obesity

As obesity is continuing to rise, it has become the most prevalent metabolic disease in the devolved and developing nations, contributing to an enormous rise in other associated diseases such as diabetes, cardiovascular disease and even cancer (Castro

et al., 2016). Obesity, defined as a body mass index (BMI) above thirty kg/m^2, is primarily caused by a disproportion of intake and spending of calories. As recommended by the WHO and many other experts, fighting obesity has to start at the personal level with reduction of the energy intake from high calorie fats and sugars and an increase in exercise, accompanied by the reduction of the level of fats and sugars added by the food industry to their products (Allan, 2004).

Unfortunately for some patients these recommendations alone may not work, and for these medication is required. Current treatment works primarily by two different mechanisms of action, appetite suppression and interference with the body's ability to absorb specific nutrients (*e.g.* fats). Appetite suppressants work by exploiting the action or production of neuroendocrine satiety signals such as Cholecystokinin, which is secreted by the duodenum in response to the process of eating and digestion, to reduce meal size and frequency. The drugs most commonly used for this are catecholamines like amphetamines and anti-depressants like bupropion (Bray, 1993). A glucagon-like peptide, liraglutide is being trialed as to ascertain its ability to do just this (Rang *et al.*, 2015). To inhibit the absorption of fat, orlistat is usually recommended, which is an inhibitor of enzyme lipase causing fat malabsorption in the intestine. Orlistat is derived from lipstatin, a bioactive molecule first isolated from the Actinobacteria, *Streptomyces toxytricini* (Weibel *et al.*, 1987). Orlistat is generally considered safe and is replacing many centrally acting appetite suppressants, which have been reported to cause series of adverse effects, like addiction and pulmonary hypertension (Rang *et al.*, 2015). Regrettably, many of these pharmacological approaches to treat obesity seem to fall short, as a meta-analysis by Viner and colleagues seems to indicate that orlistat together with behavioral support has limited utility as a weight reduction treatment in adolescents, producing a small effect with frequent gastrointestinal side effects (Viner *et al.*, 2010).

Research on the stilbene 3,5,4′-trihydroxy-trans-stilbene, also named Resveratrol, has provided new leads to treat obesity. Dietary calorie restrictions on mammals was known to, not only reduce body mass, but also restore insulin sensitivity and increasing lifespan (Koubova & Guarente, 2003). The mechanism through which these actions occurred was later discovered to be the result of the activations of the silent mating type information regulation 2 homolog 1 (SIRT1) protein, that caused fat mobilization and had a role in the protection on oxidative stress (Lin *et al.*, 2000) and inhibition of the inflammation factor nuclear factor kappa B (NF-kB) (Franceschi & Campisi, 2014). Resveratrol was found to reduce the synthesis of lipids in rat liver (Arichi *et al.*, 1982) and 3T3-L1 adipocytes (Picard *et al.*, 2004), by activation SIRT1 proteins and mimicking the effects of calorie restriction (Howitz *et al.*, 2003). Resveratrol, given as dietary supplement, could

reproduce this effects on mice (Lagouge *et al.*, 2006). Regrettably, Resveratrol seems not to show any effect on humans. In a randomized placebo-controlled, double-blinded, and parallel-group design trial, 24 obese men were given high doses of Resveratrol, with no effect on blood pressure, resting energy expenditure, oxidation rates of lipid, ectopic or visceral fat content and no change in inflammatory and metabolic biomarkers (Poulsen *et al.*, 2013). Another study seems to confirm this same result, with the authors stating that Resveratrol lacked any adjuvant effect on energy restriction for obesity treatment purposes (Alberdi *et al.*, 2014). New anti-obesity drugs are thus still needed to fight against a more and more "fat" society.

Objectives

With this Master's Thesis, developed in the Laboratório de Ecofisiologia Microbiana da Universidade do Porto (LEMUP-CIIMAR), in Porto, Portugal and in conjunction with the Institut für Pharmazeutische Mikrobiologie Universität Bonn, in Bonn, Germany as part of the Erasmus+ Estágio program, we aimed to uncover more about the secondary metabolism of thirty-three bacteria isolated from sponges that previously showed signs of great potential for production of useful bioactive compounds, with particular emphasis in the search for new potential antimicrobials. To achieve this, we have attempted to extract any possible bioactive molecules with several different liquid-to-liquid extraction protocols allied with bio-guided assays and powerful bioanalytical techniques like liquid chromatography.

Furthermore, the opportunity arose for the screening for the capacity to affect lipid metabolism and obesity of extracts from these bacteria to be also performed utilizing zebrafish (*Danio rerio*) larvae.

Materials and Methods

1. Biological material

1.1. Bacterial strains and selection criteria used

Based on previous studies conducted in Laboratório de Ecofisiologia Microbiana da Universidade do Porto (LEMUP), 17 Actinobacteria strains were selected, with 16 other strains (15 belonging to the phylum Gammaproteobacteria and 1 belonging to phylum Firmicutes) later added. The thirty-three strains were chosen either because of the presence of the PKS-I and/or NRPS gene clusters or for having showed prior antimicrobial activity against bacterial targets. All thirty-three strains were previously isolated and characterized (Table 1) (Graca *et al.*, 2013; Graca *et al.*, 2015).

Table 1 - Selected bacteria strains used for testing.

Isolate	Related strain	Phylum	PKS-I / NRPS	Previous activity
#91_17	*Dermacoccus* sp.	Actinobacteria	NRPS	CA
#91_29	*Microbacterium foliorum*	Actinobacteria	N/A	CA
#91_31	*Microbacterium hydrocarbonoxydans*	Actinobacteria	N/A	CA
#91_34	*Microbacterium esteraromaticum*	Actinobacteria	N/A	CA
#91_35	*Microbacterium phyllosphaerae*	Actinobacteria	PKS-I	CA; BS
#91_36.1	*Rhodococcus equi*	Actinobacteria	PKS-I; NRPS	CA; VA; EC
#91_36.2	*Microbacterium foliorum*	Actinobacteria	N/A	CA; VA
#91_37	*Microbacterium foliorum*	Actinobacteria	N/A	CA; VA
#91_40	*Microbacterium foliorum*	Actinobacteria	PKS-I	CA; VA
SM 115	*Agrococcus* sp.	Actinobacteria	PKS-I	NA
SM 116	*Agrococcus* sp.	Actinobacteria	PKS-I	NA
B01-119c	*Microbacterium* sp.	Actinobacteria	N/A	VA; VF
B02-22.2	*Gordonia* sp.	Actinobacteria	PKS-I	BS
B02-29	*Gordonia* sp.	Actinobacteria	N/A	BS
B02-78	*Gordonia terrae*	Actinobacteria	PKS-I; NRPS	BS
B02-79	*Microbacterium* sp.	Actinobacteria	N/A	BS
B02-79a	*Microbacterium* sp.	Actinobacteria	N/A	BS
#118_14	*Proteus mirabilis*	Gammaproteobacteria	N/A	CA; VA; EC
#118_17	*Psychrobacter celer*	Gammaproteobacteria	N/A	CA; VA; EC
#118_20	*Proteus* sp.	Gammaproteobacteria	PKS-I	CA; EC
#118_19	*Enterococcus faecalis*	Firmicutes	PKS-I	CA; VA; EC
#91_10,2	*Pseudoalteromonas* sp.	Gammaproteobacteria	N/A	CA; VA; EC
#91_13	*Pseudoalteromonas* sp.	Gammaproteobacteria	N/A	CA; VA; EC

B01-7	*Pseudovibrio* sp.	Gammaproteobacteria	PKS-I	BS
B01-9	*Pseudovibrio* sp.	Gammaproteobacteria	PKS-I	BS
B01-77	*Pseudovibrio* sp.	Gammaproteobacteria	N/A	BS; MRSA
B02-8.1	*Pseudovibrio* sp.	Gammaproteobacteria	PKS-I	BS; MRSA
B02-9.1	*Pseudovibrio* sp.	Gammaproteobacteria	N/A	BS; MRSA
B02-10.a	*Pseudovibrio* sp.	Gammaproteobacteria	N/A	BS; MRSA
B02-10.b	*Pseudovibrio* sp.	Gammaproteobacteria	N/A	BS; MRSA
B02-10.c	*Pseudovibrio* sp.	Gammaproteobacteria	N/A	BS; MRSA
B02-10.1	*Pseudovibrio* sp.	Gammaproteobacteria	N/A	BS; MRSA
B02-61	*Pseudovibrio* sp.	Gammaproteobacteria	PKS-I	BS; MRSA

N/A = Not Applicable; NA = No Activity; CA = *Candida albicans*; BS = *Bacillus subtilis*; VA = *Vibrio anguillarum* VF = *Vibrio fisheri*; MRSA = Methicillin-Resistant *Staphylococcus aureus*

1.2. Target bacteria used for testing antimicrobial properties

The selected targets used in this work are a clinical isolate of *Candida albicans* (filamentous yeast), an environmental strain of *Escherichia coli* (Gram$^-$) and *Bacillus subtilis* ATCC 6633 (Gram$^+$), all belonging to the LEMUP collection.

Other targets used were *Micrococcus luteus* DSM 1790 (Gram$^+$) *Bacillus subtilis* 168 and *Chlamydia trachomatis* D/UW-3/CX. Furthermore, there are four reporter strains of *B. subtilis* used for testing the mechanisms of action (Table 2). *C. trachomatis* are obligate intracellular parasites. For this reason, *C. trachomatis* requires a host for maintenance and to conduct assays, therefore, HEp-2 cells were also used. HEp-2 are a cell line derived from the Human Epidermoid carcinoma (HeLa). HEp-2 cells were previously grown using Dulbecco's Modified Eagle Medium. All these targets belonged to the collection of the Institut für Pharmazeutische Mikrobiologie.

Table 2 – *B. subtilis* 168 reporter strains specifying the vectors present and the cellular target of each particular vector.

Reporter Strain	Vector	Target
B. subtilis 168	pAC6-*Pypu*A	Cell Wall
	pAC6-*Pyor*B	DNA Synthesis
	pAC6-*PyheI*	Protein Synthesis
	pAC6-*Pyvg*S	RNA Synthesis

1.3. Zebrafish as models for testing anti-obesity properties of extracts

For this assay, due to time constrains only strains #91-36.1, #91-36.2, #118-20, B02-8,1, B02-61, B02-22.2, B02-78 and B02-79 were able to be tested. Strains #91-36.1, #118-20, B02-8,1, B02-61, B02-22.2, B02-78 were chosen for possessing either PKS-I or NRPS genes. Strain #91-36.2 was included due to its good results in the antimicrobial assays, even though this strain lacked any PKS-I or NRPS genes. Additionally, strain B02-79 was also able to be added to the testing.

Fig 1 - Adult zebrafish swimming in an enclosed pen on the nursery aquarium with eggs on the bottom of the aquarium. Female zebrafish can be distinguished from males by the shape of their upper body, with females having a significantly larger abdomen giving them a more of a teardrop shape while male zebrafish are torpedo-shaped, with a smaller abdomen.

Anti-obesity properties of the selected strains were tested using zebrafish (*D. rerio*) larvae. To obtain the larvae, twenty individuals, ten males and ten females were first selected by size and collected from the stock at CIIMAR and separated into two different pens. Males and females can be differentiated by their body shape. Individuals were selected by size to validate the maturity of the individuals. Before collection, fish were feed generic aquarium fish feed. The day before collection, by the late afternoon, male and female adults were combined. The morning after, the eggs were collected and cleaned. Live eggs were placed on a five liters tank at 25ºC for hatching. Seventy-two hours after, the eggs hatched and larvae were collected on to a petri dish. Then they were washed from the egg shell and placed in forty-eight well plates with freshwater, seven per well. To avoid extreme size disparities between larvae that can skew results, larvae were sorted by size.

2. Media used for testing

2.1. Media for maintaining target bacteria

Different media were used to grow and maintain the bacteria. *C. albicans*, *E. coli* and *B. subtilis* ATCC 6633 were cultivated in petri dishes with Luria Broth (Table 3) at 37ºC in the dark. *M. luteus and B. subtilis* 168 were cultivated in petri dishes with Mueller-Hinton Broth (Table 3). While *B. subtilis* 168 was grown at 37ºC, *M. luteus* was cultivated at 30ºC, all of them in the dark. All liquid cultures of target bacteria were shaked at 200 rotations per minute (r.p.m).

Table 3 - Media composition used for maintenance and assays with the target bacteria.

Reagents	Medium	
	Mueller-Hinton Broth	Luria Broth
	Units per liter	
Beef Extract Infusion	2.0 grams	—
Casein Hydrolysate	17.5 grams	—
Starch	1.5 grams	—
Tryptone	—	10.0 grams
Yeast Extract	—	5.0 grams
NaCl	—	10.0 grams
Deionized water	1000.0 mL	1000.0 mL
Agar	16.0 grams	16.0 grams

2.2. Media used for maintenance and growth of selected strains

The selected strains used for extraction were all grown/maintained in petri dishes containing Marine Broth and incubated at 25ºC in the dark. All liquid cultures were also shaked at 200 r.p.m. while incubating (Table 4). The natural sea water used in the media described above was sampled off the coast in Matosinhos, Porto, Portugal. As part of the work done was performed in Bonn, Germany, which is approximately 300km from the nearest source of sea water (the North Sea, in the Netherlands), natural sea water was unavailable. For this reason, artificial sea water, using Sea Salts from SIGMA® was prepared.

Table 4 - Media composition used for maintenance of strains and their culture for extraction.

Reagents	Medium		
	Marine Broth	Nutrient Broth	M13 Modified Broth
	Units per liter		
Peptone	5.0 grams	5.0 grams	0.25 grams
Yeast extract	1.0 grams	1.0 grams	0.25 grams
Tris-HCl	—	—	50.0 mL
Deionized water	—	1000.0 mL	10.0 mL
[1,2]Glucose solution (2,5%)	—	—	10.0 mL
[1,3]Vitamins solution	—	—	10.0 mL
[1,4]Hutner's basal salts solution	—	—	20.0 mL
[5]Natural sea water/artificial sea water	1000.0 mL	—	900.0 mL
Agar	16.0 grams	16.0 grams	16.0 grams

[1]Sterilized through a 0.22 µl pore filter and added to the medium after sterilization

[2] Solution prepared with sterilized water

[3]0,1 µg/mL cobalamin, 2.0 µg/mL biotin, 5.0 µg/mL thiamine-HCl, 5.0 µg/mL Ca-pantothenate, 2.0 µg/mL folic acid, 5.0 µg/mL riboflavin and 5.0 µg/mL nicotinamide.

[4]Cohen-Bazire et al. (1957)

[5] Sea water was previously filtered through a 0,45 µm pore filter

2.3. Assessment of the effect of chemical and biological stress on the extracts

Secondary metabolism in bacteria is often associated to the presence of stressful situations, like nutrient deprivation, osmotic stress and presence of competitors or predators. Because of this, several modifications to the growth media and conditions of the strains were tested.

Nutrient Broth is a complex medium crafted with deionized water. For this, it was used to test the lack of salinity. Sea water contains 23.926 g/L of sodium chloride (NaCl), but it is usually rounded to 24 g/L (Kester *et al.*, 1967). With this in mind, Marine Broth supplemented with NaCl to a concentration of 26 g/L, was used to test higher salt concentrations (Table 4). This represents an approximate 15 % increase compared to natural seawater.

To analyze the effect of limiting nitrogen and organic compounds, M13 Modified Broth was used. M13 Modified Broth is a much less chemically complex medium than Marine Broth and contains lower levels of available nitrogen for the cells (Table 4).

To simulate the presence of potential competitors, strains were exposed to an autoclaved culture of *Bacillus subtilis* and *Escherichia coli*, in a 1:100 ratio in Marine Broth.

3. Extraction protocols used throughout this work

3.1. Extraction protocol using acetone and dimethyl sulfoxide

This protocol was based on Graca *et al*, 2013. Incubation of the stains was done on a petri dish with Marine Broth for approximately three to five days at the usual conditions of 25ºC in the dark. Afterwards, 20 mL liquid cultures in Marine Broth were prepared for all thirty-three strains and incubated for five days. At the end of the incubation period, each culture was used to prepare four types of extracts. One was the aqueous phase extract (F) while the other three were organic phase extracts, designated as crude (C), pellet (P) and supernatant (S). For the aqueous phase extract, 2 mL portion of the culture were centrifuged at 13000 r.p.m. for five minutes and the supernatant collected, filtered and sterilized through a 0.22 µm cellulose acetate syringe filter. As for the organic extracts, an organic mixture made of 10% dimethyl sulfoxide (DMSO) in Acetone was premade. For the crude extracts, the organic mixture was added to a 2-mL portion of culture in a 1:1 ratio and incubated for one hour at 200 r.p.m. in the dark. For the two other organic extracts, the supernatant and the pellet, another 2 mL of culture was retrieved and centrifuged at 13000 r.p.m. for five minutes. The resulting supernatant and pellet were separated and the organic mixture was added in the same proportions as the previous organic extract and incubated in the same conditions for one hour. After the 1-hour incubation, 2 mL of the upper phase of all the three organic extracts were retrieved and placed in a 2 mL Eppendorf and dried overnight until half or more of the volume was desiccated. In the morning after, the volume of the pellet extracts was completed up to a volume of 1 mL with sterile deionized water. To increase both durability and to concentration of the extracts produced, lyophilization was performed and extracts were then stored at -20ºC. Prior to use, extracts were dissolved in 1 mL of DMSO.

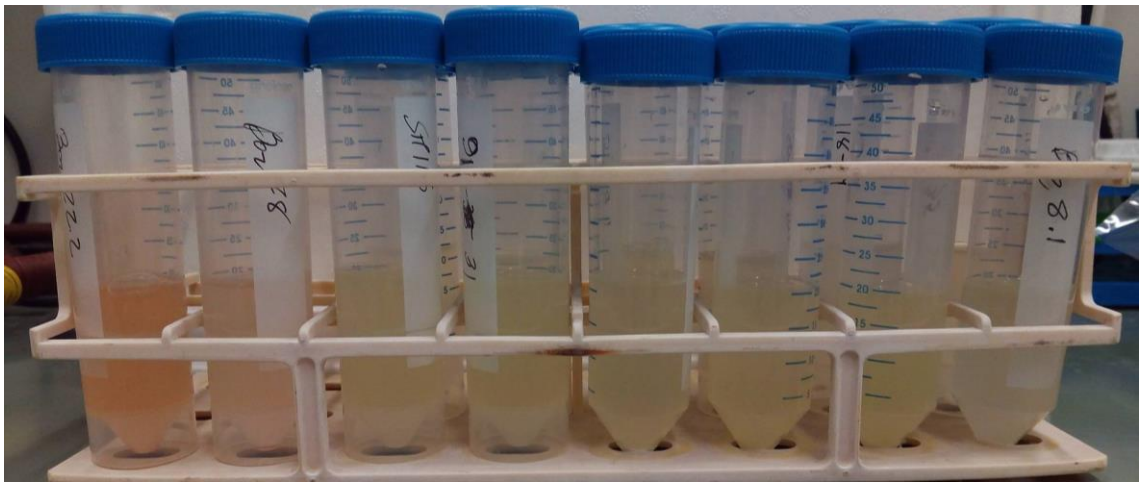

Fig 2 - Twenty mL culture of strains ready for extraction after the 5 days incubation period.

3.2. Extraction protocol using ethyl-acetate

In the attempt to improve yields and thus inhibition effect of the extracts, a more powerful solvent was chosen. Ethyl-acetate is the ester of ethanol and acetic acid and has two main characteristics that facilitate the extraction of compounds: immiscibility in water and low boiling point, 77°C at atmospheric pressure or 9ºC at near vacuum (6,7 kPa). The immiscibility in water allows for a better separation of the aqueous phase from the organic, removing the need of lyophilizing the culture prior to extraction. The low boiling point allows for the use of softer conditions for drying the organic phase, thus avoiding damage of any compound extracted.

For this extraction, cultures of the strains were again grown up to five days. At the end of the incubation period, ethyl-acetate was added twice to the cultures in a 1:1 ratio, mixed for thirty minutes in a separatory funnel and then phase separation was permitted to occur. The aqueous was discarded and the organic was collected. In higher scale extractions, since the separatory funnel maximum volume was 500 mL, ethyl-acetate and the cultures were mixed in a separate vial and only then transferred to the separatory funnel. The collected organic phase was then dried in a Rotavapor® R-100 from BUTCHI, a rotatory vacuum evaporator at 25ºC at 100 mbar. After the ethyl-acetate was completely removed, 2 mL of DMSO was added to the balloon to dissolve the solid residues that formed on the bottom of the balloon if the extracts were used for antimicrobial assays. The residue in the balloon was dissolved in 2 mL of methanol instead of DMSO if the extract was used for liquid chromatography.

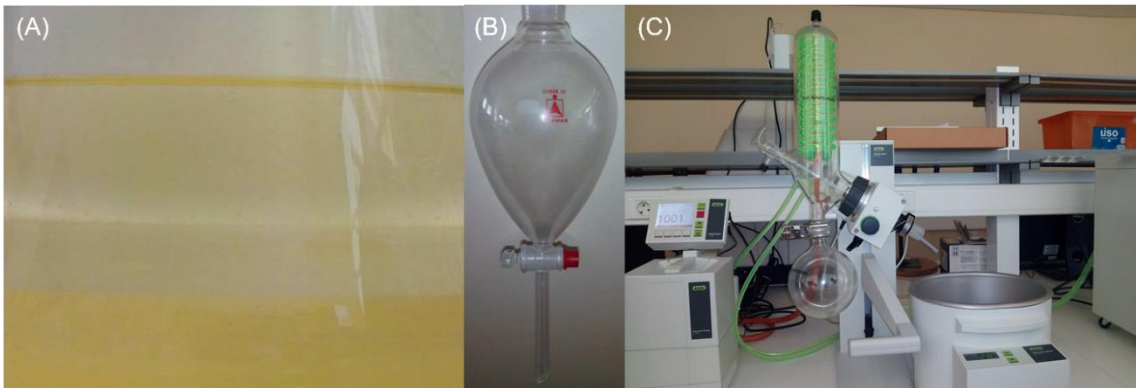

Fig 3 – (A) Close-up of phase separation in a 500 mL culture extract with ethyl acetate on top and the aqueous phase on the bottom. (B) Example of the 500 mL separatory funnel. (C) Rotatory evaporator from BUTCHI used for drying organic extracts.

3.3. Amberlite^{TM} XAD16N resin extraction with ethyl-acetate

Amberlite^{TM} XAD16N, from MERCK, is a non-ionic resin specifically designed to adsorb and hold hydrophobic compounds up to 40,000 MW, and specifically antibiotics. For extraction with Amberlite, a two liters culture of the strain #91-36.2 was grown for the five days at 25ºC in Marine Broth with the mixture of autoclaved culture of *Bacillus subtilis* and *Escherichia coli* at 200 r.p.m. in the dark. At the third day of culture, previously autoclaved Amberlite^{TM} resin was added to the culture, in a total of 30 g for the two liters. At the end of the incubation period, the resin was filtered out using cellulose filters. The culture was then centrifuged, and the pellet was collected and was extracted in the same fashion as used with the ethyl-acetate previously. To the resin, 300 mL of ethyl acetate were added and mixed for 1 hour. After this period, the resin was separated from the ethyl-acetate and was discarded. As previously referred, the ethyl-acetate was dried in the rotary evaporator, and the solid residue was dissolved in DMSO.

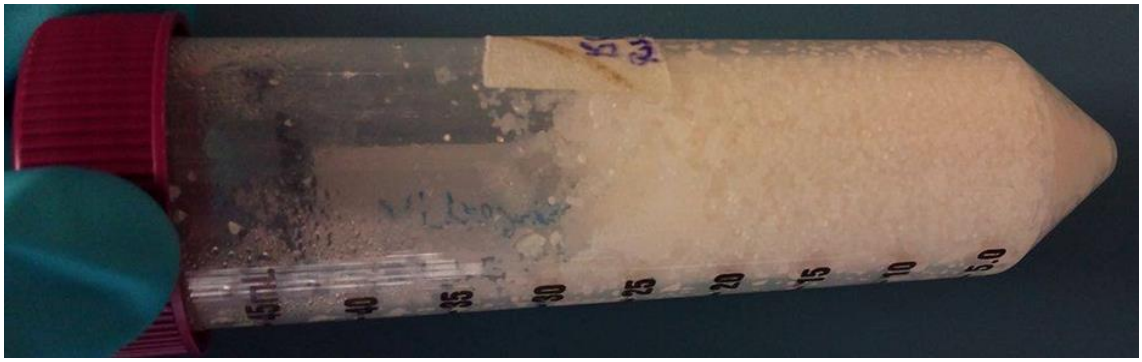

Fig 4 - Amberlite XAD16N resin autoclaved and ready to be added to the culture.

3.4. Extraction protocol using methanol and dichloromethane

To test possible anti-obesity compounds produced by the selected strains, 100 mL of the strains selected for the assay were grown in Marine Broth at 25ºC, 200 r.p.m. in the dark

for five days. At the end of the incubation period, the cultures were centrifuged and the supernatant was place at -20ºC for later use. The pellet was lyophilized to remove any trace of water. To the dried pellet, 50 ml methanol were added and mixed vigorously for 30 min. After the methanol was collected, the process was repeated for a second time. The methanol was collected and subsequently dried in the rotatory evaporator. The solid residue in the balloon was then dissolved in 3 mL dichloromethane (DCM) and place in previously weight vials. The vials were place in a fume hood and the DCM was dried overnight. Afterwards, the weight of the vials was recorded. The vials were stored at -20ºC until further use. For the anti-obesity assay a concentration to be at a specific concentration of 10 mg/mL was used. DMSO was used as solvent.

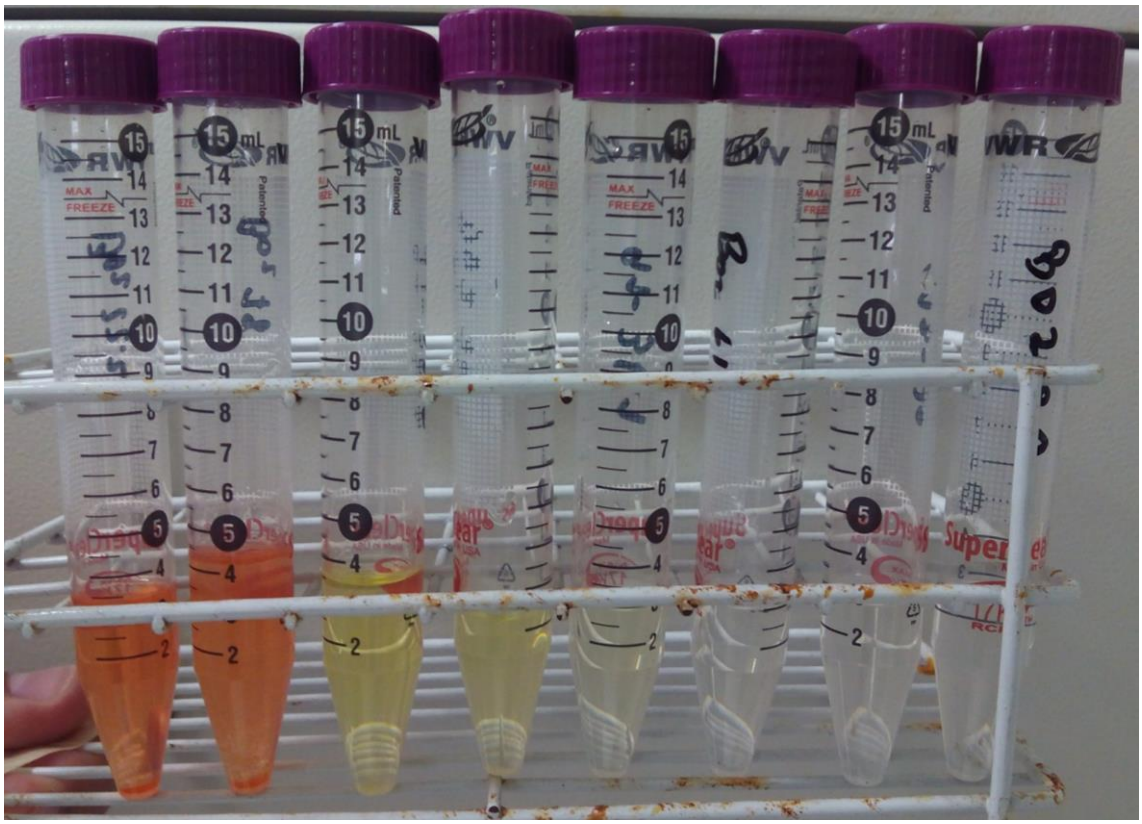

Fig 5 - Finished and dissolved extracts ready for use in anti-obesity assays.

4. Preliminary anti-microbial screenings

To assess the viability of production by the strains, the preliminary anti-microbial assays in liquid used by Graca *et al* (2013) and Graca *et al* (2015) were repeated.

4.1. Growth curves of *Candida albicans*, *Escherichia coli* and *Bacillus subtilis*

Firstly, growth curves for *Candida albicans*, *Escherichia coli* and *Bacillus subtilis* were performed. Using fresh plates of either *Candida albicans*, *Escherichia coli* or *Bacillus*

subtilis, overnight 10 mL liquid culture with Luria Broth was made and incubated at 37ºC with 200 r.p.m. . The next morning the optical density at 600 nm (OD_{600nm}) of the overnight cultures were measured and a 1:100 ratio 100 mL Luria Broth culture was made using the previous overnight culture and incubated under the same conditions. The OD_{600nm} of the 1:100 100mL culture was tested every thirty minutes and registered. Additionally, a sample was also taken, serially diluted up to a factor of 10^{-6} and seeded on a plate containing Luria agar, to see how many colonies forming units (C.F.U.) the culture possesses at the time. The plates we placed on an incubator at 37ºC and colonies were counted the day after. The growth curves for these three targets were performed in triplicates.

When the OD_{600nm} is plotted against the time, a curve with three phases appear. The first one is the lag phase, the second one is the exponential phase and finally the third one is the stationary phase. When the OD_{600nm} values between the second phase are logarithmized, the duplication time and an equation relating C.F.U.s to time for the microorganism was obtained.

4.2. Antimicrobial liquid assays

Similarly to Graca *et al* (2013), Greiner Bio-One 96-well plate were incubated overnight with each well containing 90 µL of culture of each target standardized to 2.5×10^5 and completed with 10 µL of each extract in triplicates. Moreover, 100 µL of each target organism were also grown to assess total growth of the microorganisms. Positive controls used were also done in triplicates. The OD_{600nm} of plates was measured and then incubated for 24h at 37ºC in the dark. After the incubation period, OD_{600nm} was again read. Analysis of inhibition of growth of the target microorganism was calculated, using the following formulae.

$$\mathrm{OD_R} = \mathrm{Abs_F} - \mathrm{Abs_I}$$

$\mathrm{OD_R}$= Optical density of each replica; $\mathrm{Abs_F}$= Absorbance value after 24h incubation; $\mathrm{Abs_I}$= Absorbance value prior to incubation.

$$\bar{x}_{Ext} = \frac{OD_{R1} + OD_{R2} + OD_{R3}}{3}$$

$\bar{x}_{Ext}$= Mean optical density for each extract.

$$\%_{Inhibition\ for\ each\ extract} = 100 - \frac{\bar{x}_{Ext}}{\bar{x}_C} * 100$$

$\bar{x}_C$= Mean optical density of the control negative control

Inhibition of growth above 20% was considered as a positive result.

5. Modified Kirby-Bauer assays

Using the same extraction protocol used in the liquid assays, another batch of extracts was tested. The inhibition assay in solid performed here is a modified Kirby-Bauer assay, in which Mueller-Hinton Broth is seeded with the desired number of target cells and a drop of extract is placed on the surface and incubated at 37ºC, or 30ºC for *M. luteus*, overnight. For this assay, the initial targets chosen were *M. luteus* and *B. subtilis*, as these bacteria are sensitive to low concentrations of antibiotics, thus making it easier to detect any antimicrobial activity by any extract.

As in the liquid assays, target cultures were grown overnight in Mueller-Hinton Broth liquid, at 200 r.p.m. and 30ºC for *M. luteus* or 37ºC for the other targets. The following morning, OD_{600nm} was read. To allow standardization, the following equation is used to seed the media to 0.5 McFarland:

$$\frac{\text{Volume of medium [mL]} * 10^7}{\text{OD}_{600\text{nm}} * (2.0 * 10^9)}$$

And since we are using 40 mL rectangular Nunc™ OmniTray™ plates, the equation could be simplified to:

$$\frac{1}{5 \times \text{OD}_{600\text{nm}}}$$

When regular circular petri dishes are used, which can only contain 20 mL instead of 40 mL, we can simply halve the result obtained by the previous equation. After the media was seeded, and using the Liquidator™ 96 pipette from Mettler-Toledo Rainin, forty-eight 10 µL droplets were seeded in chess-like pattern the plate, to provide adequate spacing between droplets. After overnight incubation, inhibition halos were examined and compared to the control. Positive control used was Ampicillin, at 0.1 µg/mL for *Micrococcus luteus* and *Bacillus subtilis*.

5.1. Direct cultures assay

To determine if cultures were still able to produce inhibition of growth on targets independently from extraction methods, direct interaction between strains and targets was performed. For this, 20 mL cultures of the strains were grown in Marine Broth for three days. Identically to what was done in the modified Kirby-Bauer assay, plates of Mueller-Hinton Broth were seeded with the targets. After the media was seeded, 10 µL of cultures were directly placed on top of the agar, forty-eight at a time using the Liquidator™ 96 pipette for evenly distributed space around the droplets. Plates were

placed at 30ºC, an intermediate temperature between the 25ºC and 37ºC, the optimal growth temperatures for the strains and targets, respectably, for three days. After the incubation period, plates were examined for signs of inhibition.

5.2. Well assay

The modified Kirby-Bauer assay has the drawback that only 10 µL of extract can be used for testing on the media surface and still allow for the detection of inhibition halos. This can be a problem if the production of antimicrobial metabolites is small or if the extract protocol was not able to sufficiently retain enough compound to achieve inhibition.

Therefore, to bypass these problems, wells were made in the testing plate. These wells allow up to 100 µL of extract to be tested, while also permitting a more evenly spaced area between droplets than if 10 µL were placed by hand. Positive controls used were again Ampicillin, at 0.1 µg/mL for *M. luteus* and *B. subtilis*.

5.3. Diffusion assays with filter disks

To prevent both evaporation and diffusion complications on possible antimicrobial compounds in the extracts that may conceal their action in the modified Kirby-Bauer assays, a new assay was devised. Comparably to assays done before, target cultures were grown overnight and seeded, using the same formula, in Mueller-Hinton broth with 0.6% agar instead of the normal 1.6%. To add rigidity to the media, before placing this layer, a first coating with 1.6% agar Mueller-Hinton was made on the petri dish. Sterilized filter discs were then placed above the surface of the agar and filled with 100 µL of each extract. Positive control was Ampicillin at 1 mg/mL. Negative controls used were DMSO or methanol, depending on which one was used as solvent. For both controls, filter discs were filled with 10µL of the respected solvent. Finally, and like in the previous assays, plates were incubated at 37ºC for twenty-four hours. Plates were examined for inhibition halos around the discs.

5.4. Modes of action of the extracts

For the modes of action (MoA) assays, the reporter strains of *Bacillus subtilis* 168 (Table 2) were used. These strains were genetically engineered with plasmids containing a set of promotors that can identify which one of the four main biosynthesis pathways has the extract or compound affected. The promotors used were PypuA for cell wall, PyorB for DNA synthesis, PyvgS for RNA synthesis and PyheI for protein synthesis. These promotors are attached to a copy of the LacZ gene on the pAC6 vector plasmid. LacZ encodes for the a β-galactosidase, which can hydrolyze X-Gal into galactose and the indigo blue compound, 5-bromo-4-chloro-3-hydroxyindole. So, when an extract is being

tested, if the extract damages one of the four biosynthesis pathways, the corresponding promoter is activated. Following its activation, the LacZ gene is transcribed and any X-Gal incorporated by the cell from the medium is hydrolyzed and a dark blue hue is created, indicating the effect of the extract on the pathway.

For the assay, the four reporters were grown overnight at 37ºC and 200 r.p.m. in Mueller-Hinton Broth containing 5 µg/mL chloramphenicol until the OD_{600} reached 0.5 to 1. The five different strains were incorporated in Mueller-Hinton agar supplemented with 5 µg/mL chloramphenicol and X-Gal (80 µg/mL in reporters pAC6-*Pypu*A, pAC6-*Pyor*B, pAC6-*Pyvg*S and 104 µg/mL in reporter pAC6-*PyheI*), using the same formula as the one used for the modified Kirby-Bauer assays. Ten µL of the extracts were then tested on the plates containing the five reporters. The plates were incubated at 37 ºC for twenty-four hours and each assay was done in duplicate. Positive controls used were Ampicillin for cell wall, Rifampicin for RNA synthesis, Tetracycline for protein synthesis and Ciprofloxacin for DNA synthesis.

6. Evaluation of anti-*Chlamydia trachomatis* activity

Chlamydia trachomatis can alternate between the extracellular cell, infectious elementary body and the intracellular, non-infectious reticulate body throughout their life cycle (Bastidas *et al.*, 2013) and are responsible for one of the most prevalent sexually transmittable diseases. Not only due to their intracellular nature, *C. trachomatis* is able to, under stress conditions, form persistent aberrant bodies. These aberrant bodies are much more resistant to stress, and even thought not showing signs of infection, *C. trachomatis* can reactivate after stressful conditions have disappeared. These two facts make *C. trachomatis* extremely difficult to treat.

To evaluate the bioactivity of extracts against *C. trachomatis*, the following assay was performed. Two hundred µL of Hep2 host cell suspension was incubated in 96-well plates for forty-eight hours. Following the incubation period, 50 µL of *C. trachomatis* D/UW-3/CX were added and infection was allowed for two hours at 37 °C and 5% CO_2. At the end of the incubation period, the medium was replaced with 100 µL of fresh medium along with two dilutions of the #91-36.2 extract, one of 2.56 µL and another of 1.28 µL of DMSO and water. This dilution was done so that DMSO would not kill the host cells, as eukaryotic cells are susceptible to it. Afterwards, the cultures were incubated for 30 h at 37 °C, 5% CO_2 and then fixed with ice cold methanol for 5 min. Finally, the samples were stained using 100 µL of fluorescein-conjugated antibodies specific for chlamydial lipopolysaccharide (LPS), The “Pathfinder Chlamydia Conformation System” from BioRad, and incubated for 30 minutes. Cytoplasm was stained with Evans blue, as part

of the kit. Next, the cultures were stained with DAPI at 3 mg/mL for one minute and washed two times with PBS for 10 minutes each. The samples were then ready for analysis by fluorescence microscopy.

7. Testing anti-obesity properties of extracts from selected strains

To conduct the testing on the zebrafish larvae, a solution of salts was first prepared to accommodate the eggs. This egg water consists of marine sea salts dissolved in deionized water, up to a concentration of 60 µg/mL. The egg water was then mixed with Phenylthiocarbamide (PTU), to a final concentration of 200 µM. PTU inhibits melanogenesis in zebrafish by blocking tyrosinase-dependent steps in the melanin pathway (Karlsson *et al.*, 2001) making the larvae transparent. Using a 1 mL syringe, the water in the wells where the larvae were placed previously was removed, leaving behind a dead volume of 125 µL. Unfortunately, during this step, larvae can be sucked by the syringe causing irreversible damage to the larvae. To the well were then added 625 µL of the egg water and PTU mixture in two 312.5 µL portions. Finally, 0.75 µL of extracts was added to the well. DMSO and Resveratrol were used as controls at the same concentration as the extracts (10 mg/mL). Each extract and controls were tested in duplicate. Twenty-four hours after the first exposure, the egg water in the wells was renewed following the previous steps. Fifteen µL of Nile red, a fluorescent dye that stains both membrane and intracellular neutral lipids, was also added. The larvae were again exposed for another twenty-four hours. At the end of this 48-hour period, the larvae were anesthetized with a solution of 0.3% tricaine, collected into a concave slide and arranged with the abdomen facing up. In this position, the fat reserves of the larvae were more visible. Fluorescence was induced on the Nile red using a Leica DM6000 microscope with an excitation wavelength of 552 nm. The images were saved for analysis of the fluorescence intensity were measured using the software ImageJ.

The levels of the inhibitory capacity against lipid production and accumulation were calculated using the intensity of fluorescence of the Nile red in conjunction with any lipids. The mean intensity of the negative control larvae, exposed only to DMSO, was considered as 100% of lipid accumulation. Inhibitory capacity was established as the inverse of the lipid accumulation. Positive control larvae were exposed to resveratrol, that mimics dietary calorie restrictions, stimulating metabolic rates and thus show an abnormally low lipid buildup in the larvae and no fluorescence in the larvae is detected.

7.1 Statistical treatment

To assess whether the differences amongst the control group and the treatment groups was significant, one-way analysis of variance (ANOVA) test was done on the mean

intensity values. ANOVA test was done in Microsoft® Office Excel, with alpha level was set at 0.5. After ANOVA, the Dunnett's test was also done to compare each of the of treatments with the DMSO control. The Dunnett's test is a multiple comparison procedure (many-to-one comparison) to compare each of the treatments to the DMSO control. The Dunnett's test was also performed in Microsoft® Office Excel.

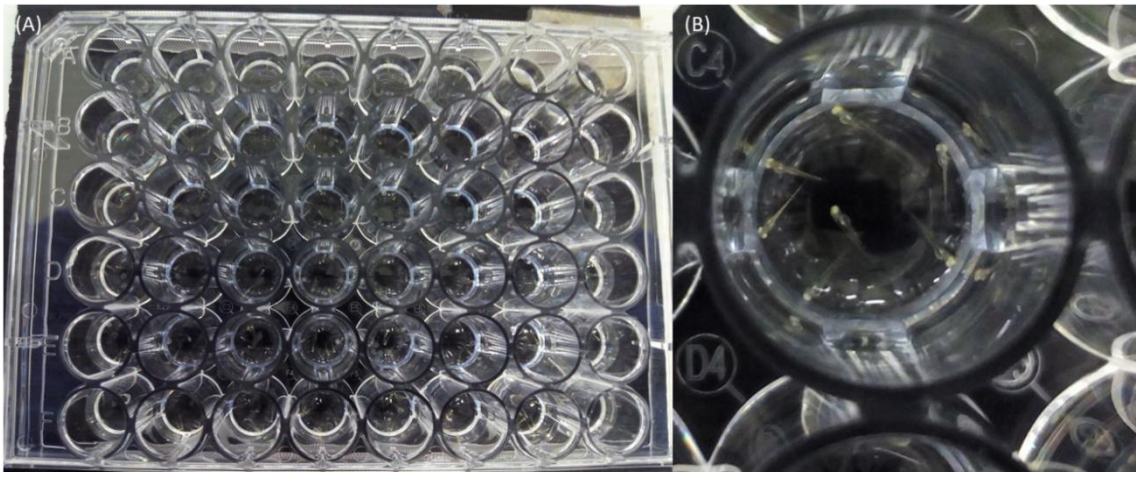

Fig 6 - Forty-eight well plate with (A) all hatched zebrafish larvae and close-up (B) of a well with 7 larvae in it.

8. Gel permeation chromatography

Extracts are complex mixtures of organic molecules. Due to their complexity, identifying the molecule that triggers the action seen in the assays can be complicated. Therefore, breaking down the extract into fractions with less complexity is the best option. To achieve this, gel permeation chromatography (GPC) was chosen. GPC is a type of size exclusion chromatography, that separates analytes based on size. GPC uses an organic solvent passing through a column packed with a gel, usually cross-linked polyacrylamide or agarose gel.

For GPC, only the two more promising strains, #91-36.1 and #91-36.2 were chosen. Five hundred mL of each strain were incubated for 5 days at 200 r.p.m. with Marine Broth supplemented with the autoclaved *E. coli* and *B. subtilis* at 25ºC. After the incubation period, extraction with ethyl acetate was performed. After drying on the rotatory evaporator, solid residues in the balloon were dissolved with methanol. A sample for testing was taken and tested using the modified Kirby-Bauer assay. GPC was performed on these extracts.

GPC was performed using a Sephadex® LH20 column, with a GILSON® pump at a velocity of 2 mL/min, with fraction collected every fifteen minutes, up to a total of 3 hours. The Sephadex® LH20 column was chosen as the stacionary phase as Sephadex® LH20

was specially designed for separating terpenoids and small weight peptides, the principal category of molecules produced in secondary metabolism. The solvent used was methanol. After GPC, every fraction was dried in the rotatory evaporator using 100 mbar at 25ºC, with the solid residues being dissolved in 2 mL of methanol with the exception of the first 2 portions that were discarded. Finally, every fraction was tested for antimicrobial activity using again the modified Kirby-Bauer assay.

Fig 7 – Chromatograph used for GPC. (A) Gilson 307 pump running at 2mL/min. (B) Sephadex LH20 column used.

Results and Discussion

1. Preliminary antimicrobial assays

All the preliminary antimicrobial assays were performed using fresh extracts from 2 mL cultures in acetone plus DMSO, except for extraction with ethyl-acetate which used 20 mL of culture.

1.1. Antimicrobial liquid assays in 96-wells plates

Liquid antimicrobial assays in 96-wells plates seemed to show results consistent to the ones showed by Graca *et al* (2013) and Graca *et al* (2015). Most of the strains were able to inhibit growth the of *Bacillus subtilis* above the 20% level (Table 5). Several extracts from different strains induced an inhibition level above 50% and in general, the more effective extracts were the ones obtained from the cell pellet (P) (26 positive extracts). The less effective extracts were the aqueous ones (F), indicating that the majority of the molecules produced were more soluble in organic solvents.

Table 5 – Levels of inhibition produced by the 33 bacterial extracts against the 3 targets.

Isolate	Levels of activity (in %)											
	B. subtilis				*E. coli*				*C. albicans*			
	F	C	P	S	F	C	P	S	F	C	P	S
#91_17	-96.3	-130.3	32.4	58.4	-13.0	3.2	5.8	-1.3	-6.5	1.6	2.9	-0.7
#91_29	-92.6	35.8	52.2	38.1	3.6	5.2	3.8	0.4	1.8	2.6	1.9	0.2
#91_31	-118.0	-118.7	19.1	-5.9	-11.9	1.9	-0.6	-1.2	-5.9	1.0	-0.3	-0.6
#91_34	-99.5	30.4	67.3	30.6	3.0	6.7	3.1	0.3	1.5	3.4	1.5	0.2
#91_35	-151.6	-114.6	52.7	24.7	-11.5	5.3	2.5	-1.1	-5.7	2.6	1.2	-0.6
#91_36.1	-8.3	21.0	29.7	22.5	2.1	3.0	2.3	0.2	1.1	1.5	1.1	0.1
#91_36.2	19.1	17.7	19.9	2.9	1.8	2.0	0.3	0.2	0.9	1.0	0.1	0.1
#91_37	-100.7	-80.2	36.7	36.2	-8.0	3.7	3.6	-0.8	-4.0	1.8	1.8	-0.4
#91_40	13.6	22.9	28.7	-9.5	2.3	2.9	-1.0	0.2	1.1	1.4	-0.5	0.1
SM 115	16.3	36.5	49.3	38.9	3.7	4.9	3.9	0.4	1.8	2.5	1.9	0.2
SM 116	21.7	54.9	39.0	43.1	5.5	3.9	4.3	0.5	2.7	2.0	2.2	0.3
B01 119c	23.7	24.2	45.2	25.1	2.4	4.5	2.5	0.2	1.2	2.3	1.3	0.1
B02 22.2	4.6	26.5	56.2	13.0	2.7	5.6	1.3	0.3	1.3	2.8	0.7	0.1
B02 29	54.8	20.9	-6.5	51.9	2.1	-0.7	5.2	0.2	1.0	-0.3	2.6	0.1
B02 78	-123.8	41.0	58.4	26.8	4.1	5.8	2.7	0.4	2.1	2.9	1.3	0.2
B02 79	24.4	2.7	41.3	16.0	0.3	4.1	1.6	0.0	0.1	2.1	0.8	0.0
B02 79a	-9.6	31.6	42.7	34.6	3.2	4.3	3.5	0.3	1.6	2.1	1.7	0.2
#118_14	-110.2	-90.6	55.5	26.1	-9.1	5.6	2.6	-0.9	-4.5	2.8	1.3	-0.5
#118_17	-131.5	40.7	45.2	35.5	4.1	4.5	3.6	0.4	2.0	2.3	1.8	0.2
#118_20	-104.4	45.0	43.1	66.1	4.5	4.3	6.6	0.5	2.3	2.2	3.3	0.2
#118_19	-129.7	35.0	58.0	11.8	3.5	5.8	1.2	0.4	1.8	2.9	0.6	0.2
#91_10.2	17.5	22.9	29.5	15.3	2.3	3.0	1.5	0.2	1.1	1.5	0.8	0.1
#91_13	-105.6	-67.3	53.6	10.3	-6.7	5.4	1.0	-0.7	-3.4	2.7	0.5	-0.3
B01 7	14.3	23.8	22.9	7.1	2.4	2.3	0.7	0.2	1.2	1.1	0.4	0.1
B01 9	18.9	11.9	31.2	3.5	1.2	3.1	0.4	0.1	0.6	1.6	0.2	0.1
B01 77	-0.8	25.1	34.5	12.8	2.5	3.5	1.3	0.3	1.3	1.7	0.6	0.1
B02 8.1	30.0	13.4	19.8	16.7	1.3	2.0	1.7	0.1	0.7	1.0	0.8	0.1
B02 9.1	-81.3	-139.0	56.0	28.9	-13.9	5.6	2.9	-1.4	-7	2.8	1.4	-0.7
B02 10.a	20.2	25.8	24.6	16.9	2.6	2.5	1.7	0.3	1.3	1.2	0.8	0.1
B02 10.b	0.6	13.7	16.8	17.5	1.4	1.7	1.8	0.1	0.7	0.8	0.9	0.1
B02 10.c	0.2	12.8	17.9	18.1	1.3	1.8	1.8	0.1	0.6	0.9	0.9	0.1
B02 10.1	-122.1	42.7	52.2	14.1	4.3	5.2	1.4	0.4	2.1	2.6	0.7	0.2
B02 61	18.6	19.3	14.0	12.7	1.9	1.4	1.3	0.2	1.0	0.7	0.6	0.1

F = Aqueous phase extract; C = Crude organic extract; P = Pellet organic extract; S = Supernatant organic extract. Positive hits (more than 20% inhibtion) highlighted in green.

In total 18% of culture filtrate (F) extracts, 58% of the crude organic extracts (C), 79% of the cell pellet organic extracts (P) and 48 culture supernatant organic extracts (S) were bioactive. Of these, 1 filtrate, 1 crude organic extract, 10 pellet organic extracts and 1

supernatant organic extract were above the 50% level of activity against *B. subtilis*. For strains SM 116 and B01-119c, all the extracts were positive regarding bioactivity. These preliminary results seemed to indicate a good bioactive potential of the strains screened, showing promising capabilities.

Even though high bioactivities were obtained against *B. subtilis*, *Candida albicans* and *Escherichia coli* showed no signs of inhibition. These results may be explained by two factors: 1) the high sensibility of *B subtilis* to antimicrobial compounds (Adimpong *et al.*, 2012), especially when compared to the *E coli* used in this study, an environmental strain which has revealed high resistance in various antimicrobial screening (Graca *et al.*, 2013; Graca *et al.*, 2016; Graca *et al.*, 2015), and 2) the need for extraction protocol refinement, because extracts might have possessed an insufficient concentration of bioactive molecules for detecting inhibition.

1.2. Modified Kirby-Bauer assays

This assay is the more traditional assay to determine both antimicrobial resistance and to test effectiveness of extracts and newly developed bioactive molecules. Due to the previous lack of inhibitions in both *E. coli* and *C. albicans*, these targets were not tested in this assay. The selected targets were *B. subtilis* and *Micrococcus luteus,* another Gram^{+} bacterium. *B subtilis* was chosen based on the preliminary results and *M. luteus* was chosen for its sensibility to bioactive compounds (Dischinger *et al.*, 2009). However, similar extract to the previous ones assayed in 96-well plates did not induced inhibition (Fig. 8).

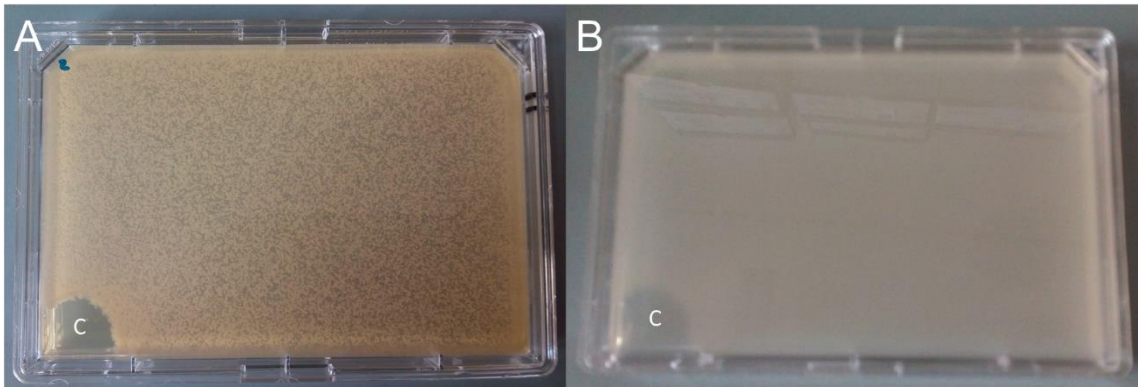

Fig 8 – Kirby-Bauer assay of Acetone plus DMSO extracts from several bacteria against *M luteus* (A) and *B. subtilis* (B). The control used (C) was ampicillin at 0.1 mg/mL. No extract from the 33 strains showed inhibition

To validate these results, this assay was repeated twice and similar results were obtained. The lack of bioactivity by these strains was not in agreement with the

preliminary results in 96-well plates and the genetic potential of the chosen strains as previously reported (Graca *et al.*, 2013; Graca *et al.*, 2015).

1.2.1. Well assays with Acetone plus DMSO extracts

As no inhibition was detected with 10 µL droplets of extract, 100 µL of each extract (in total 132 from the 33 bacteria) were tested using the well assays. With 10-fold increase of overall extract, it was expected to find bioactivity at least in some extracts.

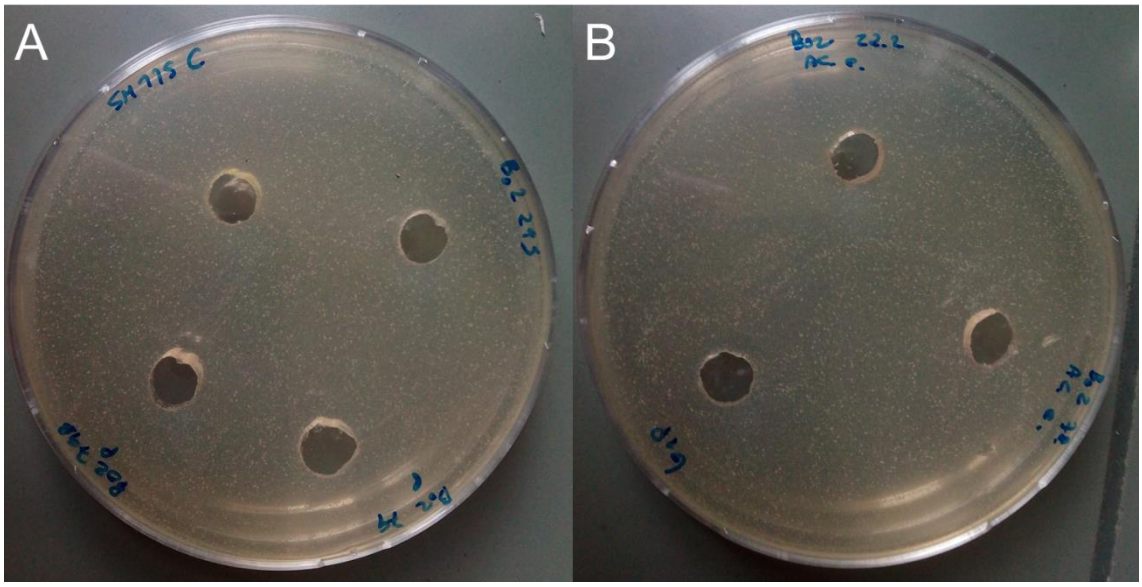

Fig 9 - Example of two plates of *M. luteus* used in the well assay where 100 µL extract were used. (A) Extracts from 2 mL culture in Acetone plus DMSO. (B) Extracts from 20 mL culture obtained with ethyl-acetate.

However, even with the higher volume of extract used, no inhibition halos were detected for any of the strains screened against *B. subtilis* or *M. luteus* (Fig 9 A).

1.2.2. Disk assay using Acetone plus DMSO extracts

The diffusion effectiveness in a given agar mesh decreases with the increase of the molecular weight of the diffusible molecules (Lebrun & Junter, 1993). To bypass any diffusion problem that might have occurred in the two previous assays, the agar concentration in the media was decreased from 1.6% to 0.6%. To avoid the drawback of loss of rigidity and structural integrity of the media, which in turn could impact the results, a second, more rigid layer of media was placed below the softer layer. Using fresh acetone plus DMSO extracts of 2 mL of culture, filter disks were soaked with 100 µL extract and placed on top of the media with a lower concentration of agar. Despite all the modification to the assay, inhibition halos were not detected (Fig 10).

With all possible variations done to the assays and inconsistency or lack of bioactivity results obtained so far, other variables like extraction solvent or stress inducing agents were tested for the obtainment or production of bioactive molecules.

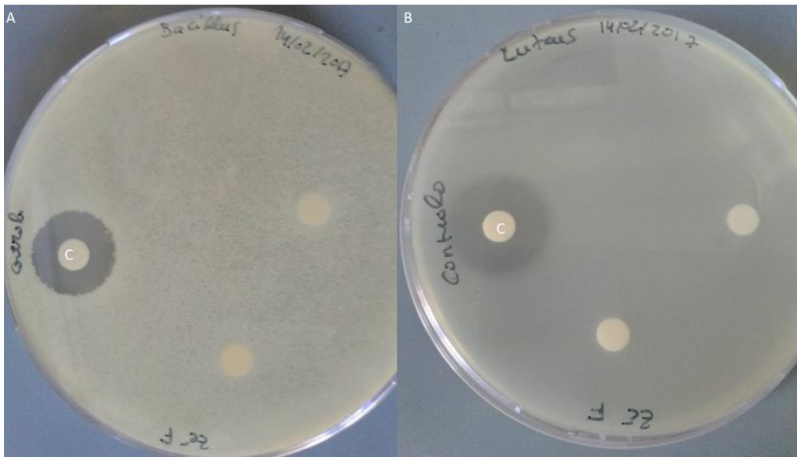

Fig 10 – Two examples of the disk assay performed with two media layers (1.6% and 0.6% agar) against *B. subtilis* (A) and *M. luteus* (B). The control used (C) was ampicillin at 0.1 mg/mL. No extract from the 33 strains showed inhibition.

1.2.3. Modified Kirby-Bauer assays of 20 mL culture ethyl-acetate extracts

Fig 11 – Kirby-Bauer assay of Acetone plus DMSO extracts from several bacteria against *M luteus*. The control used (C) was ampicillin at 0.1 mg/mL. No extract from the 33 strains showed inhibition.

It was then decided to change the extraction protocol. So, extracts were done using 20 mL of culture instead of 2 mL, a 10-fold increase in concentration and instead of acetone plus DMSO, ethyl-acetate was used as solvent.

As the lack of bioactivity of all the extracts assayed persisted (Fig 9 B and 11), it was hypothesized that the strains could have changed their secondary metabolism and stopped or decreased their production of bioactive molecules.

1.2.4. Direct culture assays

To guarantee that bacterial strains were still fit to produce antimicrobial compounds in a non-extracted way, it was decided to co-culture them with the different targets. Culturing them in Mueller-Hinton medium was also making stress on bacteria, as Mueller-Hinton is a salt-less medium.

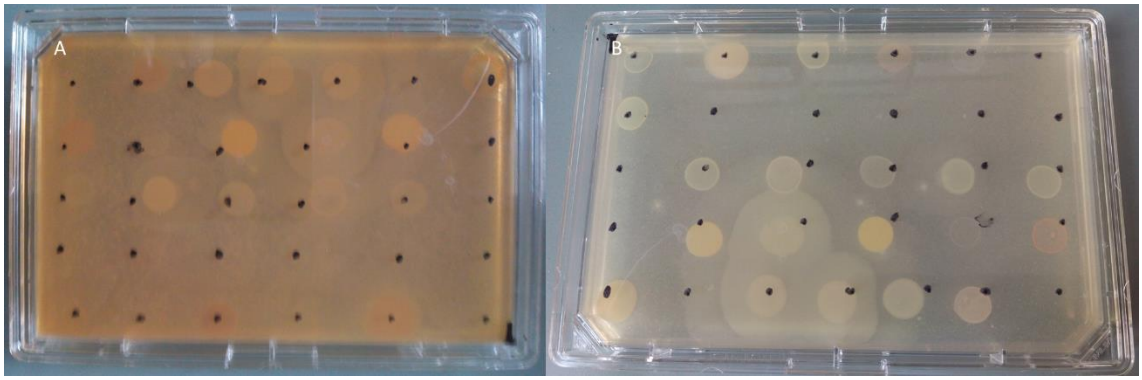

Fig 12 – Co-culturing assay where 31 bacterial strains were cultivated for 3 days in Mueller-Hinton medium with targets incorporated. (A) Plate of *B. subtilis*. (B) Plate of *M. luteus*.

However, the co-cultured strains were not effective in producing any visible sign of inhibition in *B. subtilis* (Fig 12 A). *M. luteus* unfortunately did not grow normally, producing unusually transparent colonies instead of the normal yellow. Regardless, *M. luteus* did not demonstrated any sign of inhibition (Fig 12 B).

1.2.5. Potentiation of bioactivity production by biological stress

In order to stimulate secondary metabolism and subsequent production of defense molecules, all the strains (Table 1) were cultured in 20 mL cultures supplemented with autoclaved *B. subtilis* and *E. coli*. Extracts were made using the acetone plus DMSO protocol.

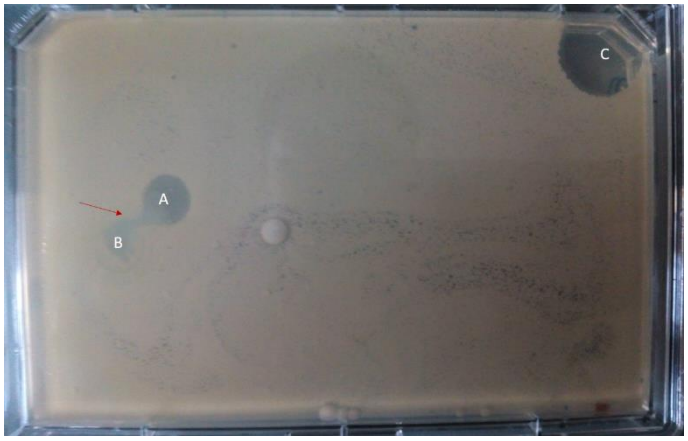

Fig 13 - Kirby-Bauer assay of 48 Acetone plus DMSO extracts *B. subtilis* showing two inhibition halos ((A) Extract #91-36.2 F. (B) Extract #118-20 F. Arrow points to possible mixing of the extracts). The control used (C) was ampicillin at 0.1 mg/mL.

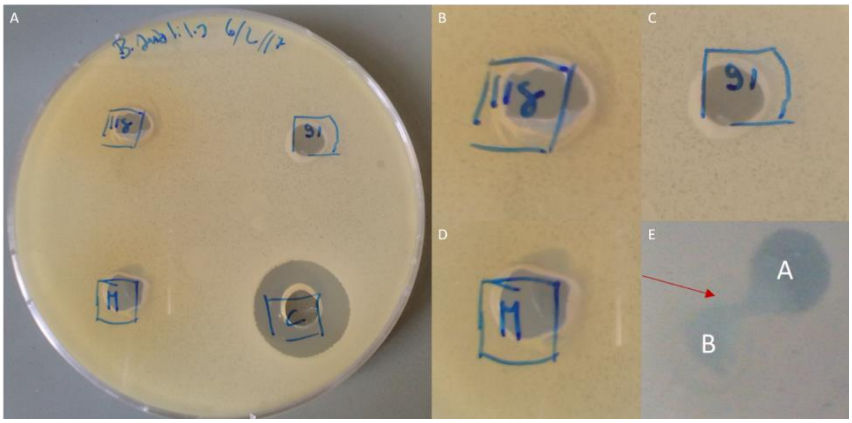

Fig 14 – Well assay of extract #91-36.2 F and extract #118-20 F and mixture of the two extracts. (A) Plate with *B. subtilis*. (B) Close up of extract #118-20 F. (C) Close up of extract #91-36.2 F. (D) Close up of mixed extracts. (E) Close up of extracts #91-36.2 F and #118-20 F from the previous plate (arrow points to possible mixing of the extracts).

Inhibition halos were detected for extracts #91-36.2 from the filtrate of the culture (F) and #118-20 F (Fig. 13), but the two extracts mixed in the plate (see arrow in Fig. 13). To undoubtedly validate these two bioactivities, the two extracts were screened independently and in a mixture of 1:1. The extracts volume was increased 10-fold. Inexplicable, the previous results were unable to be reproduced, even using 10 times more extract (Fig. 14).

1.2.6. Induction of bioactivity by chemical stress

Three different incubation conditions of all bacterial strains were evaluated on the production of bioactive compounds. A 15% increase on NaCl in the Marine Broth medium (salt stress) was tested to potentiate induction of bioactive molecule production. Cultivation using non-saline Nutrient Broth was also performed (osmotic stress). Furthermore, the effect of lower levels of nutrients compared to Marine Broth on bioactive production was tested using M13 Modified Broth (Marine Broth: 5% of peptone and 1 % of yeast extract. M13 Modified Broth: 0.25% of peptone and 0.25% of yeast extract). Two mL of culture were used for the extraction with Acetone plus DMSO.

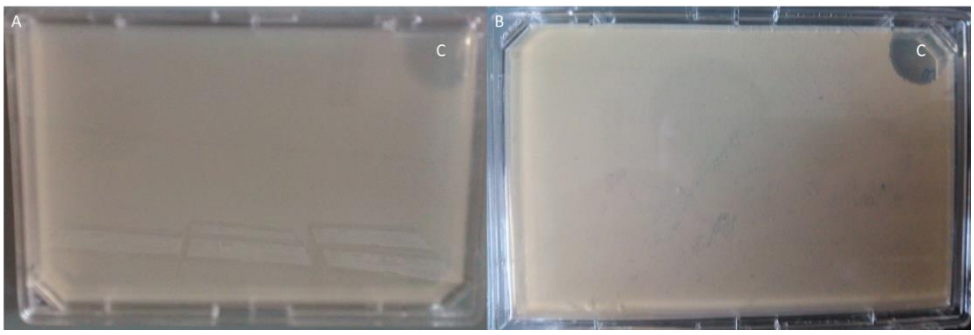

Fig 15 – Kirby-Bauer assay of 48 acetone plus DMSO using cultures grown in Nutrient Broth (osmotic stress) extracts against *M luteus* (A) and *B. subtilis* (B). The control used (C) was ampicillin at 0.1 mg/mL.

All strains were able to grow under all these conditions, however no bioactivity was observed for any of the extracts from all the stress conditions evaluated (Fig. 15).

2. Assays with higher extraction volumes in ethyl-acetate

2.1 Modified Kirby-Bauer assays

As overall almost no bioactivity was obtained with small-scaled cultures, it was decided to attempt higher extraction volumes. Passing from a 2 mL to 500 mL cultures, obtaining a 250-fold increase, it was expected to potentially guarantee a much higher concentration of the bioactive molecules extracted. The extraction protocol was also revised, being chosen the simpler and more efficient extraction with ethyl-acetate (Siek, 1978). Using higher culture volumes, it comes with the drawback that only a smaller number of strains can be tested in a reasonable timeframe.

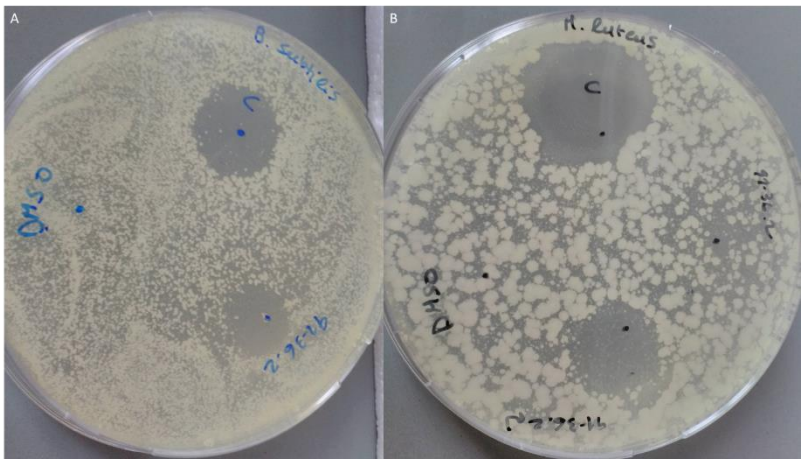

Fig 16 - – Kirby-Bauer assay of DMSO extract #91-36.2 using 500 mL of culture and ethyl-acetate for the extraction against *B. subtilis* (A) and *M. luteus* (B). The control used (C) was ampicillin at 0.1 mg/mL. Extract #91-36.2 sucessfully inhibited growth of both *B. subtilis* and *M. luteus*.

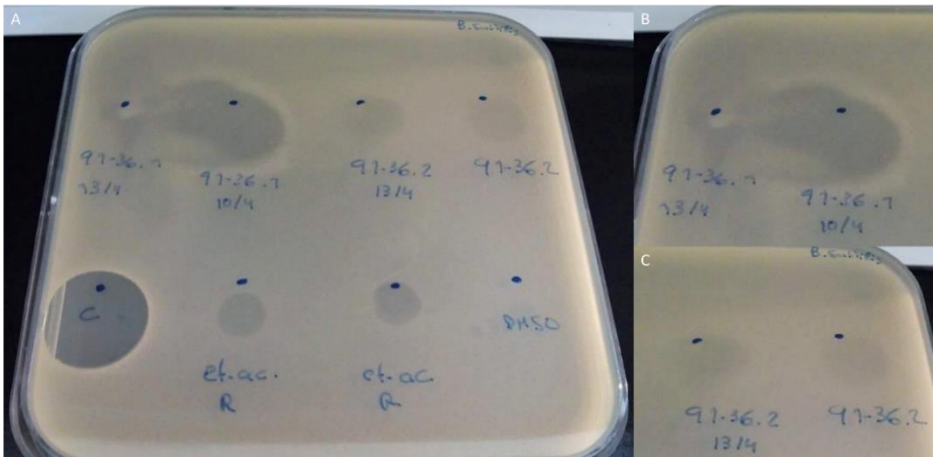

Fig 17 - – Kirby-Bauer assay of DMSO extracts #91-36.1 and #91-36.2 using 500 mL of culture and ethyl-acetate for the extraction against *B. subtilis* (A). The control used (C) was ampicillin at 0.1 mg/mL. Both extracts #91-36.1 and #91-36.2 sucessfully inhibited growth of *B. subtilis*.

Since the aqueous extract of #91-36.2 (F) showed antimicrobial bioactivity in the potentiation of bioactivity production by biological stress (see section 1.2.5), this strain was initially chosen. Incubation was done in 500 mL of Marine broth supplemented with autoclaved *B. subtilis* plus *E. coli* cultures, to mimic conditions described in section 1.2.5 (biological stress). After incubation, 500 mL of culture were extracted using the ethyl-acetate protocol and the dried residue was dissolved in DMSO. Extracts were tested using 10 µL droplets on top of the agar.

With this methodology the extract was able to produce inhibition halos on both *B. subtilis* and *M. luteus* (Figs. 16 and 17). Moreover, extracts remained bioactive, although less, after 2-week periods. Furthermore, strains #91-36.1 and B02-78 were also tested. These were chosen primarily by the presence of both PKS-I and NRPS genes in their genome.

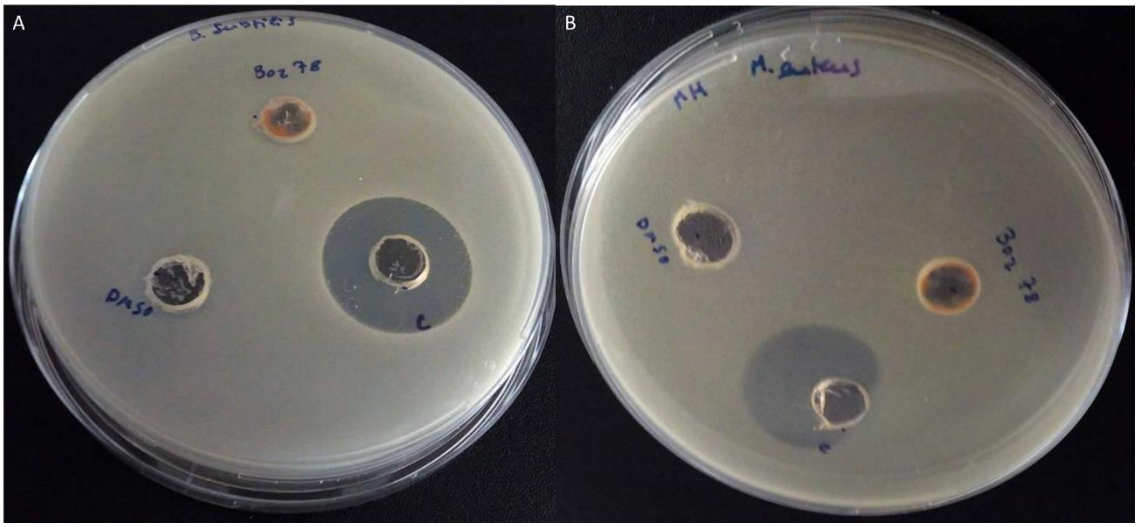

Fig 18 - Well assay of DMSO extract B02-78 using 500 mL of culture and ethyl-acetate against *B. subtilis* (A) and *M. luteus* (B). No inhibition was observed in either two targets. The control used (C) was ampicillin at 0.1mg/mL

As shown in Figure 17, strain #91-36.1 was able to produce an extract with antimicrobial effects on *B. subtilis*. The extract from strain B02-78 had a bright orange/red color, which can be seen in Figure 18. The extract was tested using the wells assay protocol described in section 1.2.1. This strain did now show any inhibition against both *B. subtilis* and *M. luteus*, as can be seen in Figure 18.

2.2. Mode of action assays

The mode of action (MoA) assays allow, using specific targets (Table 2), the identification of the metabolic pathway through which a bioactive compound interacts with cells leading to their death or impairing their growth. The specific targets were *B. subtilis* PyorB reporter for DNA, *B. subtilis* Pyhel reporter for Protein, *B. subtilis* PypuA reporter for Cell Wall and *B. subtilis* PyvgS reporter for RNA. Due to time constraints, this assay was

performed using only extracts from strain #91-36.2. In a first trial, no development of color, even in the controls used was observed which, indicated, perhaps, that the X-Gal was either insufficient or was possibly decayed (Fig. 19).

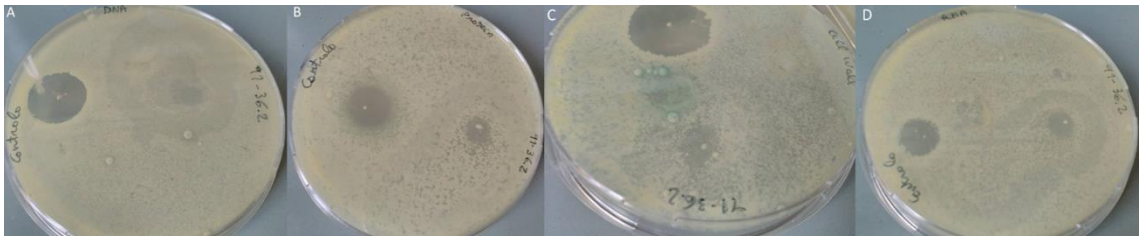

Fig 19 – Mode of action screening with *B. subtilis* PyorB reporter for DNA (A), *B. subtilis* Pyhel reporter for Protein (B), *B. subtilis* PypuA reporter for Cell Wall (C) and *B. subtilis* PyvgS reporter for RNA (D).

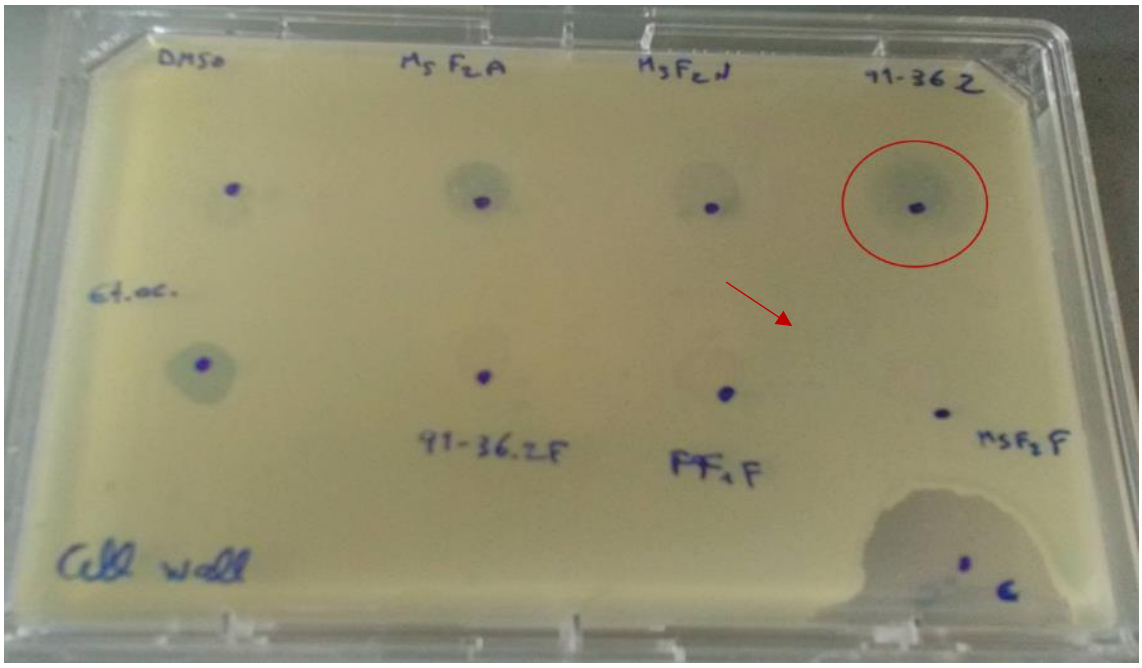

Fig 20 - Mode of action screening with *B. subtilis* PypuA reporter for Cell Wall with positive result circled in red. Arrow points to bluish coloration around the control. The control used was ampicillin at 0.1m mg/mL.

In a second attempt, where fresh X-Gal was used, a faint but noticeable blueish coloration appeared in the place where the 10 µL droplet was placed (Fig. 20). As it was evident with strain *B. subtilis* PypuA, the extract is effective against the cell wall. No blue color was noticed in any of the other three reporters.

3. Anti-*Chlamydia trachomatis* activity of ethyl-acetate extracts from #91-36.2

Once again and due to time constrains, the search for bioactivity against *Chlamydia trachomatis* was only done with extracts from strain #91-36.2.

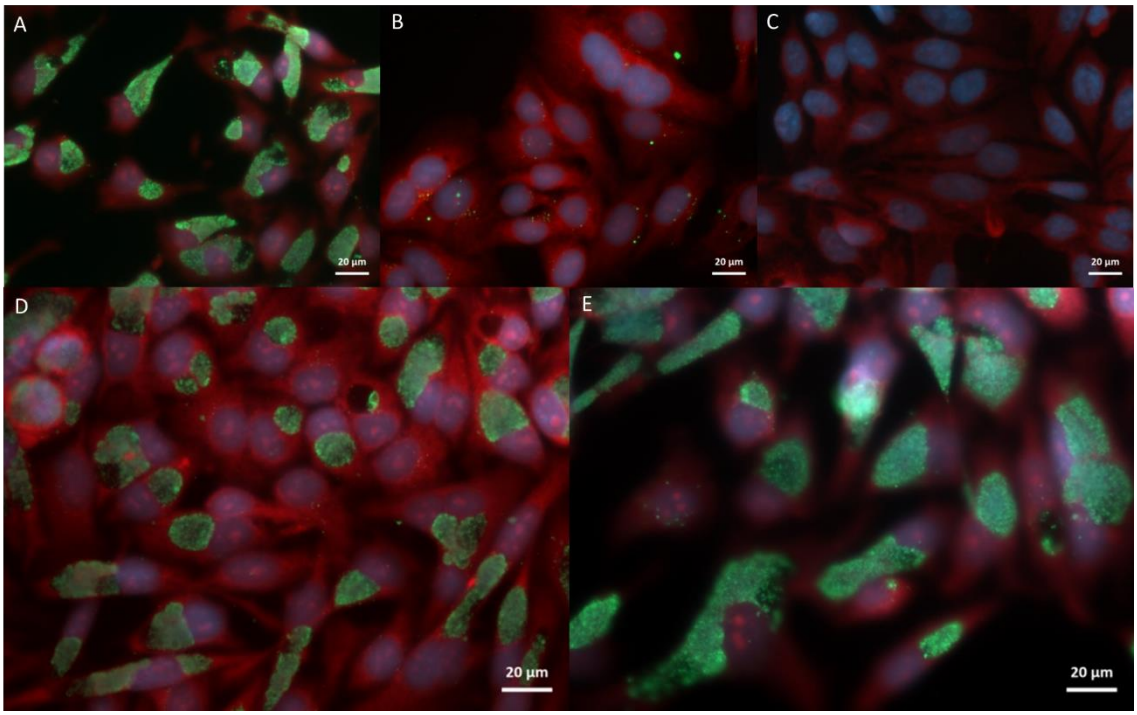

Fig 21 – Assays performed with HEp-2 cells. (A) Infected HEp-2 cells with *C. trachomatis.* (B) Infected HEp-2 cells with *C. trachomatis* treated with Ciprofloxacin at 1.0 µg/mL. (C) Uninfected HEp-2 cells. (D) HEp-2 cells infected with *C. trachomatis* treated with 1.28 µL of #91-36.2 extract. (E) HEp-2 cells infected with *C. trachomatis* treated with 1.28 µL of DMSO.

The uninfected HEp-2 cells did not stain with the fluorescein-conjugated antibodies specific for chlamydial lipopolysaccharide (LPS) as they do not possess *C. trachomatis* intracellularly (Figs. 21 C; 22 C). When HEp-2 cells were infected with *C. trachomatis* they showed an intense green labelling because of the fluorescein-conjugated antibodies attached to the chlamydial LPS on the cell surface (Figs. 21 A; 22 A). When infected cells were treated with Ciprofloxacin, no green labelling was visible, because the chlamydial infection was controlled (only small dots of the remaining infection could be seen) (Figs. 21 B; 22 B). When infected HEp-2 cells were treated with 1.28 µL of #91-36.2 extract, chlamydial aberrant bodies formed, indicating that the extract was inducing stress on *Chlamydia*. Under this volume (1.28 µL), no effect was visible due to cell incubation with DMSO (the #91-36.2 extract solvent) (Fig 21 E). When the infected HEp-2 cells were treated with a higher volume of #91-36.2 extract (2.56 µL) a strong reduction of *Chlamydia* inside the HEp-2 cells was observed. The small labelling dots that could be seen (Fig. 22 D) on the cells treated with this extract were comparable to the ones seen after treatment with ciprofloxacin (Fig 21 B, 22 B). These results seem indicative of anti-chlamydial activity of strain #91-36.2. However, as DMSO in the same concentration (2.56 µL added) also induced a decrease of infection as well as of the production of

chlamydial aberrant bodies, caution must be taken regarding the conclusions of these results.

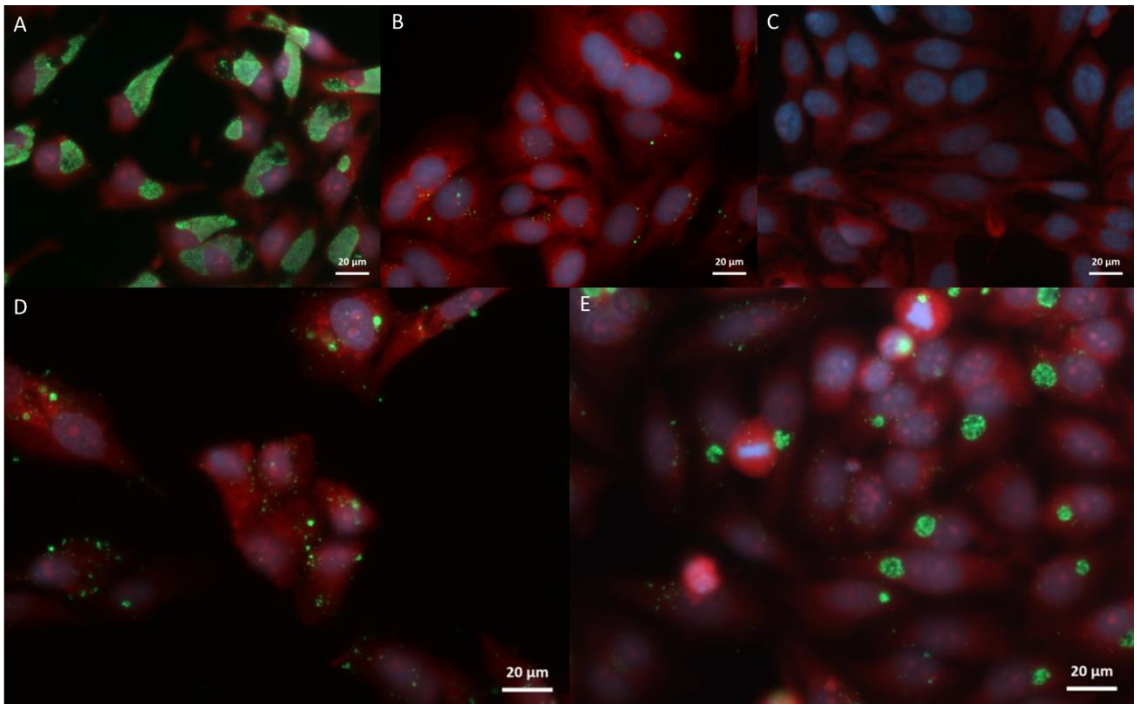

Fig 22 – Assays performed with HEp-2 cells. (A) Infected HEp-2 cells with *C. trachomatis.* (B) Infected HEp-2 cells with *C. trachomatis* treated with Ciprofloxacin at 1.0 µg/mL. (C) Uninfected HEp-2 cells. (D) HEp-2 cells infected with *C. trachomatis* treated with 2.56 µL of #91-36.2 extract. (E) HEp-2 cells infected with *C. trachomatis* treated with 2.56 µL of DMSO. Chlamydial aberrant bodies were formed with this treatment.

4. Gel permeation chromatography (GPC) of #91-36.1 and #91-36.2 extracts

Extracts are characteristically extremely complex mixtures of different molecules, with the majority being comprised of primary metabolic products and a smaller amount of secondary metabolic products. To identify the constituents of the extracts capable of inducing inhibition it is required to fractionate the extracts, thus separating them into less complex mixtures and ultimately individual molecules. GPC has several advantages such as a good separation of large from small molecules with a minimal volume of eluate, good sensitivity and minimal loss of samples due to negligible interaction with the stationary phase (Skoog *et al.*, 2007). This technique, combined with the Sephadex LH 20 column seemed to be a good candidate for separation of the extracts.

Extracts from #91-36.1 and #91-36.2 with biological stimulation were chosen for GPC. Since both extracts could inhibit both M. luteus and B. subtilis, fractions were tested only against B. subtilis. As the first two fractions corresponded with the initial volume of the

column and thus having no real bioactive relevance for testing, fractions testing started at fraction 3 (F3) and end in fraction 15 (F15).

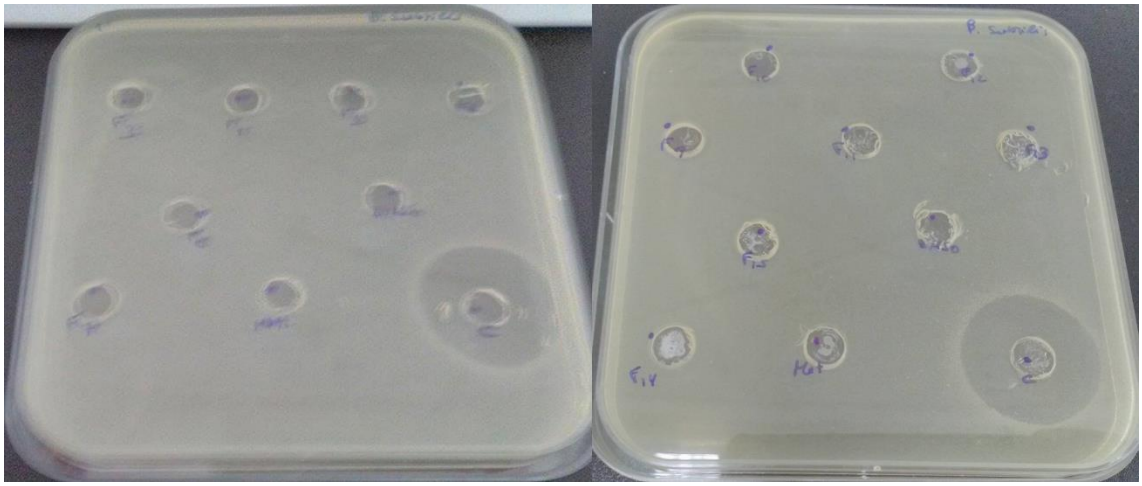

Fig 23 - Well assay with *B. subtilis* with fractions from GPC of extract #91-36.1. The control used was ampicillin at 0.1 mg/mL

None of the fractions from both strains showed inhibition (Fig. 23). Probably these results are due to the dilution of the bioactive compound.

5. Ethyl-acetate extraction with Amberlite™ XAD16N resin

In view of the previous lack of bioactivity in GPC attempt, an extraction with an adsorption resin was assayed. Amberlite™ XAD16N is used to remove hydrophobic compounds up to 40,000 MW and separation of large organic molecules. As it has a high surface area, it increases its effectiveness on extraction.

A 2-liter culture of strains #91-36.1 and #91-36.2 with biological stimulation was made. At the third day of incubation, 30 grams of Amberlite™ (1.5%) were added and collected after the 5 days incubation period using cellulose filters. The resin was then submerged in ethyl-acetate for 1 hour with shaking for extraction. The rest of the culture was then centrifuged and the cell pellet collected and extracted with ethyl-acetate. After dried, the residues from the extractions were dissolved in DMSO. The Kirby-Bauer assay of the four extracts against *B. subtilis* and *M. luteus* showed no inhibitory effects as shown for #91-36.2 (Fig. 24). Similarly, the extracts from strain #91-36.1 yielded do inhibitions against both *B. subtilis* or *M. luteus*.

Although antimicrobial inhibition was observed in several times and various assays, it has not been constant over time. This made it impossible to isolate potential bioactive molecules besides inducing great frustration due to lack of results.

Loss of bioactivity by the screened strains may be due to (1) changes in their secondary metabolism for unknown reasons and (2) low levels of production of bioactive molecules under the conditions tested.

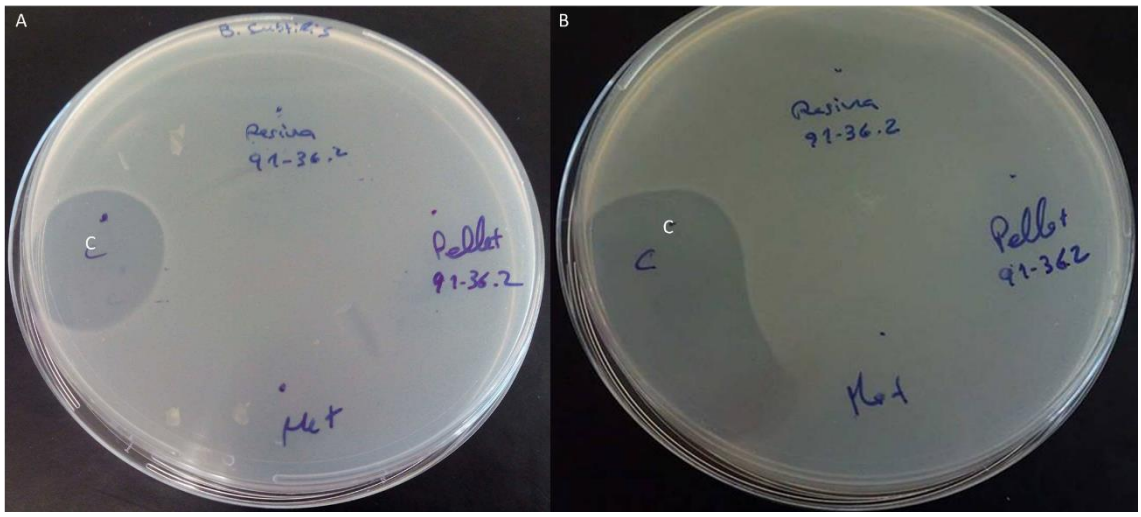

Fig 24 – Kirby-Bauer assay of extracts #91-36.2 from 2-liters of culture using Amberlite^{TM} and the cell pellet with ethyl-acetate against *B. subtilis* (A) and *M. luteus* (B). No inhibitions were observed in both targets. The control used (C) was ampicillin at 0.1 mg/mL.

6. Anti-obesity activity from extracts of selected strains

Parallelly to the screening of the strains for new antimicrobial molecules we had the opportunity to screen for molecules with the capacity to affect lipid metabolism. This screening is important due to the fact that a real pharmacological approach that is both safe and effective is needed to fight against obesity.

The assay was performed with 8 of the 33 bacterial strains. The 100 mL culture methanol extracts after dried were dissolved in DMSO in a final concentration of 10 mg/mL. Their effect on zebrafish larvae was assessed with Nile Red after 2 days of exposure to the extracts. Larvae incubated with only DMSO (negative control) showed an intense red staining which was considered as the 100% level of lipid content (Fig 25 A and B). DMSO has low toxicity and is commonly used solvent in the fish embryo test (Kais *et al.*, 2013) and as Jones *et al* showed, DMSO has no effect on lipid accumulation on zebrafish larvae. When larvae were treated with 10 mg/mL of Resveratrol no staining was visible in their bodies, showing a complete reduction of their lipid accumulation and 100% lipid inhibition (Fig 25 C and D, Fig 26).

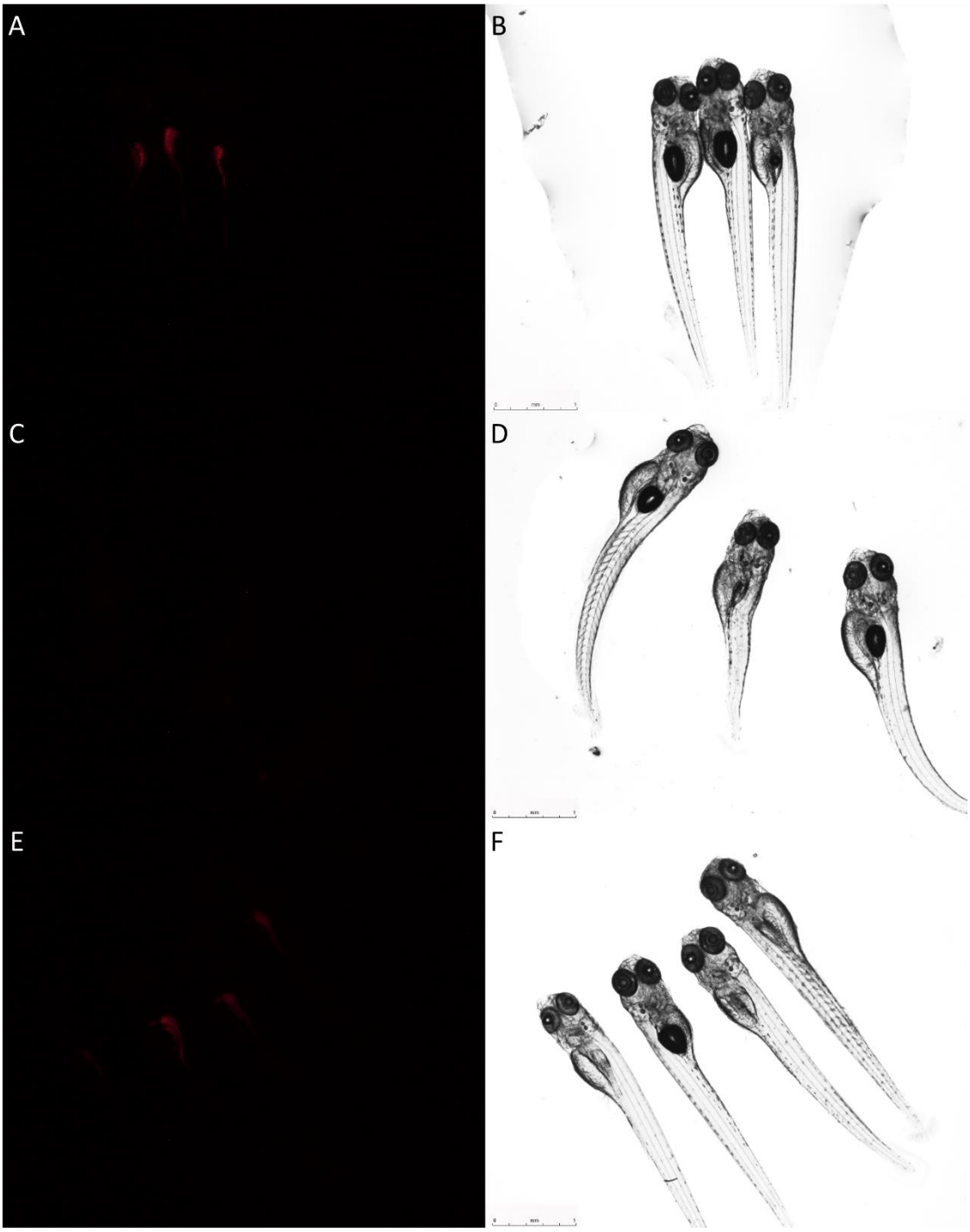

Fig 25 – Zebrafish larvae under fluorescence (A, C, E) and contrast-phase microscopy (B, D, F). Negative control larvae, exposed only to DMSO (A, B). Larvae show a normal lipid buildup, situated mostly among the lower abdomen. Positive control larvae, exposed only to Resveratrol (C, D). Larvae do not show lipid buildup among the lower abdomen. Larvae exposed to extract B02-61 (E, F). Lipid accumulation in the abdomen is occurring although slightly reduced when compared to DMSO.

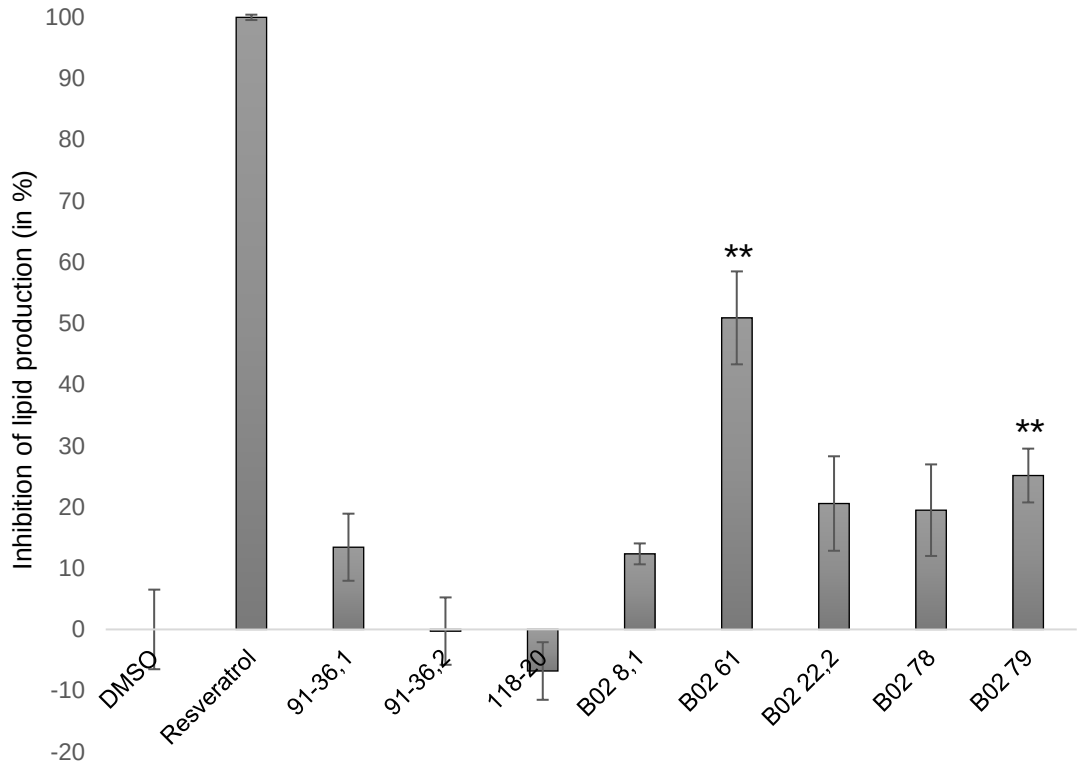

Fig 26 - Percentage of inhibition of lipid production and accumulation in zebrafish larvae. Error bars are representing standard error of the mean (in general n ≥ 5). ** represents significant differences between the treatments and the control (DMSO) (= $p < 0.01$).

Zebrafish larvae treated with extracts from selected strains showed red staining of lipid drops in Figure 25 (E and F). This means that no total inhibition of the lipid accumulation was achieved by the extracts. However, six strains induced a reduction in larvae lipid content (Fig 26). Despite this, only strains B02-79 and B02-61, showed a significant reduction of about 25% and 50% reduction, respectably. Statistical analysis showed these results to be statistically different from the control ($F_{[8,65]} = 6.98$; $p < 0.001$). No effect on lipid production was observed with extracts for strains #91-36.2 and #118-20 (Fig. 26).

Although a complete inhibition of the lipid production was not obtained with the bacterial extracts, we could achieve a significant lipidic reduction. These results are very promising, suggesting that further studies should be done with increased concentration of our extracts. Subsequent extract fractionation would allow the isolation of the bioactive compound. Due to time constrains, this could not have been done.

7. Overall discussion

The bacteria used in this work (17 strains of Actinobacteria, 15 strains of Gammaproteobacteria and 1 strain of Firmicutes) belong to phyla known for their potential to produce bioactive compounds (Aleti *et al.*, 2015; Holmström & Kjelleberg, 1999; Radjasa *et al.*, 2007). They were chosen based on their previous bioactive potential as well as by their genetic potential in secondary metabolism production (Graca *et al.*, 2013; Graca *et al.*, 2015). They have the presence in their genomes of PKS-I and NRPS genes. These are responsible for many biosynthesized compounds of interest. PKSs are a family of enzyme complexes that catalyze polyketides by extending linear carbon extensions, similarly to fatty acid biosynthesis (Dutta *et al.*, 2014). NRPS, similarly to PKSs, are a cluster of modular enzymes. NRPSs on the other hand synthesize peptide products, through complex regiospecific and stereospecific reactions and using a variety of proteinogenic and non-proteinogenic amino-acids (Strieker *et al.*, 2010). Both these genes are arranged in a single operon with different modules. Furthermore, the tested bacteria showed initially in this study, a good bioactive potential in the small-scale screening in 96-well plates and based on all the data, we were confident to obtain a successful outcome in this study. However, several subsequent screenings gave contrary results, which made it impossible to reach the major goal of this thesis.

Several kinds of antimicrobial screenings and extraction protocols were tested in order to overcome the lack of consistent results. Increase of culture extraction volume resulted in an increased level of bioactivity and ethyl-acetate proved to be the best solvent. Ethyl acetate, the ester of ethanol and acetic acid, can extract nonpolar drugs quite efficiently. Additionally, ethers, like ethyl acetate are also hydrogen bond acceptor molecules being able to extract electron donor solutes. Ethyl-acetate is also relatively safe and inexpensive. These factors make ethyl-acetate one of the most versatile solvents and most used solvent for general purpose extractions (Siek, 1978). Ethyl-acetate is also one of the more commonly used organic solvents (Schulz *et al.*, 1995; Selvameenal *et al.*, 2009; Zhang *et al.*, 2015). Despite good results with ethyl acetate, future work must be done to refine the extraction protocol. These modification should include the testing of other solvents (or solvent mixtures, like MeOH/DCM) and conditions (for example, the effect of “salting-out”) (Siek, 1978).

Using higher culture extraction volume, bioactivity against *B. subtilis* and *M. luteus* was achieved by strains *Microbacterium foliorum* (#91-36.2) and *Rhodococcus equi* (#91-36.1). These results were encouraging. Unfortunately and unexpectedly, none of the medium modifications introduced (salt stress, osmotic stress and nutrient limitation)

stress favored bioactivity. Biological competition was somewhat able to favor bioactivity, although at the end its effect was lost. As the secondary metabolism is not necessary for the growth or survival of the producer, regulation is highly sensitive to the conditions stimulating their production. Because of this, medium composition is vital for the production of bioactive molecules (Bibb, 2005). It is also fundamental that when competing with other microorganisms, production levels of metabolites increase, to protect the producer from competitors (Firn & Jones, 2003). As such, when cultured in nutrient rich, osmotically balanced media or grow unchallenged by other microorganisms, wild-type bacteria do not produce any secondary metabolites or their production is reduced (Vining, 1990).

R. equi is a soil-dwelling pathogenic actinomycete that can cause pulmonary infections specially in young foals (horses aged six months or lower) and immunocompromised humans. *R. equi* can be a facultative intracellular parasite of macrophages and its virulence is dependent on the presence of virulence plasmids. Despite the genus *Rhodococcus* being well known for its various applications, due to their extraordinary metabolic versatility and ability to biodegrade polyaromatic compounds and polychlorinated phenols and production of drugs and hormones (Ahmad *et al.*, 1991; Yam *et al.*, 2010), no antimicrobial bioactive secondary metabolite has been reported in the genus (McLeod *et al.*, 2006). Nevertheless, several studies on the genus *Rhodococcus*, and in particular *R. equi*, show that it possesses a great genetic potential. Besides having the characteristically large genome of Actinobacteria, several strains of *R. equi* have numerous assemblies of both PKS-I and NRPS genes (Ayuso-Sacido & Genilloud, 2005; Doroghazi & Metcalf, 2013; McLeod *et al.*, 2006). Rhodococci have high tolerance to stress, specifically desiccation (LeBlanc *et al.*, 2008), fact that may justify the lack of bioactive stimulation by stressful conditions in our strain #91-36.1. The results obtained here indicate that *R. equi* might be indeed producing a bioactive metabolite.

While first isolated from the phyllosphere of grass plants, *M. foliorum*, has also been found in other environments like marine sponges (Behrendt *et al.*, 2001; Graca *et al.*, 2013; Graca *et al.*, 2015). Although literature on the genus *Microbacterium* is scarce, *M. foliorum* contributes to aroma development in cheese during the ripening process (Deetae *et al.*, 2009). Moreover, *M. foliorum* did not show any PKS-I or NRPS genes in their genome (Graca *et al.*, 2013; Graca *et al.*, 2015). Strain #91-36.2 of *M. foliorum* was able to inhibit *B. subtilis* and *M. luteus*. Furthermore, its extract seemed to have the ability to interfere with the cell wall. These results indicate that *M. foliorum* capacity to produce antimicrobial metabolites should be due to a gene different from a PKS-I or a NRPS.

Future work with these strains must include further genetic analysis, with a more complete characterization of the gene/genes responsible for this activity. Work must also focus on the constant obtainment of the bioactive molecules in enough quantity to induce inhibition, assess or confirm their mode of action, viability against other targets and isolation and characterization of the bioactive molecules. Regardless, the results achieved during this thesis are prove of the biotechnological potential that *R. equi* and *M. foliorum* possess.

Somehow encouraging bioactivities were obtained against *C. trachomatis.* Once again, *M. foliorum* (strain #91-36.2) was bioactive against this intracellular parasite. Unfortunately, since DMSO also had a smaller but noticeable effect at the highest concentration tested, we must be careful regarding the conclusions of these results. At the lowest concentration tested, *C. trachomatis* developed aberrant bodies. Aberrant reticulate bodies contain non-dividing cells and continue to synthesize proteins and replicate DNA. The resulting inclusions of viable but nonculturable cells result in a prolonged infection (Hogan *et al.*, 2004). This effect is undesirable, because *C. trachomatis* can fail to respond to antibiotic treatment while also leading to a long term phenotypic resistance to otherwise effective antibiotics (Wyrick & Knight, 2004). Combined to this, the emergence of homotypic antibiotic resistance in human *Chlamydiae* remains as a threat that requires the development of novel drugs active against this bacterium. However, since *M. foliorum* #91-36.2 is bioactive against *C. trachomatis*, *M. foliorum* seems to be a good candidate for searching for bioactive anti-chlamydia molecules. Confirmation should be done by testing the extract in another solvent, to prevent the interfering seen with DMSO.

Regarding the anti-obesity assay, *Pseudovibrio sp.* (B02-61) and *Microbacterium sp.* (B02-79) were bioactive against lipid accumulation in zebrafish. Zebrafish larvae are a very useful tool for identifying non-toxic small molecules for developing molecular therapeutics for treating clinical obesity (Jones *et al.*, 2008). Obesity, already in 1995 imposed a cost of $100 billion in the USA (Wolf & Colditz, 1996). Recent estimates suggest total annual economic costs associated with obesity over $215 billion (Hammond & Levine, 2010). Unfortunately, Resveratrol does not seem to show any adjuvant effect on energy restriction for obesity treatment purposes on humans (Alberdi *et al.*, 2014). With all pharmacological approaches available today carrying safety concerns, including carcinogenic, cardiovascular, hepatotoxic, and psychiatric adverse effects (Hainer & Hainerova, 2012), newer therapy drugs are required. The results achieved by *Pseudovibrio sp.* and *Microbacterium sp.* make them promising candidates in the search for new anti-obesity bioactive compounds. Future work should focus in

obtaining refined fractions, with a higher concentration of bioactive compound to attempt a total inhibition of lipid accumulation.

The work presented in this thesis opens the field for future approaches. Since we had limited time to perform the laboratory work, needed surveys to confirm results, isolate and characterization the metabolites behind these bioactivities are required.

Conclusions

This work showed that the bacteria tested demonstrated bioactivity that needs to be further explored and that to achieve effective bioactivity induction, an increasing in the culture extraction volume is required. Of the various solvents tested, ethyl-acetate was the most effective one.

Antimicrobial activity of the Actinobacteria *Microbacterium foliorum* (#91-36.2) and *Rhodococcus equi* (#91-36.1) was observed against two Gram$^+$ bacteria, *B. subtilis* and *M. luteus*. *Microbacterium foliorum* was also bioactive against *C. trachomatis.* Furthermore, extracts from *Microbacterium foliorum* showed to act by targeting the cell wall.

The Gammaproteobacteria *Pseudovibrio sp.* (B02-61) and the Actinobacteria *Microbacterium sp.* (B02-79) were somehow effective in the reduction of lipid accumulation in larvae of zebrafish.

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