# EXPLOITING MARINE BIODIVERSITY: THE POTENTIAL OF UNCULTIVABLE MICROORGANISMS FOR THE IDENTIFICATION OF NOVEL ANTIMICROBIAL COMPOUNDS

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*"The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day."*

*Albert Einstein*

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## Summary

Antimicrobial resistance has spread dramatically in last 60 years leading to an increase in the number of deaths due to infection diseases. The excessive and often inappropriate use of antimicrobial drugs has led to the development of a new group of microorganisms, the Multidrug Resistant (MDR) bacteria, which show resistance toward the most common antibiotics. This phenomenon is becoming a serious threat to the public health and the economy. The bioprospecting of marine and extreme environments has yielded a noteworthy number of novel molecules with biotechnological applications from a wide range of macro and microorganisms, representing a very promising strategy to counteract MDR bacteria. The main gap is to have access to the real microbial biodiversity because less than 1% of microorganisms is cultivable in the laboratory conditions.

In this project, new antimicrobial compounds have been discovered from bacteria and fungi by different strategies. In the first work, following a bioprospecting pipeline, Antarctic shallow water sediments were used to isolate microorganisms that were screened for their capability to inhibit the growth of selected MDR bacteria. A bioassay-guided purification approach allowed the identification of rhamnolipids (a class of glycolipids well known as biosurfactants) produced by a *Pseudomonas gessardii* strain able to strongly inhibit MDR strains, in particular Gram-positive bacteria. These molecules have many biotechnological applications, especially in bioremediation field and, over last years, as antimicrobial compounds. The second work focuses the attention on the improvement of cultivation methods, exploiting a new device, the Miniaturized Culture Chip (MCC), for the isolation of “not-common” or novel bacteria. The innovation of this system is the possibility to grow microorganisms directly in their natural habitat simulating environmental conditions. By using the MCC an unexplored Antarctic strain, *Aequorivita* sp., was isolated. A genome mining approach on *Aequorivita* sp. was applied identifying the main biosynthetic gene clusters. The evaluation of its bioactive potential led to the discovery of three new intracellular aminolipids showing antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and anthelmintic activity against the nematode *Caenorhabditis elegans*. This was the first report that demonstrated the bioactivity of this strain and stimulates the research of new cultivation method by which obtain new and unexplored sources of compounds. The third work instead, gave more attention to marine fungi, well-known producers of secondary metabolites. Extracts of nine fungi isolated from the green alga *Flabellia petiolata,* collected from the Mediterranean Sea, resulted to be active against some pathogen bacteria. In particular, the chemical profiling of three marine fungi, *Beauveria bassiana* (MUT 4865), *Knufia petricola* (MUT 4979) and a new fungal species (MUT 4861) expressed a high compounds variability, novelty and activity.

The successful results of this project that combines the bioprospecting of hostile environments with the optimisation of the critical step of the “biodiscovery pipeline” confirmed the huge potential of microorganisms as producers of novel bioactive compounds and demonstrated that this research field is far from to be fully exploited.

## Riassunto

Il principale obiettivo di questa ricerca è la scoperta di nuovi composti bioattivi da macro e microrganismi marini. In particolare, considerando la preoccupante diffusione di batteri "Multidrug Resistant" (MDR), cioè resistenti alla maggior parte degli antibiotici presenti attualmente sul mercato, negli ultimi anni è nata l'esigenza di scoprire nuovi composti ad attività antimicrobica. Il problema ha proporzioni mondiali aumentando il numero di decessi ed i costi per la sanità pubblica; esso non è limitato ai soli batteri patogeni, ma anche a funghi, virus e parassiti. Recenti studi hanno evidenziato il potenziale di batteri e funghi isolati da ambienti estremi e da fonti marine come risorsa di nuovi composti bioattivi. Le motivazioni di questa scelta risiedono nell'evidenza che questi microrganismi devono affrontare condizioni molto dure per la sopravvivenza (alte pressioni, scarsità di nutrienti e di luce, temperature estreme) e l'elevata pressione selettiva può aver portato alla sintesi di diversi composti con potenziale antimicrobico e di notevole interesse biotecnologico e farmaceutico. Uno dei maggiori problemi che si riscontra nella lotta ai batteri multi-resistenti è la difficoltà di scoprire antibiotici che non siano già stati studiati. In particolare, l'uso di tecniche di isolamento tradizionali porta all'identificazione di microrganismi comuni, i quali, con alta probabilità, produrranno molecole note. Una possibile soluzione potrebbe essere l'isolamento di microrganismi nuovi e quindi poco studiati per la produzione di composti antimicrobici. Il principale "gap" però è rappresentato dalla "non-coltivabilità" *in vitro* della maggior parte delle specie microbiche esistenti che, quando estrapolati dal proprio ambiente naturale, non riescono a crescere nelle condizioni riprodotte in laboratorio. Sono quindi necessari diversi approcci per l'isolamento di nuovi microrganismi come fonte di nuove molecole bioattive. L'accesso alla vera biodiversità microbica potrebbe aprire la strada a nuove importanti scoperte in campo scientifico.

**L'obiettivo di questo progetto di ricerca** è stato l'isolamento di nuove molecole ad azione antimicrobica impiegando diverse strategie. Nella prima è stato sviluppato e ottimizzato un processo (Drug-discovery pipeline) che comprende la raccolta dei campioni ambientali da ambienti estremi, l'isolamento dei microrganismi, lo screening per la bioattività, l'estrazione, la purificazione e l'identificazione di molecole ad attività antibiotica ed interessanti dal punto di vista biotecnologico. Oltre alla ricerca in ambienti estremi, applicando nuove metodologie di isolamento e screening dei microrganismi, è stato possibile selezionare batteri poco studiati per la produzione di composti bioattivi (antimicrobici e antiparassitari). Oggetto della ricerca sono stati anche i funghi marini notoriamente noti come produttori di metaboliti secondari di interesse industriale.


## Capitolo 1. Batteri da ambienti estremi: una risorsa di molecole antimicrobiche

Nel primo capitolo, sono stati utilizzati sedimenti marini antartici per l'isolamento di microrganismi. Per esplorare la biodiversità presente nei campioni, i sedimenti raccolti sono stati diluiti in quattro terreni di coltura differenti, sia in liquido sia in solido, e incubati per un tempo massimo di 45 giorni. Dopo il periodo di incubazione le colonie sono state raccolte usando stuzzicadenti sterili, aggiunte singolarmente in una piastra multi-pozzetto e incubate a 20 ° C per 10 giorni in agitazione (220 rpm). Tutte le colonie isolate sono poi state conservate a -80 °C con l'aggiunta di glicerolo.

Con l'obiettivo di selezionare microrganismi produttori di composti antimicrobici è stato eseguito uno screening primario mediante esperimenti di cross-streaking. Questo metodo permette di valutare l'attività antimicrobica di un ceppo batterico definito "tester" (nuovi isolati) contro un panel di batteri "target" (batteri resistenti agli antibiotici). Il ceppo "tester" viene strisciato su una metà di una piastra Petri contenete un terreno di coltura solido e incubato per 5 giorni ad una determinata temperatura. Durante il periodo di incubazione il batterio raggiungerà una fase stazionaria in cui produrrà e diffonderà all'interno del terreno di coltura le sue molecole, tra cui i metaboliti secondari. Al termine del periodo di incubazione i batteri "target" vengono strisciati perpendicolarmente sull'altra metà della piastra e incubati per 2 giorni a 37 °C (temperatura ottimale per i ceppi "target"). Alla fine verrà valutato il livello di crescita dei batteri "target". La mancata crescita di uno o più batteri indicherà che il ceppo "tester" ha prodotto molecole in grado di inibirne la crescita. In questo lavoro il cross-streaking è stato ottimizzato per lo screening di batteri marini i quali non sono in grado di crescere sullo stesso mezzo di coltura dei batteri "target". Per far fronte quindi a questo limite è stata messa a punto un'unica piastra Petri contenente due terreni di coltura differenti, uno adatto per la crescita di batteri marini e l'altro adatto per i batteri patogeni. Una volta selezionati potenziali produttori di molecole bioattive, questi batteri sono stati cresciuti in liquido variando il mezzo di coltura e altre condizioni di crescita per stimolare la produzione di composti antimicrobici. I mezzi di coltura esausti sono stati poi estratti con solventi organici al fine di creare una libreria di estratti da analizzare attraverso appositi saggi contro i target stabiliti. L'attività antimicrobica è stata valutata mediante saggi di inibizione in liquido contro batteri multiresistenti calcolando la minima concentrazione inibente (MIC). Gli estratti positivi per l'attività antimicrobica sono stati poi prodotti in larga scala per effettuare una purificazione. Al fine di ottenere composti puri e identificare le molecole biologicamente attive, gli estratti sono stati frazionati applicando una procedura di estrazione in fase solida (SPE), le frazioni sono poi state saggiate per la loro attività e quelle attive ulteriormente purificate tramite HPLC (cromatografia liquida ad alta prestazione). L'ultimo step ha riguardato la determinazione della struttura dei composti tramite l'utilizzo di NMR (Risonanza Magnetica Nucleare) e spettrometria di massa. Inoltre una nuova tecnica di co-coltivazione di microrganismi è stata utilizzata al fine di indurre l'espressione di geni silenti riportando promettenti risultati.

# Risultati conseguiti

Circa 200 batteri antartici sono stati isolati e conservati in glicerolo alla temperatura di -80 °C. Una parte di questi batteri è stata sottoposta ad uno screening preliminare mediante il metodo del "Cross-streaking". Questo metodo qualitativo permette di osservare interazioni antagoniste tra un ceppo "tester" e diversi ceppi "target". Mediante questo metodo due batteri identificati come *Pseudomonas gessardii* C5 e *Pseudomonas fluorescens* T28 hanno mostrato un'attività antimicrobica contro alcuni patogeni target. Il ceppo *P. gessardii C5* è stato selezionato per successivi studi in liquido. In particolare, questo batterio è stato inoculato in terreno liquido e dopo 5 giorni di incubazione il materiale extracellulare è stato estratto mediante etile acetato, evaporato utilizzando un rotavapor e l'estratto così generato è stato impiegato per saggi di inibizione in liquido contro un panel di patogeni più ampio. Per questi saggi, i batteri patogeni sono stati incubati in piastre multiwell da 96 pozzetti in presenza dei vari estratti a diverse concentrazioni e la loro crescita è stata valutata misurando

l’assorbanza a 600 nm dopo 24 ore di incubazione a 37°C. L’estratto prodotto da *P. gessardii* C5 ha mostrato una potente attività antimicrobica contro diversi batteri target, in particolare l’attività più elevata è stata rilevata contro batteri Gram-positivi. Questo estratto è stato poi sottoposto ad una procedura di purificazione guidata dalla bioattività, mediante un frazionamento preliminare utilizzando colonne C18 SPE reverse phase che ha permesso di individuare la frazione attiva, la quale è stata ulteriormente purificata mediante HPLC. Analisi chimiche di NMR e spettrometria di massa hanno permesso di identificare le molecole attive. Si tratta di ramnolipidi, glicolipidi molto noti come biosurfattanti, ma con una potente attività antimicrobica. I ramnolipidi trovano applicazione in diversi settori industriali. Vengono utilizzati per il biorisanamento, nei detergenti e negli ultimi anni la loro attività antimicrobica, specialmente contro batteri Gram-positivi ha attirato l’attenzione delle aziende farmaceutiche. Al fine di incrementare la produzione di ramnolipidi o di indurre l’espressione di altri metaboliti, un particolare sistema di co-coltivazione è stato applicato. Quattro batteri diversi, ma isolati dallo stesso ambiente sono stati coltivati in colture miste e gli estratti ricavati sono stati valutati per la loro capacità di inibire la crescita di batteri resistenti agli antibiotici. Uno dei batteri selezionati ha mostrato una spiccata attività antimicrobica quando cresciuto in presenza di altri batteri rispetto alla coltura pura. Inducendo cambiamenti nell’espressione genica, questa tecnica potrebbe permettere l’isolamento di nuove sostanze da parte di batteri e funghi.

## Capitolo 2. Isolamento mediante “Miniaturized Culture Chip” di un batterio Antartico *Aequorivita* sp. come fonte di nuovi composti bioattivi

Nel secondo capitolo è stato impiegato un nuovo approccio per l’isolamento di microrganismi mediante l’utilizzo del Miniaturized culture Chip (MCC). Il numero di specie batteriche esistenti va da $10^7$ a $10^9$, ma la maggior parte non è mai stata osservata o coltivata. La metagenomica ha dimostrato l'enorme ricchezza della biodiversità nel mondo microbico. La "sfida dei microrganismi incoltivabili" è un argomento che coinvolge gli scienziati da molti anni. Le ragioni dell'incoltivabilità sono diverse e molte ancora sono sconosciute. È evidente che alcuni batteri richiedono requisiti specifici per la crescita, presenti solo nel loro habitat, come la presenza di alcune comunità microbiche o la necessità di specifiche caratteristiche fisiche o chimiche dell'ambiente naturale (luce, ossigeno, pH, pressione, temperatura ecc.). Tra le strategie per la coltivazione di microrganismi nuovi o rari, la simulazione *in vitro* dell'ambiente naturale è sicuramente una delle più promettenti. L’innovazione portata dal MCC è proprio la possibilità di crescere i microrganismi direttamente nel proprio habitat naturale. L’MCC è un sottile chip di alluminio formato da migliaia di micro-pozzetti che possono alloggiare microrganismi diversi. Utilizzando una comune piastra Petri il chip viene posto direttamente sui sedimenti raccolti dall’ambiente, poi una soluzione degli stessi sedimenti viene diluita sulla superficie del chip e il tutto è incubato per permettere la crescita dei microrganismi, i quali per poter crescere dovranno necessariamente sfruttare i nutrienti provenienti dai sedimenti naturali e avranno la possibilità di comunicare con i microorganismi presenti all’interno dei sedimenti stessi su cui il chip è adagiato. L'uso di dispositivi innovativi in grado di riprodurre condizioni naturali aumenta la possibilità di coltivare diverse specie microbiche le quali possono essere impiegati nel settore farmaceutico e biotecnologico.

## Risultati conseguiti

Mediante l'utilizzo del MCC è stato possibile isolare 19 microrganismi da sedimenti Antartici. Le colonie, ben visibili mediante un microscopio a bassa risoluzione, sono state raccolte e utilizzate per analisi filogenetiche e microbiologiche. Diverse specie di batteri sono state isolate, evidenziando quanto sia fondamentale l'utilizzo di sedimenti/campioni naturali all'interno dei terreni di coltura formulati per l'isolamento. Tra tutti i batteri identificati l'attenzione è stata focalizzata sull'isolato *Aequorivita* sp. Oltre ad articoli riguardo la tassonomia di questo genere non sono presenti in letteratura altre informazioni, soprattutto riguardo la produzione di composti bioattivi da parte di questi batteri. Per prima cosa, in collaborazione con l'istituto Pasteur di Parigi il ceppo in esame è stato depositato nella loro collezione e ne è stato sequenziato il genoma completo. Quindi su *Aequorivita* sp. e altri batteri appartenenti al genere *Aequorivita,* depositati all'istituto Pasteur, è stata applicato un approccio di "Genome mining" identificando tutti i principali cluster biosintetici (indipendentemente dalla loro effettiva espressione) presenti nel genoma di questi batteri. Successivamente è stata valutata la capacità di inibire batteri resistenti agli antibiotici mediante saggi di inibizione in liquido e *Caenorhabditis elegans* come modello per trovare composti antiparassitari. I risultati hanno mostrato una promettente attività antimicrobica, soprattutto verso *S. aureus* meticillina resistente (MRSA) e antiparassitaria dell'estratto intracellulare (ottenuto distruggendo meccanicamente le cellule ed estraendo mediante acetato di etile) e la totale assenza di attività da parte dell'estratto extracellulare (ottenuto estraendo direttamente il brodo esausto mediante acetato di etile). Lo "scale-up" della coltura ha permesso di ottenere materiale sufficiente per la procedura di purificazione. L'uso di NMR e spettrometria di massa ha permesso l'identificazione dei composti attivi. In particolare sono stati identificati 8 amminolipidi di cui 5 noti e 3 non riportati in letteratura. Negli ultimi anni l'interesse per questa classe di molecole ad azione antimicrobica ha riscontrato un notevole incremento perché meno suscettibili all'insorgenza di resistenza da parte di batteri patogeni. L'approccio utilizzato in questo lavoro, combinando la ricerca in ambienti estremi con nuovi metodi per l'isolamento di nuove specie batteriche ha permesso l'identificazione di nuovi prodotti di possibile interesse industriale e farmaceutico e l'esplorazione di ulteriori approcci che uniranno tecniche omiche e microbiologiche potrebbero migliorare i metodi di coltivazione attuali risultando nella scoperta di nuove molecole bioattive.

## Capitolo 3. Analisi del potenziale antimicrobico di funghi marini isolati da alghe per contrastare i batteri multi-resistenti: diversità filogenetica e caratterizzazione chimica.

Nel terzo capitolo è riportata l'analisi del potenziale antimicrobico di alcuni funghi marini. Questi ultimi rappresentano un'importante fonte di metaboliti secondari. Si stima che il potenziale bioattivo di questi organismi sia notevolmente sottovalutato in quanto molto spesso i geni codificanti per composti bioattivi sono silenti e quindi non espressi in determinate condizioni. I funghi in esame sono stati isolati dall'alga verde *Flabellia petiolata* raccolta nel Mar Mediterraneo. Diversi terreni di coltura sono stati utilizzati per la crescita dei funghi in modo da selezionare le migliori condizioni di crescita ed eventualmente, di produzione. Acetato di etile ed acetone sono stati impiegati per effettuare le estrazioni dei brodi esausti e dei miceli preceduti da un

trattamento con azoto liquido necessario per rompere la parete cellulare. Tutti gli estratti sono poi stati aggiunti alle piastre contenenti i batteri target calcolando la minima concentrazione inibente. Gli estratti positivi sono poi stati sottoposti ad un'analisi chimica che ha permesso l'identificazione di diverse molecole attive.

## Risultati conseguiti

I funghi sono stati selezionati in base alla presenza di geni codificanti per polichetide sintasi (PKS) e peptidi non-ribosomiali sintasi (NRPS). I funghi isolati (classificati con il codice MUT, in quanto appartengono alla Mycoteca Universitatis Turinensis) sono stati identificati a livello molecolare e assegnati alle classi Dothideomycetes, Sordariomycetes e Eurotiomycetes. Al fine di selezionare i migliori mezzi di crescita per la produzione di composti antimicrobici, sono state eseguite estrazioni preliminari e saggi antimicrobici su colture fungine in piccola scala coltivate in diversi mezzi di crescita. Queste analisi hanno dimostrato che il MeCl (20 g di estratto di malto, 17 g di NaCl) era il mezzo migliore per l'espressione dei composti bioattivi e pertanto è stato scelto per i successivi esperimenti. Inoltre, sono stati confrontati i potenziali antimicrobici degli estratti extracellulari e intracellulari e i risultati hanno mostrato una maggiore resa e attività di quest'ultimi. Partendo da questi risultati, gli estratti sono stati usati per lo screening antimicrobico contro un gruppo di batteri patogeni umani. I più attivi e promettenti erano MUT 4861 appartenente alla famiglia Microascaceae, MUT 4865, identificato come *Beauveria bassiana* e MUT 4979 identificato come *Knufia petricola*. In particolare, gli estratti in acetato di etile prodotti da MUT 4861 e MUT 4865 sono stati in grado di inibire l'intero gruppo di patogeni, con una percentuale di inibizione superiore al 50%. Inoltre, MUT 4861 è stato in grado di inibire fortemente *B. metallica* e *P. aeruginosa* con inibizione del 92% e 86% rispettivamente, mentre MUT 4865 ha mostrato una forte attività verso *B. metallica* (100%) e *S. aureus* (86%). L'estratto del MUT 4979 ha mostrato un'elevata attività (> 80% di inibizione) contro 3 dei quattro patogeni, con l'unica eccezione di *K. pneumoniae*. Una completa analisi del profilo chimico degli estratti più promettenti ha permesso l'identificazione di nuove molecole bioattive ad azione antimicrobica. MUT 4865 è una specie ben nota per la produzione di metaboliti secondari e nonostante ne siano stati isolati molti composti bioattivi, continua ad essere una notevole fonte di nuove molecole dimostrando una notevole variabilità genetica. Per quanto riguarda MUT 4861 e 4979 questo è il primo lavoro che ne evidenzia il ruolo di produttori di molecole antimicrobiche.

# General Introduction

## 1. The antibiotic resistance crisis

"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body...and by exposing his microbes to non-lethal quantities of the drug make them resistant". Already in 1945, during his Nobel Prize lecture, Alexander Fleming pronounced these words and warned of the danger of resistance. Since this wise "prediction" the worldwide population successfully faced infectious diseases discovering many new antibiotics with a different mode of actions. Until the sixties, the world was in what is called "Golden Age" of antibiotic discovery. Moreover, by using chemical synthesis the number of new molecules exponentially increased, modifying and optimising natural products [1]. Antibiotics have successfully contributing to improve clinical surgery preventing or treating infections that can occur in patients who are receiving chemotherapy treatments; who is affected by chronic diseases such as diabetes, end-stage renal disease, or rheumatoid arthritis; or who have had complex surgeries such as organ transplants, joint replacements, or cardiac surgery [2-4]. For more than 20 years' human being hold and used these powerful molecules to defeat bacteria, but then, between the 1960s and 2000, no new major classes of antibiotics were discovered (**Fig. 1**) and furthermore, pharmaceutical companies reduced microbial screening programs (one of the best source of bioactive compounds) due to their declining productivity and challenging regulatory barriers [5, 6]. The development of new antimicrobial agents had become a more complex, costly and lengthy process. On an average, research and development of anti-infective drugs take around 15-20 years and can cost more than 1 billion dollars [7]. The politics of the regulatory authorities, like the US Food and Drug Administration (FDA) have also contributed to the problem by failing to approve drugs endowed with non-inferior properties.

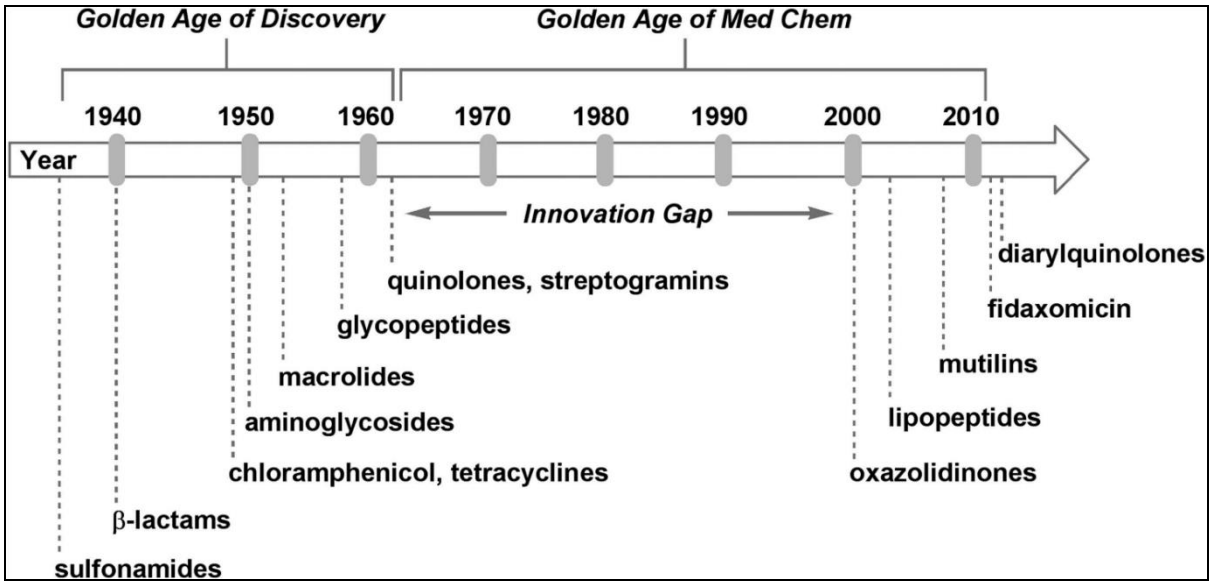


**Figure 1:** The timeline shows the 'Golden Age' of antibiotic discovery (1940–1960) and the 'Golden Age' of antibiotic medicinal chemistry (from 1960 to present). No new structural classes of antibiotics were introduced between 1962 and 2000, representing a serious innovation gap during the genomic era.

After this period defined "Innovation gap" few new drugs were developed, but microorganisms exploited their flexible metabolic power to adapt to existing drugs. It is not surprising that many bacteria became resistant to antibiotics because in billion

years of evolution they developed different strategies for survival, but what is alarming is their capability to acquire resistance in a very short time. Resistance to antibiotics was reported a few years later their approval as showed in **Table 1**. For example, Linezolid approved in 1999 and used for the treatment of infections caused by Gram-positive bacteria registered a *Staphylococcus aureus* resistant strain in the same year [8]. The same happened with Fidaxomicin the first in a new class of narrow spectrum macrocyclic antibiotic drugs approved in 2011 and active especially against clostridia [9].

| *Antibiotic* | *Year deployed* | *Clinical resistance observed* | *Ref.* |
|---|---|---|---|
| **Sulfonamides** | **1930s** | **1940s** | **[10]** |
| **Penicillin** | **1943** | **1946** | **[10]** |
| **Streptomycin** | **1943** | **1959** | **[10]** |
| **Chloramphenicol** | **1947** | **1959** | **[10]** |
| **Tetracycline** | **1948** | **1953** | **[10]** |
| **Erythromycin** | **1952** | **1988** | **[10]** |
| **Vancomycin** | **1956** | **1988** | **[10]** |
| **Methicillin** | **1960** | **1961** | **[10]** |
| **Ampicillin** | **1961** | **1973** | **[10]** |
| **Cephalosporins** | **1960s** | **Late 1960s** | **[10]** |
| **Nalidixic acid** | **1962** | **1962** | **[11]** |
| **Fluoroquinolones** | **1980s** | **1980s** | **[12]** |
| **Linezolid** | **1999** | **1999** | **[8]** |
| **Daptomycin** | **2003** | **2003** | **[13]** |
| **Retapamulin** | **2007** | **2007** | **[14]** |
| **Fidaxomicin** | **2011** | **2011** | **[9]** |
| **Bedaquiline** | **2013** | **2013** | **[15]** |
| **Ceftazidime/avibactam** | **2015** | **2015** | **[16]** |

**Table 1:** Evolution of resistance to clinical antibiotics

One of the explanations for this phenomenon is the uncontrolled use of antimicrobial agents and the adaptation strategies of microbes. The overuse of antibiotics and many times the incorrect prescription of them contribute to the developing of resistance [17, 18]. A huge amount of antibiotics (80% in the U.S. according to estimations) is used in livestock to promote growth and to prevent infections and this significantly contributes the developing of some resistances [6, 19]. Moreover, in the bacterial world, the horizontal gene transfer allows the exchange of genetic material (plasmids carrying genes for antibiotics resistance) intra and inter-species conferring resistance to many classes of antibiotics [18]. In addition to acquired resistance, microorganisms can be intrinsically resistant to different molecules [20].

The selective pressure caused by the use of antibiotics led to the development of a new class of bacteria, Multidrug Resistant (MDR) bacteria. Most of them are resistant to at least three classes of antibiotics and represent a serious threat to public health [21]. Today, more than 70% of pathogenic bacteria are resistant to most antibiotics on the market, and the frequency of multi-drug resistance in the community have extended the resistance problem beyond the confines of the hospital [22]. Reappearing “old pathogens” and new re-emerging opportunistic pathogens could

potentially recreate the pre-antibiotic era. So diseases and disease agents that were once thought to be controlled by antibiotics are returning in new leagues resistant to these therapies [22, 23]. Recently, February 2017, World Health Organization (WHO) published a list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria that pose the greatest threat to human health (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/). The list was drawn up in a bid to guide and promote research and development (R&D) of new antibiotics, as part of WHO's efforts to address growing global resistance to antimicrobial medicines. Some of the most problematic MDR organisms include genera like *Acinetobacter*, *Pseudomonas, Enterobacter, Staphylococcus* and others. These MDR bacteria can cause severe and often deadly infections and they are very often found in hospitals [24]. The issue of MDR bacteria has global dimensions, but poor life condition of developing and underdeveloped countries aggravates this problem. Resistance to common drugs is not limited to bacteria but also to fungi, viruses and especially parasites. Among parasites, a prominent role is played by nematodes. Gastrointestinal nematodes, such as the blood-sucking *Haemonchus contortus*, are major parasites of ruminants that cause substantial economic losses to livestock production worldwide. Inevitably, drug resistance has emerged in human and livestock pathogenic helminths against each class of antihelmintic compounds [25].
The "never-ending story" between antibiotics and resistant organisms cannot be stopped, but understanding the mechanisms of resistance and focusing the attention on unexplored areas, we can increase the possibilities to discover new effective drugs.

## 1.1 Mechanisms of antibiotic resistance

Bacteria were one of the first life forms appearing on Earth, about 3.8 billion years ago and if they survived until now, it is due to their ability to adapt to the external world, fighting for food and evolving strategies to resist to antimicrobial agents produced by competitors. Recently, many bacteria become resistant to most of the antibiotics released on the market employing several mechanisms in attaining multidrug resistance. Resistance obviously has a genetic basis: it can be intrinsic, via mutation of an endogenous chromosomal gene, or acquired by incorporation of the foreign genetic material into their chromosome by conjugation, transduction, and transformation [26, 27]. A brief description of main mechanisms for antibiotic resistance in bacteria are summarised below [28] and showed in **Figure 2**;
**Impermeability:** antibiotics with intracellular target have to penetrate outer and/or cytoplasmic membrane in order to exert their function. Bacteria can decrease the uptake of antimicrobial compounds by membrane molecules, such as lipopolysaccharide (LPS) [29]. Especially in Gram-negative bacteria, changes in surface hydrophobicity, outer membrane ultrastructure, outer membrane protein composition, and change in outer membrane fatty acid composition, all changes that decrease the permeability of membrane barriers.
**Efflux systems pump:** drug efflux systems pump out a broad range of chemically and structurally unrelated compounds from bacteria in an energy-dependent manner, without drug alteration or degradation. This mechanism was first described for tetracycline in the late 1970s [30].
**Target alteration:** through this mechanism antibiotic are not able to bind the target. In some cases, the binding site is protected by other molecules, like in the case of tetracycline resistance [31]. Other mechanisms include the modification of target site

by point mutations, enzymatic alterations of the binding site (e.g. addition of methyl groups), and/or replacement or bypass of the original target.

**Antibiotic modification or destruction:** bacteria evolved the capability to produce enzymes able to inactivate or degrade the antibiotic itself. One of the best examples of resistance via modification of the drug is the presence of aminoglycoside remodelling enzymes (AMEs) that covalently modify the hydroxyl or amino groups of the aminoglycoside molecule, while enzymes like β-lactamases destroy the amide bond of the β-lactam ring, rendering the antimicrobial ineffective [32].

**Resistance due to global cell adaptations:** complex mechanisms are used by bacteria to regulate cell wall synthesis and membrane homeostasis. Although the exact mechanism mediating this phenomenon has not been fully elucidated, it appears to involve alterations in cell wall metabolism that results in changes in surface charge producing electrostatic repulsion and avoiding the interaction with particular charged antibiotic molecules such as Daptomycin [33].

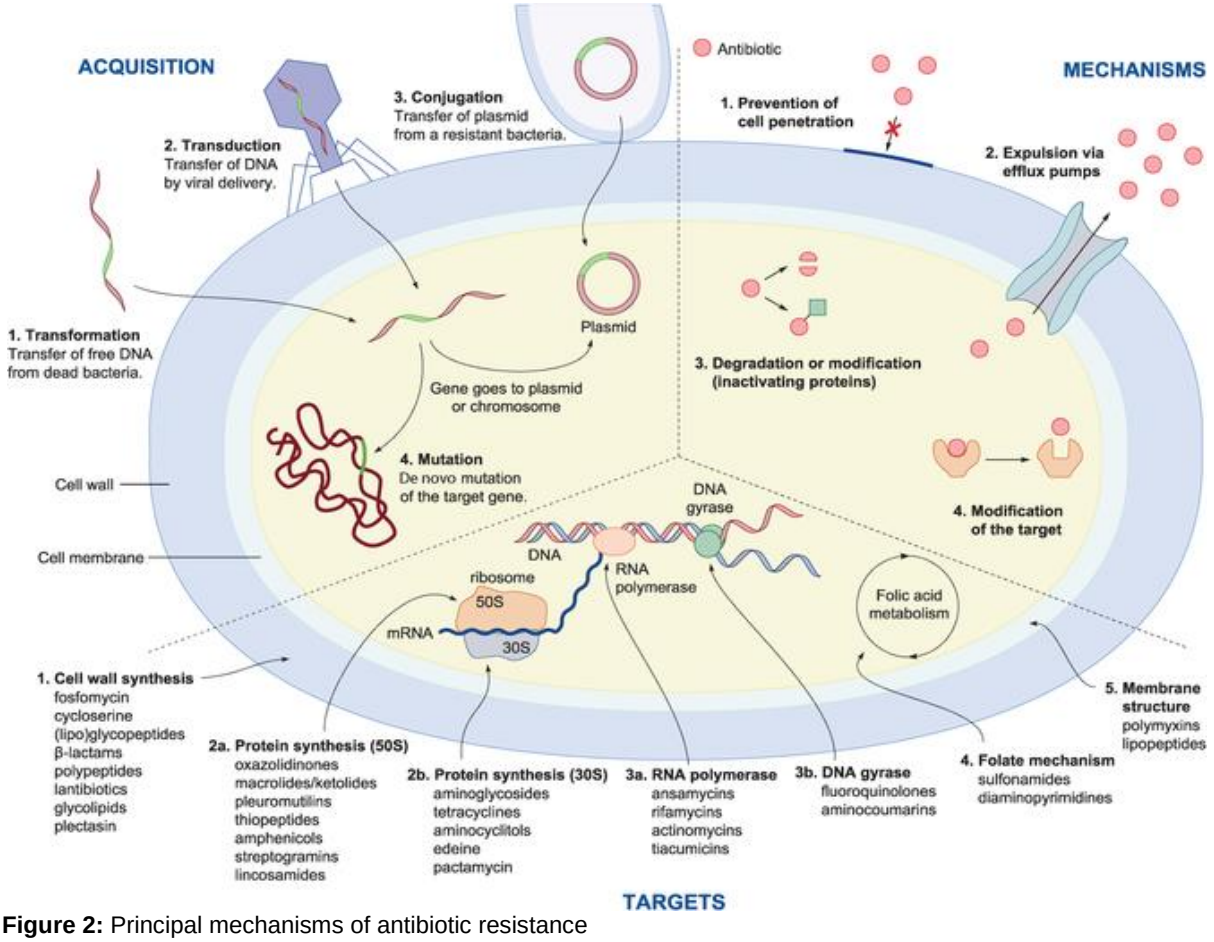


**Figure 2:** Principal mechanisms of antibiotic resistance

Despite the presence of some mechanisms of resistance, molecules targeting bacterial membrane still seems to be the best choice to face the antibiotic resistance. One emerging class of compounds, called AMLPs (antimicrobial lipopeptides), shows promise features that make these molecules good candidates for drug development. In particular, they are less vulnerable to evolved resistance because they disrupt the structure and function of microbial membranes. Microbes, to evolve drug resistance, would require many big changes to alter the mixture of lipids composing the membrane. AMLPs are small and stable lipophilic molecules and could represent a promising alternative to traditional antibiotics. Past studies have shown that these

synthetic compounds have potent activity against a range of pathogens and can clear infections in mice [34].

## 2. Bioprospecting of natural products from marine and extreme environments

The concomitant relentless increase in antibiotic resistance infections, coupled with the failure to find new antibiotics, signals a return to the pre-antibiotic era. To counteract this phenomenon, different strategies are needed. The bioprospecting of unexplored environments like extreme and marine habitat could lead to the discovery of new Natural Products (NP). NP represent the largest source of new antibiotic molecules, covering about two-thirds of new antibacterial therapies approved between 1980 and 2010 [35, 36]. Thinking about extreme habitat, Antarctica is one of the most extreme places on Earth. Although most of the continent is covered by glacial ice sheets, ice-free areas comprising approximately 0.4% of the continental land mass are discontinuously distributed around the coastal margins. Some areas are polar deserts characterized by extremely low annual precipitation (<100 mm) and an absence of vascular plants and vertebrates; most of the biological activity is limited to the top four or five inches by the permanently frozen ground below. Mean summer high and low winter temperatures in the dry valleys are −5 °C and −30 °C [37, 38]. There is a high level of UV exposure and long periods of light/dark. The isolated and unique nature of Antarctica attracted the attention of scientists. The reasons are obvious; in comparison to many over-explored tropical areas, Antarctica and the ocean that surrounds it is home to a large number of unknown organisms adapted to the “extreme cold and conditions”. These “extremophiles” exhibit physical and chemical adaptations not found elsewhere on the planet [39]. As a fact, some Antarctic places are studied for the exploration of Extra-terrestrial life because of its peculiar environment [40]. In some way, marine environments could be considered as an extreme habitat. They include a wide range of temperature, salinity, light and pressure encountered by life. Within the oceans, habitats can range from tropical sunlight surface waters to ocean trenches with 110 MPa pressure at 11 km below sea level. Furthermore, temperatures in the ocean can be over 350 ºC in pressurised fluids in hydrothermal vents and as low as -35 ºC in channels within the sea ice. If we consider the vastness of the aquatic environment, it is possible to appreciate the great importance of focusing scientific efforts on marine bioprospecting to look for new molecules. Although the diversity of life in the terrestrial environment is extraordinary, marine environment is estimated to be the richest source of biodiversity in the world [41]. In recent years, bioprospecting applied to marine and extreme environments have led to the development of a wide variety of natural compounds, especially secondary metabolites with unique biological activities [42] and high potential for biotechnological and pharmaceutical applications. Furthermore, areas beyond the limits of national jurisdiction, such as open seas and Antarctica, are still not strictly regulated by Nagoya Protocol (which aims at sharing the benefits arising from the utilization of genetic resources in a fair and equitable way) [43] and it makes less complicated the access to the biological resources. From 2001 to 2016, a vast amount of new biological natural compounds with various activities, such as antibacterial, antitumor, antiviral and so on, have been isolated from polar and marine organisms including microorganisms, lichen, moss, bryozoans, cnidarians, echinoderms, molluscs, sponges and tunicates [44]. Several compounds showing the same bioactivity were discovered in different places in the marine environment [45-47]. For example, anthracimycin, a new antibiotic produced by a marine-derived

actinomycete has been discovered [48]. This molecule shows significant activity against *Bacillus anthracis* and several Gram-negative bacteria. Another antimicrobial compound named Exophilin A, which inhibits the growth of Gram-positive bacteria has been found from a marine fungus [49]. One of the best sources of NP remain microorganisms, including bacteria and fungi and going to unexplored areas could increase the chances to discover different species able to produce novel bioactive compounds.

## 2.1 Antarctic bacteria

Bacteria able to grow at low temperatures are classified as psychrophiles, which can grow at temperatures not exceeding approx. 15 °C and psychrotrophs (or psychrotolerants) that tolerate a broader range of temperatures, between 15 °C and 30°C [50]. Microorganisms evolved different mechanisms which permit they to tolerate extremely low temperatures. These mechanisms include the increasing of membranes fluidity, the ability to accumulate compatible solutes (e.g. glycine, betaine and trehalose), the expression of cold shock, antifreeze and ice-nucleating proteins, as well as the production of cold-active enzymes [50-52]. Studies have shown that Antarctic bacteria are an incredibly rich source of unique enzymes, proteins and other bioactive compounds that can benefit industry [53]. More attention has been focused on cold-adapted enzymes, but recently several new NPs showing antimicrobial activity have been isolated from Antarctic bacteria. An intracellular biomolecule similar to anthraquinone and indane derivatives of a diterpenoid isolated from an Antarctic cyanobacterium showed antibacterial activity towards Gram-positive *Mycobacterium tuberculosis*, *S. aureus*, Gram-negative *Salmonella typhi*, *Pseudomonas aeruginosa* and three MDR strains of *Escherichia coli* [54]. Two pigments named violacein and flexirubin were isolated from two Antarctic bacterial strains. The two compounds displayed antibacterial activities against some mycobacteria with low MIC values (ranging from 2.6 to 34.4 µg/mL) and might be valuable natural lead compounds for new antimycobacterial drugs used for tuberculosis chemotherapy [55]. Two new rhamnolipids showing inhibition effects against *Burkholderia spp*. and *S. aureus* were isolated from an Antarctic *Pseudomonas sp*. [56].

## 2.2 Marine fungi

From the discovery of the antibiotic cephalosporines in 1948 [57], marine fungi have been considered an excellent source of bioactive compounds and since the 1980s, the number of new antimicrobial compounds discovered from marine fungi is increased. They represent a huge reservoir of biologically active secondary metabolites that are often produced by multifunctional enzyme complexes such as PolyKetide Synthases (PKSs) and Non-Ribosomal Peptide Synthetases (NRPSs). Metabolomic and genomic studies on marine fungi have shown the presence of many unexpressed biosynthesis genes which could encode for unknown metabolites [58]. Several studies by Kong et al. demonstrated the tremendous potential of marine fungi as producers of unknown molecules with novel scaffolds [59], which might be useful for drug discovery [60] but in contrast to bacteria, the basic knowledge of marine fungi, such as distribution and ecological role is still scarce. These promising data encourage the scientific efforts towards the exploitation of the marine fungi and considering that a large part of the marine ecosystem is still unexplored, we can speculate that a huge number of new molecules is waiting to be discovered. Marine fungi have been retrieved from various marine habitats from superficial waters

mangroves, marine algae, and salt marshes (mostly in association with marine invertebrates) until the deep subseafloor. True marine fungi can grow and sporulate exclusively in seawater, where facultative marine fungi are able to adapt away from their natural habitat [61]. The physical factors that influence the marine fungi are a) salinity and pH, b) low water potential, c) high concentration of sodium ions, d) low temperature, e) oligotrophic nutrient conditions and f) high hydrostatic pressure, the last three parameters being unique to the deep-sea environment [61]. In response to their environment, marine fungi can produce a wide range of secondary metabolites. Many reports describe the great antibacterial and antifungal activity of extracts produced by marine fungi [62-64], most which isolated from a marine host exploiting their capability to maintain mutualistic relationships with algae or sponges. An isolate of a *Pestiolata* sp. from the brown alga *Rosenvingea* sp., collected in the Bahamas islands, produced the chlorinated benzophenone pestalone, a compound that showed an inhibition effect against methicillin-resistant *S. aureus* and *Enterococcus faecium* [65]. This compound was then produced by chemical synthesis (not confirming the same level of bioactivity) [66]. Natural compounds sometimes contain minor impurities which are the real responsible for their bioactivity). From the fungal broth of a marine *Aspergillus* species isolated from the surface of the marine brown alga *Sargassum horneri* in Korea yielded a new polyoxygenated decalin derivative, dehydroxychlorofusarielin B, which was found to exhibit antibacterial activity against MDR *S. aureus* [67]. Although linking biological activities to any ecological role remains a difficult task, the amazing range of bioactivities of marine fungi suggests that these secondary metabolites could be defensive compounds and therefore is likely to play important roles in symbiosis and competition with other organisms in their habitats. Most metabolites reported from marine environment are produced by *Aspergillus* and *Penicillium* [68] meaning that they express many genes responsible for the production of antimicrobial compounds, but several times the exploitation of these strains lead to the discovery of well-known metabolites.

## 3. Microbial “uncultivability”

Although the bioprospecting of unexplored environments is a promising strategy to isolate microbial species able to produce unknown bioactive compounds, that is not enough. Most of the time, as a fact, the application of classic isolation techniques leads to the identification of very common microbial species, which produce known molecules. The result is a huge waste of time and money. The point is, that is very difficult to isolate rare or novel strains since they are not cultivable in laboratory conditions. The problem of microbial “uncultivability” is an issue that involves microbiologists for many years. Molecular-based approaches demonstrated that approximately less than 1 % of microorganisms present in an environmental sample is cultivable *in vitro* [69, 70] and it drastically limits scientists' knowledge of microbial life and their derived products. There are several explanations for this phenomenon. Certain bacteria have fastidious growth requirements including the need for specific nutrients, pH conditions, incubation temperatures or levels of oxygen in the atmosphere [71]. Another important factor is the extrapolation of microorganisms from the natural environment, which naturally contains specific nutrients, signal molecules produced by indigenous microbial communities that contribute to building a network of signals essential for the growth of many bacteria [72, 73]. Bacterial communication is an essential factor to retrieve unknown species. Many terms are used to describe microbes that are not able to grow under laboratory conditions, but the “not-yet-cultivated” microorganisms seem the correct definition. They play

important ecological roles in their habitat and sometimes are the most abundant and ubiquitous species [74], but still, lab conditions are not suitable for their growth; so they are just “not-yet-cultivated”. Significant efforts have been made in recent years developing new strategies for the isolation of new strains. The majority of culture media used to date have been nutrient-rich. This condition may favour the growth of fast-growing bacteria at the expense of slow-growing species, some of which are oligotrophic and may be inhibited by substrate-rich conventional media. Consequently, the use of dilute nutrient media and the extension of incubation time has led to the successful cultivation of previously unculturable bacteria from various aquatic and terrestrial habitats [75]. Other strategies include the use of helper strains as a source of growth factors and the simulation of natural environment using innovative devices diffusion chambers and isolation chips [76, 77]. The access to real microbial biodiversity could lead to a new era of biodiscovery with many novel antibiotics discovered and exploited against the emergence of MDR bacteria. The first step in this direction was the isolation by the iChip, a new system composed of microwells and semipermeable membranes, of a new β-proteobacteria provisionally named *Eleftheria terrae* able to produce a new antibiotic, the Teixobactin [78]. This study highlighted the importance of new organisms such as uncultured bacteria as a source of new antimicrobials.

## 4. The unexpressed potential of the genome

Recent advances in DNA sequencing technologies allow the whole genome sequencing in a rapid and cost-effective way. As a consequence, new tools for the genome analysis were developed (BAGEL for the identification of bacteriocins [79], and AntiSMASH for the identification of a wide range of biosynthetic clusters) ranging from PKS and NRPS to siderophores [80]. Genome mining approach (a rapid approach to discover new and novel secondary metabolites for drug discovery using genome data) has revealed that microorganisms contain genes to produce other natural products, but often they are silent or cryptic biosynthetic gene clusters [81, 82]. It means that they are not expressed under certain conditions. The concept of “laboratory conditions” again play a fundamental role in the discovery of new natural products. Applying the right stimuli/strategies/approaches to microbes, the overexpression of some genes or the induction of silent genes will be possible. Various strategies changing the growth conditions can be applied to induce secondary metabolites production. The OSMAC approach (One Strain Many Compounds) and co-cultivation with other organisms gave the best results regarding new bioactive compounds [83, 84]. These different approaches, mainly the co-cultivation, are based on the assumption that, in the laboratory, many microorganisms (bacteria and fungi) are not stimulated enough in comparison with their natural environment where they live in communities and have to fight for the survival. Limitations of these techniques are represented by the selection of the right conditions, but the recent advancements in the HPLC-MS/MS allow the rapid comparison of metabolites profiling and a guided selection of the best condition. The aim is to detect any variation in the microbial metabolism in response to different stress. Finally, thanks to the information obtained by genome mining and metabolomics data the NPs discovery process can be accelerated.

## 5. Marine Biotechnology

Marine (or blue) biotechnology encompasses the sustainable use of marine resources for biotechnological applications addressing global challenges of food, energy, and health. Over the last years, the impact of the marine biotechnology on the bioeconomy is considerably increased. The global market for marine biotechnology products and processes is predicted to reach US$4.8 billion by 2020, rising to US$6.4 billion by 2025 [85]. In Europe, marine biotechnology was identified by the EU Blue Growth Strategy (2012) as an enabling activity of high potential for the bioeconomy. Through EU funding, new consortia and marine infrastructure are born creating new jobs and bringing innovations. It is considered an area of great interest and potential due to the contribution for the building of an eco-sustainable and highly efficient society. Marine biotechnology has a horizontal scope encompassing very different applications. It includes techniques such as bioprocessing, bioharvesting, bioprospecting, bioremediation leading to the discovery and exploitation of new natural products of industrial interest. In particular, drug discovery represents one of the most promising research field [86]. The history of marine drug discovery is rich of molecules that entered in clinical and pre-clinical studies [87] highlighting the importance of investing in this field. An example of successful "blue business" that provides 'proof-of-concept' for exploiting marine chemical diversity in human health is demonstrated by the marine-derived natural product Trabectedin (ET-743; Yondelis®), a potent antitumor originally isolated from the Caribbean Sea squirt, *Ecteinascidia turbinata* and currently prepared synthetically [88]. The relevance and impact of the marine biotechnology on the society and bioeconomy is clear encouraging the development of new processes and technologies aimed to the discovery of new products.

## 6. Aim of the project

The aim of this project was the exploitation of microorganisms as a source of novel bioactive compounds able to counteract the spread of MDR bacteria which represent a serious threat to the public health. The project is divided into three chapters which explore different strategies for the identification of novel antimicrobial compounds:

**Chapter 1:** In this chapter, a "Drug-discovery pipeline" was applied. Classic isolation techniques were used to isolate bacteria from Antarctic sediments. Then a procedure of bioassay-guided purification was used in order to evaluate the antimicrobial potential of the most promising bacteria against a panel of MDR bacteria. Moreover, several strains were selected for co-cultivation experiments with final aim to induce the expression of "silent" bioactive compounds.

**Chapter 2:** In order to discover rare or new microorganisms, an innovative device which allowed to mimic the natural environment was used. By this method an unexplored Antarctic bacterium was isolated, the whole genome was sequenced and new antimicrobial compounds were identified from this strain.

**Chapter 3:** In this chapter, the screening of novel marine fungi, isolated from the green alga *Flabellia petiolata* collected from the Mediterranean Sea, showed the presence of fungal strains endowed with strong inhibitory activity against MDR bacteria. The chemical profiling of the most active strains led to the identification of the bioactive molecules.

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# CHAPTER 1

# Bacteria from the extreme: a source of antimicrobial compounds

# Bacteria from the extreme: a source of antimicrobial compounds


## Abstract

Recently, the uncontrolled use of antibiotics led to the widespread of multidrug resistant (MDR) bacteria becoming a serious threat to human health. The Golden Age of antibiotics comes to an end. There is the pressing need to discover novel effective antimicrobial compounds and the bioprospecting of extreme environments could be a promising strategy. In this work, Antarctic shallow water sediments were used to isolate microorganisms that were screened for their capability to inhibit the growth of selected MDR bacteria. Among the isolates, one belonging to *Pseudomonas* genus showed a high antimicrobial activity against the pathogens panel, especially against Gram-positive bacteria. The purification of molecules responsible for the bioactivity and the structure elucidation by NMR and LC-MS-MS allowed the identification of a family of rhamnolipids, an important class of bioactive compounds with many biological functions of biotechnological and pharmaceutical interest.


## 1.1 Introduction

Antimicrobial resistance has spread dramatically in last 60 years leading to an increase in the number of deaths due to infectious diseases. The excessive and often inappropriate use of antimicrobial drugs has led to the development of a new group of microorganisms, the MDR bacteria, which show resistance toward the most common antibiotics. This phenomenon is becoming a serious threat to public health and the economy [1]. In contrast, the development of new antimicrobial agents has slowed down due to lack of interest in the pharmaceutical industry. Indeed, in the last two decades, only two antibacterial drugs with a new mode of action have been progressed into the market [2]. New strategies are needed to address this problem and slow down the spread of drug resistance. For example, in 2012 FDA has implemented the Generating Antibiotics Incentives Now (GAIN) Act, a provision within the Food and Drug Administration Safety and Innovation Act (FDASIA), to promote the development of new antibacterial and antifungal drugs. The US government has issued “The National Strategy for Combating Antibiotic Resistant Bacteria”, which identifies priorities and coordinates investments to prevent, detect, and control outbreaks of resistant pathogens. Nowadays, infections caused by antibiotic-resistant bacteria, such as the “**ESKAPE**” pathogens (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa,* and ***E****nterobacter* species)*, Burkholderia cepacia* complex (Bcc) and others, are increasing human mortality [3]. Many of these bacteria are also able to produce biofilms that are relevant in a wide range of clinical domains including intensive care medicine, surgery [4] but also other sectors as the food industry [5]. In biofilms, bacteria are quite well protected against antibacterial treatments. Furthermore, biofilms can release harmful toxins and even obstruct indwelling catheters [6]. It is hence obvious that an effective screening program has become an urgent and unmet need for isolation and identification of new molecules. In that sense, bioprospecting of marine and extreme environment, like Antarctica, represents a very promising strategy for the isolation of microorganisms as a source

of new natural drug candidates. Those environments remain largely unexplored in comparison to terrestrial ecosystems and organisms. In order to survive in such environments, microorganisms have accumulated remarkable physiological and functional heterogeneity, and currently, constitute an unlimited reservoir of genetic diversity [7]. The easier access to those environments in recent years has led to the development of a wide variety of marine-derived compounds, especially secondary metabolites with unique biological activities [8] and high potential for biotechnological and pharmaceutical applications. Several antibacterial [9], antifungal [10] and antiviral [11] compounds have been isolated from marine organisms, so far. In this work, the identification of bioactive compounds of biotechnological and pharmaceutical interest was performed following a bioprospecting pipeline.

## 1.2 Materials and Methods

**Isolation of bacterial strains**

In this work, Antarctic shallow water sediments were used for the isolation of microorganisms. To explore the biodiversity present in the samples, two strategies were applied. In the first strategy, 1 g of sediment was mixed with 9 mL of sterile water, homogenized using a vortex, serially diluted ($10^{-1}$, $10^{-2}$ and $10^{-3}$) and each dilution was plated on four different solid growth media and incubated at 4 and 20 °C for 45 days. Incubation times were extended until 45 days to increase the microorganisms number and allow the development of slow-growing strains. For the second strategy, 1 g of the same sediment was added directly to four different liquid media and incubated with agitation at 150 rpm at the same temperature of solid plates. Periodically (after 1, 3, 5, 10, 20, and 45 days) 100 µL of liquid cultures were plated on the respective solid media. This procedure allowed observing the changing in the microbiome during the time. Growth media used for the experiments are listed below.

- Marine Broth (MB): 19.4g NaCl, 8.8g MgCl, 5g Bacteriological Peptone, 3.24g NaSO4, 1.8g CaCl2, 1g Yeast Extract, 0.55g KCl, 0.16g $NaHCO_3$, 0.10g Fe(III) Citrate, 0.08g KBr, 0.034g $SrCl_2$, 0.022g $H_3BO_3$, 0.008g $Na_2HPO_4$, 0.004g Na-Silicate, 0.0024g NaF, 0.0016g $NH_4NO_3$, pH 7.6 at 25°C.
- Tryptic Soy Broth (TSB): 17g Pancreatic Digest of Casein, 3.0g Papaic Digest of Soybean, 5.0g NaCl, 2.5g $K_2HPO_4$, 2.5g Dextrose, pH 7.3
- Glycerol Arginine (GA): 20g Glycerol, 2.5g L-Arginine, 1g NaCl, 0.1g $CaCO_3$, 0.1g $MgSO_4.7H_2O$, 0.1g $FeSO_4.7H_2O$ (added as solution after sterilisation)
- Starch Casein (SC): 10g Soluble Starch, 2g $K_2HPO_4$, 2g $KNO_3$, 2g NaCl, 0.3g Casein, 0.05g MgSO4.7$H_2O$, 0.02g $CaCO_3$, 0.01g $FeSO_4.7H_2O$ (added as solution after sterilisation)
- Luria-Bertani broth (LB): 10 g/L Tryptone, 5 g/L Yeast extract, 10 g/L NaCl. This medium was used for the growth of the pathogen and the scale-up of the antimicrobial producing strain.

The composition of selected media is expressed in grams per Litre of distilled water. Solid media were prepared adding 15 g/L of Agar. 50 mg/L of Nystatin was added to each medium to inhibit the growth of fungi.

After the incubation period, colonies were picked using a sterile wooden toothpick added individually on a 96 multi-well plate and incubated at 20°C for 10 days under agitation (220 rpm). Each well contains 200 µL of liquid growth medium (the same medium used for the isolation). The isolated bacteria were then suspended in glycerol (20% final volume) and stored at -80°C.

**MDR bacteria used as target**

The following strains of human pathogens were used in this work: *Pseudomonas aeruginosa* Pa01, *Staphylococcus aureus* 6538P, *Klebsiella pneumonia* DF12SA, *Acinetobacter baumannii* Ab13, *Propionibacterium acnes* DSM 1897*, Enterococcus faecalis* DSM 20478*, Burkholderia anthina* LMG 20980*, Burkholderia diffusa* LMG 24065 and *Burkholderia metallica* LMG 24068. The strains were routinely grown at 37°C in LB medium.

**Cross streaking**

Preliminary screening for antimicrobial activity was performed by the cross-streaking method. This method allows the observation of antagonistic interactions among a tester strain (newly isolated bacteria) and several target strains (MDR bacteria). 70 µL of overnight bacterial cultures are spread on half plate and incubated for 5 days a 20 °C. During this period, the secondary metabolites produced by the tester strain are able to diffuse into the agar. After 5 days' target strains are streaked perpendicularly the tester strain and the plate is incubated at 20 °C for 1 day and 37 °C for 2 days. To overcome the limitation of the assay that the pathogens and the tester strains are not able to grow on the same medium, a dual media Petri dish was developed. From one side of the plate, the culture medium was poured to allow the growth of Antarctic isolates and the other half of the plate was filled with LB medium, suitable for the growth of MDR bacteria. Then tester and target bacteria were added as described. A control, a plate inoculated with pathogens was also maintained without inoculating the new isolates to assess the normal growth of MDR bacteria.

**Molecular identification and phylogenetic analysis of isolated strains**

The freeze and thaw method was used to obtain bacterial genomic DNA, which was used as a template for the amplification via PCR of 16S rDNA genes. PCR was carried out in a total volume of 50 µL containing DreamTaq PCR Master Mix (a ready-to-use solution containing DreamTaq DNA Polymerase, optimized DreamTaq buffer, MgCl2, and dNTPs) and 1 µM of primer Eub27F (Forward, seq: 5'-AGAGTTTGATCCTGGCTCAG-3') and Univ1492R (Reverse, seq: 5'-GGTTACCTTGTTACGACTT-3') [12]. The reaction conditions used were: one cycle (93 °C for 2 min), 30 cycles (92 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min), with a final extension of 5 min at 72 °C. PCR products were then purified, sequenced and submitted to BLAST for the phylogenetic analysis.

**Extract preparation**

A single colony of a bacterial isolate was used to inoculate 3 mL of liquid SC and LB media in sterile bacteriological tubes. After 48 h of incubation at 20°C at 200 rpm, the pre-inoculum was used to inoculate 125 mL of the same media in 500 mL flasks, at an initial cell concentration of 0.01 $OD_{600}$/mL. The flasks were incubated up to 5 days at 20°C at 200 rpm. The cultures were then centrifuged at 6800 x g at 4°C for 30', and the exhausted culture broths were collected and stored at -20°C. The exhausted culture broths were subjected to organic extraction using 3 volume of ethyl acetate in a 500 mL separatory funnel. The organic phase collected was evaporated using a Laborota 4000 rotary evaporator and the extracts were weighted, dissolved in 100% DMSO at 50 or 100 mg/mL and stored at -20°C. The extract for the scale-up of the culture was obtained using the same protocol increasing the volume up to 3 L.

**Liquid Inhibition assay**

The produced crude extracts were checked for the ability to inhibit the growth of a selected panel of human pathogens using the Minimal Inhibitory Concentration (MIC) assay. The following MDR bacteria were used for the antimicrobial screening: *Burkholderia metallica* LMG 24068, *Pseudomonas aeruginosa* PA01, *Klebsiella pneumoniae* DF12SA, *Acinetobacter baumannii* Ab13 and *Staphylococcus aureus* 6538P. All the bacteria were routinely grown at 37°C in Luria-Bertani broth. The extracts were placed into each well of a 96-well microtiter plate at an initial concentration of 2 mg/mL and serially 2-fold diluted using the appropriate medium. Wells containing no compounds represent the negative control. DMSO (2% v/v) was also used as a control to determine the effect of the solvent on bacterial growth. For the plate inoculum, a single colony of each pathogen strain was used to inoculate 3 mL of liquid medium in sterile bacteriological tubes. After 5-8 h of incubation, growth was measured by monitoring the absorbance at 600 nm and about 40000 CFU were dispensed into each well of the prepared plate. Plates were incubated at 37°C for 20 hours and growth was measured by using a VICTOR X Multilabel Plate Reader (PerkinElmer, Waltham, MA) by monitoring the absorbance at 600 nm.

**Purification of ethyl-acetate crude extract**

The crude extract obtained from the scale-up was subjected to fractionation using Chromabond SPE C18 column cartridges (Macherey-Nagel, Duren, Germany) and selectively eluted with different percentages of a methanol-water system. Five fractions were obtained: 25%, 50%, 75%, 100%, and 100% methanol with 0,04% TFA. Each fraction was dried, weighted and assays were carried out to determine the most active fraction. The active fraction was further purified by reverse-phase HPLC on a C18 column. HPLC separations were carried out using a 5µm Nucleodur reversed-phase HTec (C18, 250/10 mm, L × i.d.) column connected to an UltiMate 3000 series pump and monitored using an UltiMate photodiode array detector. Detection was carried out at 220, 254, 280 and 320nm. The gradient was ran using Buffer A (95% H2O, 5% Acetonitrile+0.04% TFA and Buffer B 5% $H_2O$ 95% Acetonitrile+0.04% TFA) at a flow rate of 3.00mL/min. Used gradient as described in **Table 1**.

| Time (min) | Flow rate (mL/min) | % of mobile phase A[a] | % of mobile phase B[b] |
|---|---|---|---|
| Initial | 3.0 | 100 | 0 |
| 7.00 | 3.0 | 80 | 20 |
| 28.00 | 3.0 | 60 | 40 |
| 35.00 | 3.0 | 40 | 60 |
| 42.00 | 3.0 | 0 | 100 |
| 56.00 | 3.0 | 0 | 100 |

**Table 1:** HPLC gradient used for the purification of antimicrobial compounds

**Identification of the antimicrobial compounds**

Isolated molecules were chemically characterized by a combination of by 1D and 2D omo- and heteronuclear NMR spectroscopy and by HRESIMS analysis in collaboration with the Department of Pharmacy of Federico II University in Naples.

**Co-cultivation experiment**
For this experiments, two special 500mL bottles were used. Each bottle has a glass neck by which is possible to make a connection with the other one. The necks are separated by a 0.22 µm filter that allowed the only exchange of molecules (from the medium or produced by bacteria) avoiding the bacterial physical contact. The system is closed using an aluminium clamp. Each bottle was filled with 150 mL of liquid growth medium and inoculated with two different bacteria. The system was incubated at 20 °C at 150 rpm for 5 days. Finally, the intracellular and extracellular material of each condition was submitted to the same extraction and screening process explained above.

## 1.3 Results

**Isolation of microorganisms**
Marine Antarctic sediments were used for the isolation of microorganisms using two approaches. Part of the sediment was serially diluted and plated on four different solid growth media MBA, SCA, GAA and TSA. In this way, the isolation of different classes of bacteria was carried out. The other approach consisted in the observation, in liquid conditions, of the microbiome variation during the time. For this purpose, 1 g of the same sediment was added directly to liquid cultures (kept in agitation) and periodically, 100 µL of cultures were plated on agar media as described in MM. The incubation time was extended to 45 days to allow the development of slow-growing strains using two temperatures, 4 and 15 °C. After 45 days, a morphological analysis was performed in order to collect the colonies. Plates at 4°C showed a less number of colonies and less biodiversity compared with plates at 15 °C. GAA resulted to be the worst medium in terms of variability and colonies number. Differences in the type, shape, number and colour were observed comparing the isolates obtained by the two approaches (solid and liquid). From liquid cultures a sort of microbial selection during the time was clear. Different microbial population appeared on plates at different times (**Fig.1)**. A remarkable variation after 45 days in TSB liquid was observed. As a fact, only two different colonies, labelled T1 and T2, were able to grow on plates (very close to each other) from liquid TSB. In total, about 200 colonies were picked and stored in 96 well-plates at -80 °C.

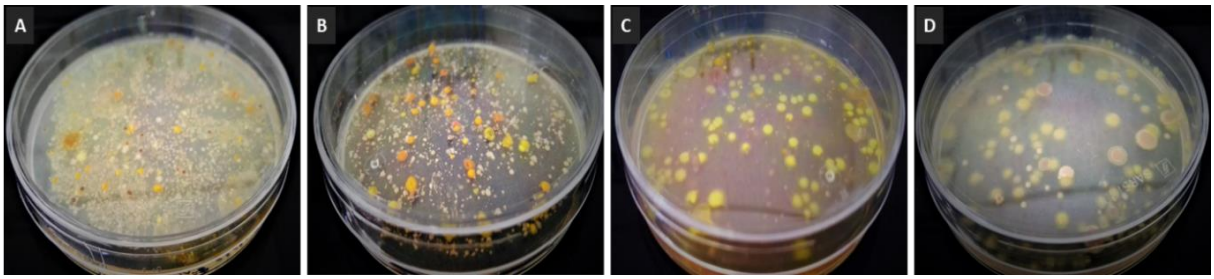

**Figure 1:** Differences in the microbial population isolated periodically from liquid Marine Broth. A= 3 days, B= 5 days, C= 10 days, D= 20 days.

**Primary screening: optimisation of cross streaking assay**
In order to check the ability of Antarctic bacteria to inhibit the growth of a panel of MDR bacteria, composed by *Burkholderia metallica* LMG 24068, *Pseudomonas aeruginosa* PA01, *Klebsiella pneumoniae* DF12SA, *Staphylococcus aureus* 6538P, and *Acinetobacter baumannii* Ab13, cross-streaking experiments were performed. The limitation of this technique is that the tester bacteria (new isolates) and the target

strains (MDR bacteria) must grow on the same growth medium. The problem is that many marine and Antarctic bacteria are not able to grow on such media. In this work, we optimise the cross streaking assay building a dual-media on the same plate as described in MM. The selected bacteria were spread onto half petri dish containing the suitable media and incubated at 15°C for five days. This ensures any secondary metabolites produced were diffused into the agar. Among 74 bacteria tested 6 showed an inhibition activity against some pathogens. Results are reported in **Table 1**. In particular, the isolate labelled as C5 and initially isolated from liquid SC (after 3 days of incubation) was able to completely inhibit the growth of *S. aureus* and *B. metallica*.

| Strain | B3 | C3 | C5 | T28 | D10 |
|---|---|---|---|---|---|
| ***S. aureus*** | **+/-** | **+/-** | **-** | **-** | **+** |
| ***P. aeruginosa*** | **+** | **+** | **+** | **+** | **+** |
| ***K. pneumoniae*** | **+** | **+** | **+/-** | **+/-** | **+/-** |
| ***A. baumannii*** | **+/-** | **+/-** | **+** | **+/-** | **+** |
| ***B. metallica*** | **+/-** | **+** | **-** | **+/-** | **+/-** |

**Table 1:** Growth of pathogen strains in the presence of Antarctic isolates in cross streaking experiment. Symbols: +, growth, +/- reduced growth, -, no growth.

### Phylogenetic affiliation of C5, T28, T1 and T2 strains

The 16S rDNA genes of isolate C5 was amplified by PCR and the obtained sequence was submitted to BLAST for the strain identification. The antimicrobial producer strain C5 showed 99% of sequence similarity with *Pseudomonas gessardii* and T28 was identified as *Pseudomonas fluorescens.* Other two isolates T1 and T2 were submitted to the same procedures and respectively identified as *Lysobacter* sp. and *Pseudoachromobacter* sp. These two strains were isolated after 45 days of incubation growing very close to each other and could be used for co-cultivation experiments.

### Antimicrobial activity of *Pseudomonas gessardii* C5

The most active strain *Pseudomonas gessardi* C5 was inoculated in liquid media LB (this medium was selected because is suitable for the growth of *Pseudomonas* strains) and after five days extracted by ethyl acetate. The produced crude extract was checked for the ability to inhibit the growth of a selected panel of human pathogens in a liquid inhibition assay, evaluating the minimal inhibitory concentration. After 24 h of incubation at 37°C the results confirmed the promising antimicrobial activity of *P. gessardii* C5 towards several pathogens, mainly Gram-positive bacteria (**Table 2,** values reported as percentage of growth inhibition). The highest antimicrobial activity was detected against *E. faecalis* with a MIC value of 15 µg/mL. Both *S. aureus* and MRSA were inhibited with a MIC value of 30 µg/mL. No significant effect was observed towards gram-negative strains except for the *Burkholderia* genus. This strain was selected for further studies.

| Extract concentration | 1 mg/mL | 0,5 mg/mL | 0,25 mg/mL | 0,13 mg/mL | 0,06 mg/mL | 0,03 mg/mL | 0,015 mg/mL | 0,007 mg/mL | 0,003 mg/mL | DMSO + cells | Cells only |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. aureus* | 91 | 91 | 90 | 91 | 87 | 83 | 52 | 0 | 0 | 0 | 0 |
| *MRSA* | 89 | 90 | 90 | 90 | 83 | 82 | 5 | 2 | 0 | 0 | 0 |
| *P. acnes* | 91 | 89 | 72 | 48 | 19 | 7 | 28 | 22 | 17 | 0 | 0 |
| *E. faecalis* | 96 | 91 | 90 | 91 | 90 | 90 | 82 | 14 | 11 | 0 | 0 |
| *P. aeruginosa* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *K. pneumoniae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. baumannii* | 14 | 9 | 8 | 7 | 1 | 7 | 1 | 1 | 1 | 0 | 0 |
| *B. metallica* | 89 | 89 | 89 | 54 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. anthina* | 87 | 87 | 87 | 44 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. diffusa* | 87 | 87 | 83 | 74 | 58 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 2:** Antimicrobial activity of *P. gessardii* C5 crude extract against a panel of MDR bacteria. Bacterial growth is measured in $OD_{600}$/mL. Red colour indicates the strong inhibition of the extract at a certain concentration while green colour indicates no inhibition.

## Bioassay-guided purification

*P. gessardii* C5 was submitted to the scale-up of the culture in order to obtain a sufficient amount of crude extract for the purification. Subsequently, the crude extract (1,5 g) was fractionated and the 4 eluted fractions were collected, dried and dissolved in DMSO to perform bioassay at a stock concentration of 50 mg/mL for MIC assay, **Table 3**. The fraction eluted at 100% methanol (89 mg) was the most active one against *S. aureus* with a MIC of 15 µg/mL (From now on, *S. aureus* was used as reference Gram-positive strain for the evaluation of antimicrobial activity).

| Extract concentration | 1 mg/mL | 0,5 mg/mL | 0,25 mg/mL | 0,13 mg/mL | 0,06 mg/mL | 0,03 mg/mL | 0,015 mg/mL | 0,007 mg/mL | 0,003 mg/mL | DMSO + cells | Cells only |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Crude Extract | 90 | 90 | 89 | 89 | 87 | 84 | 35 | 7 | 11 | 0 | 0 |
| N.B. | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25% | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50% | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 75% | 86 | 49 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 100% | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 78 | 30 | 0 | 0 |

**Table 3:** Antimicrobial activity of *P. gessardii* C5 fractions against *S. aureus*. Bacterial growth is measured in $OD_{600}$/mL. Red colour indicates the strong inhibition of the extract at a certain concentration while green colour indicates no inhibition.

The active fraction was then submitted to HPLC separation. HPLC chromatograms extracted from 200 to 400 nm presented 10 different peaks, which were separated, dried and dissolved in DMSO at a stock concentration of 10 mg/mL to perform MIC assay. Data obtained revealed a high inhibitory activity against *S. aureus* of five compounds numbered C1-5. (**Table 4**).

| | MIC | | | | |
|---|---|---|---|---|---|
| **Strain** | **C-1** | **C-2** | **C-3** | **C-4** | **C-5** |
| ***S. aureus*** | ***7 µg/mL*** | ***5 µg/mL*** | ***5 µg/mL*** | ***3 µg/mL*** | ***3 µg/mL*** |

**Table 4:** Minimal inhibitory concentration of pure compounds against *S. aureus*.

## Chemical analysis of active metabolites

According to the NMR analysis, all the isolated compounds belong to the mono-rhamno-di-lipid sub class (**Fig. 2**). This classification was easily inferred by the

presence in the $^{1}H$ NMR spectrum of only one anomeric signal ($\delta_H$ *ca* 4,80 bs) and one C5 methyl group ($\delta_H$ 1.26,d 6,4, $\delta_C$ 16.5), relative to the mono-rhamnpyranosyl unit and of two low field pentet signals at ca 5.27 ppm and 4.10 ppm relative to the 3-hydroxymethine protons of the two fatty acid chains. Compounds 3 and 5 have never been isolated so far.

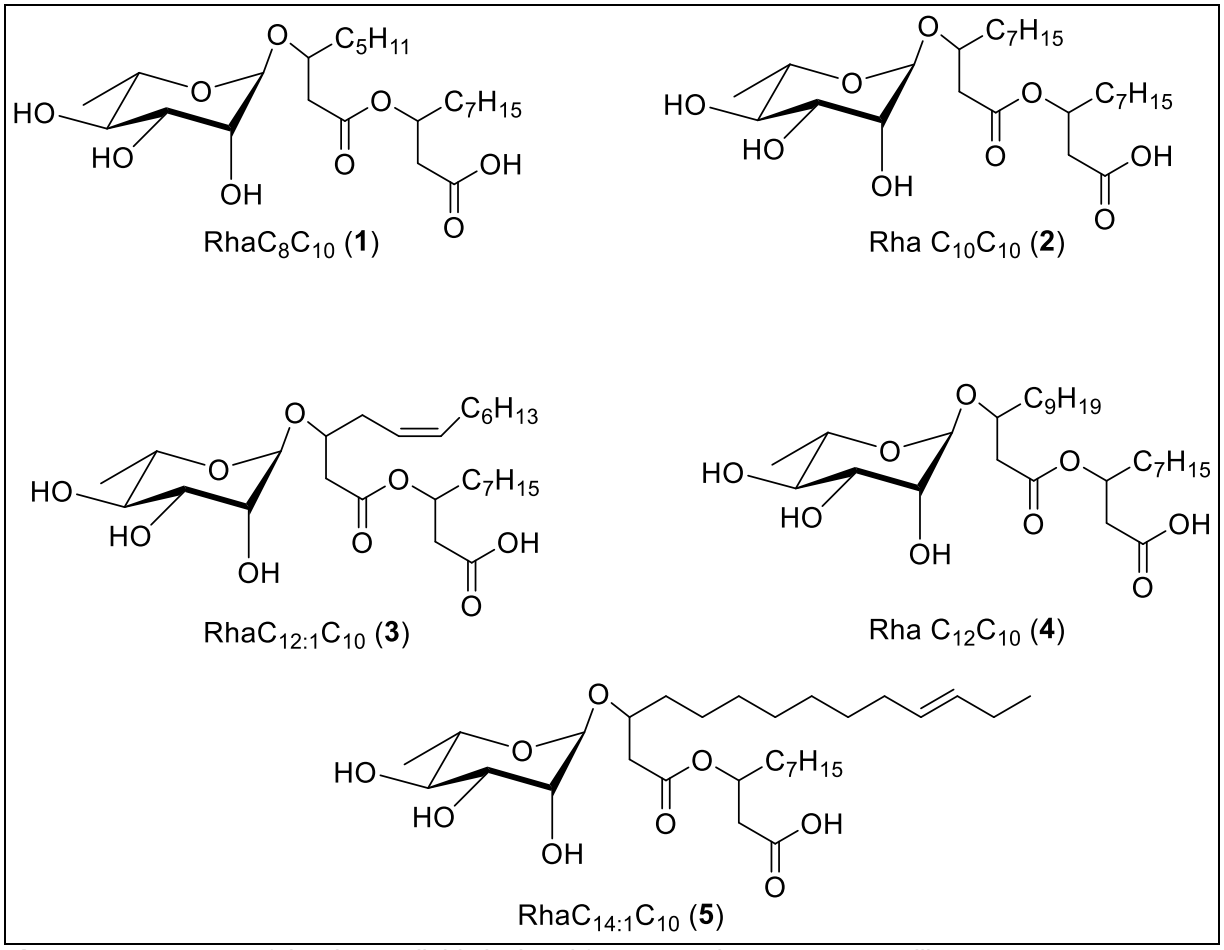


**Figure 2**: Structures of the rhamnolipids isolated from *Pseudomonas gessardii* C5

The analysis of the negative pseudo-molecular ion $[M-H]^-$, obtained by ESI-HRMS gave information on the molecular formula, whereas the analysis of the key fragment ion arising from the cleavage of the ester linkage between the two β-hydroxy fatty acid units allowed to discriminate between congeners with non-symmetric fatty acid units (**Table 5**). Fragmentation spectra are shown in section 1.6 Supplementary materials (**Fig. S1-5**). For instance, the molecular formula $C_{24}H_{44}O_9$ for compound (1) corresponds to a structure Rha-$C_8$-$C_{10}$ or Rha-$C_{10}$-$C_8$. The key fragment at m/z 305, corresponding to Rha-$C_8$ allowed to assign the structure Rha-$C_8$-$C_{10}$. Compound 2 and 4 correspond respectively to Rha-$C_{10}$-$C_{10}$ and Rha-$C_{12}$-$C_{10}$. Negative ESI–HRMS gave the main pseudo-molecular ion at m/z 529.3373 for compound 3, indicating a molecular formula $C_{28}H_{50}O_9$, consistent with Rha-$C_{10}$-$C_{12:1}$ or Rha-$C_{12:1}$-$C_{10}$. The main ion in the corresponding MS/MS spectrum at m/z 359, corresponding to the cleavage of the ester bond, allowed to assign the Rha-$C_{12:1}$-$C_{10}$ structure. The molecular formula of the compound 5 was established as $C_{30}H_{54}O_9$ by HRESIMS analysis of the pseudo-molecular ion, which implied an additional $C_2H_4$ unit, compared to compound 3. MS/MS fragmentation data evidenced two daughter ions at m/z 387.29 and 223.21 corresponding to Rha-$C_{14:1}$ and $C_{14:1}$ ($-H_2O$) negative ions,

that allowed to assign a C-$_{14:1}$ fatty acid chain linked to rhamnose unit. The $^{13}C$ and $^{1}H$ chemical shifts of compounds 3 and 5 are shown in **Table S6** in Supplementary materials. The structure of the compound, including the position of the double bond in the fatty acid and the identity and configuration of the anomeric proton of the sugar moiety, was secured by detailed NMR analysis, based on HMQC, COSY, TOCSY and ROESY experiments.

| Rha-FA-FA | Formula | $[M-H]^-$ | MS/MS fragments | Relative abundance |
|---|---|---|---|---|
| $RhaC_8C_{10}$ (1) | $C_{24}H_{44}O_9$ | 475.2916 | 305 | 1.9% |
| $RhaC_{10}C_{10}$ (2) | $C_{26}H_{48}O_9$ | 503.3227 | 333 | 4.3% |
| $RhaC_{12:1}C_{10}$ (3) | $C_{28}H_{50}O_9$ | 529.3373 | 359 | 49.3% |
| $RhaC_{12}C_{10}$ (4) | $C_{28}H_{52}O_9$ | 531.3536 | 361 | 40.4% |
| $RhaC_{14:1}C_{10}$ (5) | $C_{30}H_{54}O_9$ | 557.3376 | 387, 223, 163, 119, 103 | 4.1% |

**Table 5:** Molecular formula, pseudomolecular-ions, ion fragments and relative abundance of isolated compounds derived from HRMS/MS.

In addition, the fractionation of the crude extract also gave an enriched fraction, that contains as determined by LC-HRESIMS analysis, in positive mode, a mixture of 3-(3-hydroxyalkanoyloxy) alkanoic acids that lack the carbohydrate moiety and represent precursors of rhamnolipids (**Fig. 3**).

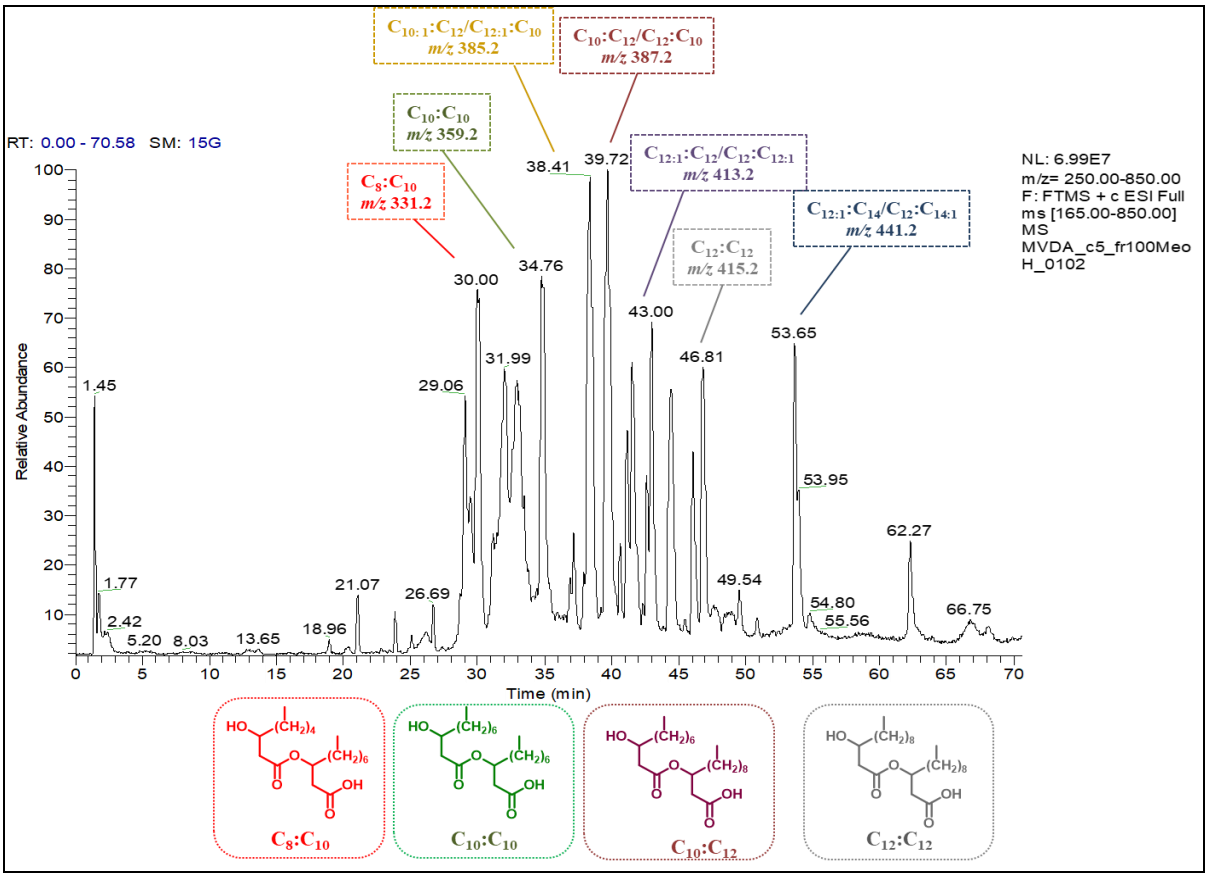


**Figure 3**: LC-HRESIMS analysis, in positive mode of a fraction containing a mixture of 3-(3-hydroxyalkanoyloxy) alkanoic acids that lack the carbohydrate moiety and represent precursors of rhamnolipids.

## Preliminary Co-cultivation experiments

During this work, many bacterial strains were isolated. Some of them showed antimicrobial activity in the conditions that we selected, but there are other approaches that could be applied to induce the expression of bioactive compounds. One of the strategies is the so-called Co-cultivation. It is based on the idea that microbes in nature don't live in axenic cultures but in communities, exchanging signal molecules, nutrients, etc. and for this experiment, we tried to simulate these conditions and to provoke variation in their metabolism. Special cultivation bottles were used for the experiment. This system is made up of two bottles connected with a glass neck and separated by 0.22 µm filter that allowed the only exchange of molecules (from the medium or produced by bacteria) avoiding the bacterial physical contact. Different co-cultivation experiments were tried involving: the rhamnolipids producer *P. gessardii* C5, the other strain, *P. fluorescens* T28 that showed weak inhibition activity during the cross-streaking experiments against almost all pathogens, then *Lysobacter sp*. T1 and *Pseudoachromobacter sp.* T2 initially isolated from TSB liquid cultures after 45 days. They were selected because of their capability to grow very close to each other on the agar plate, meaning that they communicated in some way. Co-cultivation experiments using *P. gessardii* C5 and *P. fluorescens* T28 together and with the other selected strains showed no significant variation regarding growth and bioactivity. Further studies will be performed co-cultivating *P. gessardii* C5 and *S. aureus* to increase rhamnolipids production exploiting the antagonistic effect. While the two *Pseudomonas* strains showed no variations in this system, the co-cultivation between *Lysobacter* T1 and *Pseudoachromobacter sp.* T2

displayed a positive result. Each bacterium was inoculated in one bottle and incubated for five days with agitation (**Fig. 4A**). Finally, each bottle, containing a specific strain was centrifuged and analysed separately. A different colour of the pellet of *Lysobacter* T1 strain grown in co-cultivation was immediately clear, compared with the control (**Fig. 4B, 2C**)

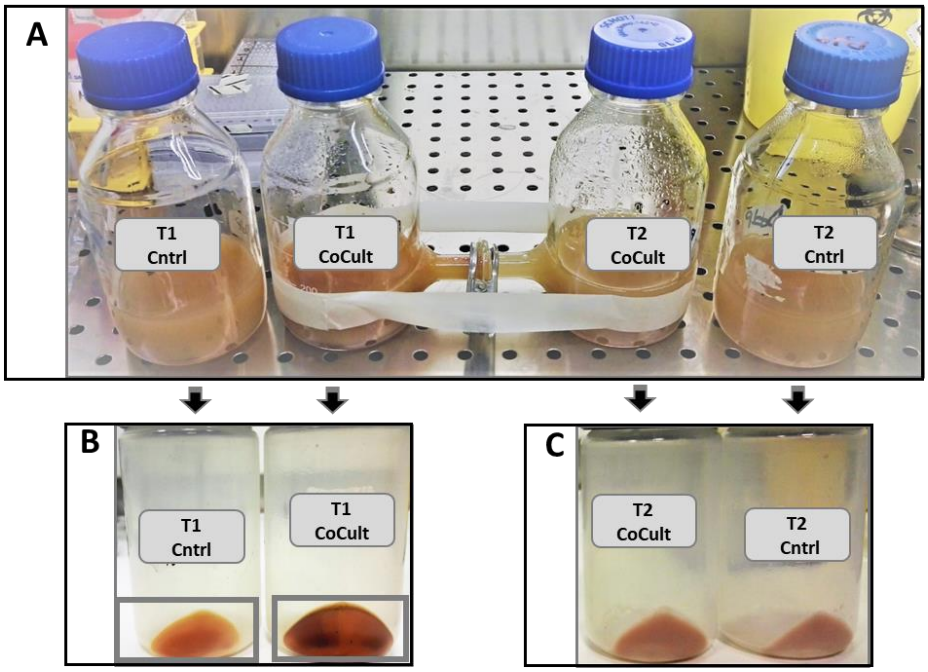


**Figure 4:** Co-cultivation bottles inoculated with *Lysobacter sp*. T1 (left bottle) and *Pseudoachromobacter sp.* T2 (right bottle) joined by a glass neck separated by 0.22 µm filter and respective controls inoculated as pure cultures in single bottles (**A**). Bacterial pellet after centrifugation with a visible colour variation of T1 grown in co-cultivation with T2, compared with the pure culture (**B, C**).

Pellet and supernatant of the two bacteria were extracted by ethyl acetate and the obtained extracts were evaluated for their capability to inhibit pathogen bacteria. Results showed an increased inhibitory effect (about 15 fold) towards *S. aureus* of the intracellular extract produced by *Lysobacter sp.* T1 co-cultivated compared with control, **Fig. 5**. The presence of *Pseudoachromobacter sp.* T2 in co-cultivation bottles induced some variation in the T1 metabolism resulting in an increased bioactivity. No significant antimicrobial effects were detected by *Pseudoachromobacter sp.* T2 and against other pathogen bacteria.

| Extract concentration | 1 mg/mL | 0,5 mg/mL | 0,25 mg/mL | 0,13 mg/mL | 0,06 mg/mL | 0,03 mg/mL | 0,015 mg/mL | 0,007 mg/mL | 0,003 mg/mL | DMSO + cells | Cells only |
|---|---|---|---|---|---|---|---|---|---|---|---|
| T1 Cntrl | 83 | 84 | 52 | 40 | 16 | 6 | 5 | 4 | 0 | 0 | 0 |
| T2 Cntrl | 35 | 18 | 16 | 16 | 11 | 7 | 7 | 7 | 0 | 0 | 0 |
| T1 CoCult | 100 | 100 | 100 | 100 | 91 | 42 | 22 | 27 | 0 | 0 | 0 |
| T2 CoCult | 49 | 19 | 11 | 9 | 7 | 6 | 23 | 34 | 0 | 0 | 0 |

**Figure 5:** Antimicrobial activity of *Lysobacter sp*. T1 and *Pseudoachromobacter sp.* T2 crude extracts against *S. aureus*. Bacterial growth is measured in $OD_{600}$/mL. Red colour indicates the strong inhibition of the extract at a certain concentration while green colour indicates no inhibition

Finally, a chemical profiling by HPLC comparing the *Lysobacter sp.* T1 intracellular extracts was performed to appreciate variations in the bacterial metabolism caused

by the co-cultivation. Results reported in **Figure 6** show a difference in the metabolites production in the most polar region, in particular between minute 8.5 – 9 an overexpression of a compound that could be the responsible for the bioactivity is clear.

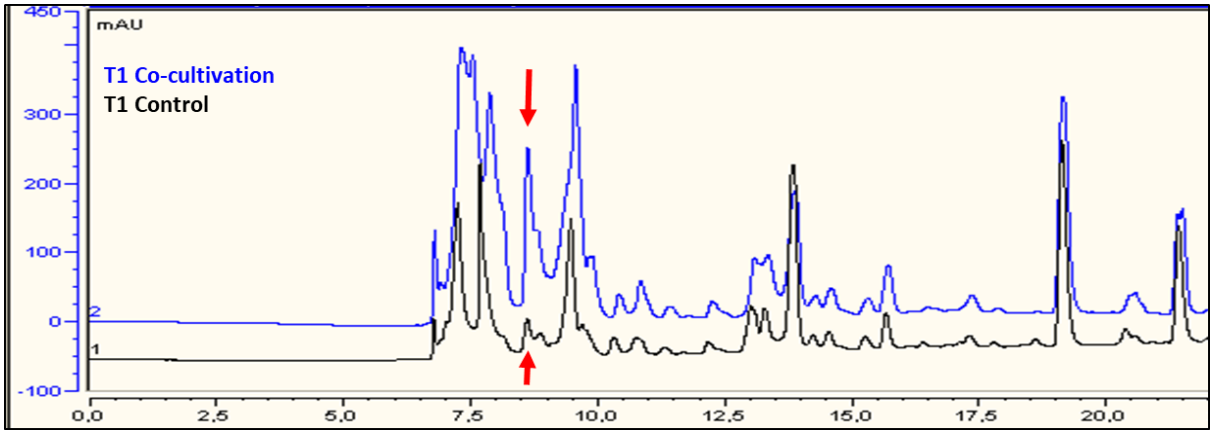


**Figure 6:** Comparison of *Lysobacter sp.* T1 HPLC chromatograms when it is grown in co-cultivation (blue line) and in pure culture (black line). The two chromatograms are exactly superimposable except for the most polar region in which there is an enhanced production of a compound in the co-cultivation condition.

## 1.4 Discussion

The alarming widespread of the antimicrobial resistance problem is an effect of the unappropriated use of antibiotics. From the discovery of antibiotics in the 1950s the worldwide population was able to successfully defeat infections disease using these powerful molecules. Followed an era defined “Golden Age” because of the discovery of numerous new and effective antibiotics [13]. Mass production of that molecules and chemical synthesis based on natural structures guaranteed protection against most of the pathogen bacteria. On the other hand, the prolonged and uncontrolled use of antibiotics led to the rapid evolution of multidrug resistant bacteria becoming a serious problem [14]. There is the pressing need to discover novel bioactive compounds and the bioprospecting of microorganisms inhabiting extreme environments could be a promising strategy. In this work, the attention has been focused on Antarctica. This extreme continent preserves a huge biodiversity and is still unexplored [15]. Shallow water Antarctic sediments have been collected and subjected to different procedures, growing microbes in solid and in liquid for 45 days to allow the development of slow-growing strains [16]. By the liquid approach, a changing in the microbial population during the time was detected. The objective of this approach was to induce the competition among microorganisms leading to the selection of the antimicrobial producers. Most of the tested strains showed no bioactivity (in the selected conditions) while four of them showed a partial or total inhibition activity against MDR bacteria. Among 74 strains subjected to cross streaking assay [17] one identified as *Pseudomonas gessardii* C5 was selected for the capability to inhibit the growth of *S. aureus* and *B. metallica*. The optimization of this assay allowed the screening of all selected bacteria overcoming the limitation in the use of the same medium for tester and target strains. Liquid inhibition assay confirmed the antimicrobial potential of this strain against a wide panel of pathogens, in particular against Gram-positive bacteria. This strain is a fluorescent, Gram-negative, rod-shaped bacterium isolated from natural mineral waters in France [18]

and based on 16S rRNA analysis, *P. gessardii* has been placed in the *P. fluorescens* group [19]. The genus *Pseudomonas* has a prominent role in the biotechnological and pharmaceutical industry for its capability to produce a wide range of bioactive compounds, but few studies focused the attention on *P. gessardii,* that is known as lipases producers [20] and as a naphthalene degrading strains [21]. The purification of the bioactive compounds produced by *P. gessardii* C5 led to the identification of a family of rhamnolipids. This class of glycolipids was discovered as a product of several bacterial strains, like *Pseudomonas aeruginosa*, *Burkholderia spp*. and others [22]. At best of our knowledge, no reports describe the purification and identification of rhamnolipids from *P. gessardii*. Although many different rhamnolipids have been described from *Pseudomonas* spp. using mass spectrometry, very often they are not purified and evaluated for the bioactivity. Compounds 3 and 5 identified from *P. gessardii* C5 have never been isolated so far, indicating that *Pseudomonas* strains are able to produce a wide range of this class of molecules. In agreement with their hydrophobic nature, these compounds eluted at a high percentage of acetonitrile (in a system Water/ACN, reverse phase C18-HPLC). As extensively reported in literature [23-25], the analysis of the negative pseudo-molecular ion $[M-H]^-$, obtained by ESI-HRMS gave information on the molecular formula, whereas the analysis of the key fragment ion arising from the cleavage of the ester linkage between the two β-hydroxy fatty acid units allowed to discriminate between congeners with asymmetric fatty acid units. In addition, the fractionation of the crude extract also gave an enriched fraction that contains as determined by LC-HRESIMS analysis, in positive mode, a mixture of 3-(3-hydroxyalkanoyloxy) alkanoic acids that lack the carbohydrate moiety and represent precursors of rhamnolipids. These compounds have been rarely isolated as components of the rhamnolipid fraction of bacteria [26]. As compared with other rhamnolipid-producing *Pseudomonas* strains, the observed relative composition of rhamnolipid components of the Antarctic of *P. gessardii* C5 revealed some distinctive features. Usually, monorhamnolipids represent a small fraction of the total amount of RL produced. Typically, *P. aeruginosa* strains produce between 75 and 80 % of di-RL and only between 20 and 25 % of mono-RL [27] and this proportion of mono-RL/di-RL is conserved through the entire RL production period. Structural analyses demonstrated a molecular population in the studied Antarctic strain composed mainly of monorhamnolipids. Because of their chemical structure and composition (they have a glycosyl head group, a rhamnose moiety, and a 3-(hydroxyalkanoyloxy) alkanoic acid (HAA) fatty acid tail), rhamnolipids perform several potential functions in bacteria: as powerful biosurfactants are involved in the uptake and biodegradation of poorly soluble substrates and are essential for surface motility and biofilm development [22]. They have numerous industrial applications, such as in agriculture, for soil remediation [28], cosmetics, as active ingredient effective for several skin treatments [29] and detergents, used in detergent compositions, laundry products, shampoos and soaps [30]. Recently, they have emerged as potential antimicrobials against a broad range of pathogens such as *Staphylococcus*, *Mycobacterium*, and *Bacillus*, and significant activity against a number of Gram-negative species, including *Serratia marcescens*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* [31-33]. Rhamnolipids act like synthetic surfactants and their proposed mechanism of action consists of intercalation into biological membranes and destruction by their permeabilising effect leading to cell death [34]. This class of compounds was isolated from *P. aeruginosa* but considering the pathogenicity of this strain novel species are of biotechnological interest for a safer production. Moreover, the capability of *P. gessardii* C5 as

producers of rhamnolipids and lipases makes this strain an important candidate for the bioremediation. For this purpose, different hydrocarbons, especially oil wastes, will be used as substrate for bacterial growth trying to stimulate the metabolism of *P. gessardii* C5 increasing the rhamnolipids production and inducing the expression of different types of rhamnolipids [35]. By this approach is possible to stimulate, induce or optimize the production of rhamnolipids but even other compounds. As a fact, it has been demonstrated that varying the growth conditions (nutrients, temperature, pH, environment) is possible to induce the expression of silent genes. Many experiments applying an OSMAC (One Strain Many Compounds) led to the discovery of previously unknown compounds [36, 37]. Beside the OSMAC approach other techniques are used. During last years, the concept of axenic cultures or classic cultivation methods was replaced by the idea of microbial community. In Nature, microorganisms interact in different ways to benefit each other within a community. Potentially, their genome can encode for a huge amount of different and novel bioactive compounds, which could be exploited for pharmaceutical and biotechnological applications [38]. Bacterial or fungal co-cultivation is a new powerful emerging tool for enhancing the chemical diversity of microorganisms [39, 40] and it seems to be a promising strategy to induce the expression of different bioactive molecules. This method can be applied between bacteria-bacteria, fungi-fungi and bacteria-fungi. The critical point for this approach is the selection of microorganisms for co-cultivation. There are no defined indications or protocols for that, but considering that communication is one of key factors, microorganisms isolated from the same environment and able to grow close to each other could be considered promising candidates for this experiment. In this work, *P. gessardii* C5 was subjected to co-cultivation experiments together with other bacteria isolated from the same environment (*P. fluorescens* T28, *Lysobacter sp*. T1 and *Pseudochrobactrum sp*. T2), but no significant results were obtained. Two Antarctic strains, instead, *Lysobacter sp*. (T1) and *Pseudochrobactrum sp*. (T2), the only bacteria isolated after 45 days of incubation on the same agar plates from Antarctic sediments, showed promising results in the co-cultivation system. In particular, *Lysobacter sp*. T1 showed and increased inhibition activity, of about 15 fold, against *S. aureus* when it grows in presence of *Pseudochrobactrum sp*. T2 compared with the control. The metabolism variation corresponded to a changing in bacterial colour, resulting darker than the pure culture. Genome sequence analysis revealed that each *Lysobacter* species has gene clusters for the production of 12-16 secondary metabolites showing a high chemical variability [41]. The problem is that many of these genes are silent. According to these preliminary results and the recent papers about *Lysobacter sp*. as an important source of new compounds, this strain will be subjected to further studies applying co-cultivation and OSMAC approach.

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## 1.6 Supplementary materials

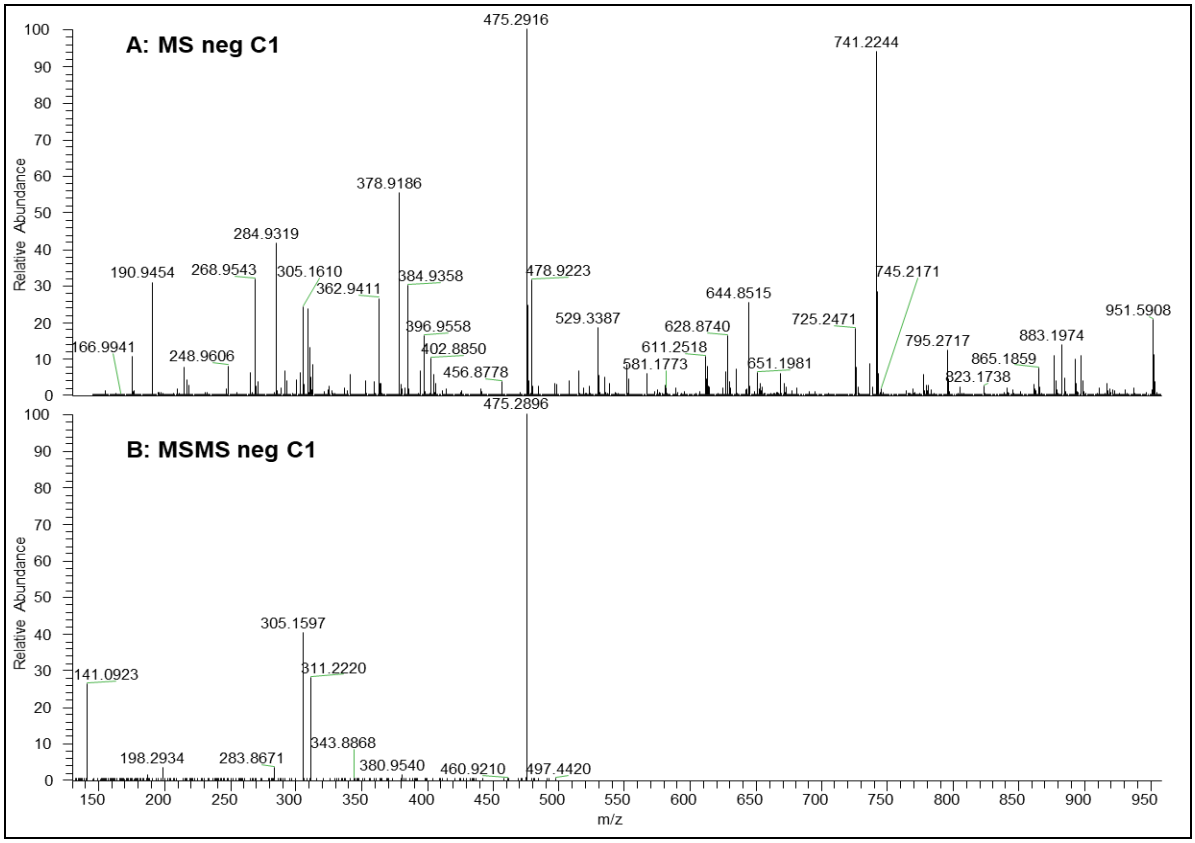



**Figure S1:** A) HRESIMS negative Compound 1. B) HRESIMSMS negative Compound 1.

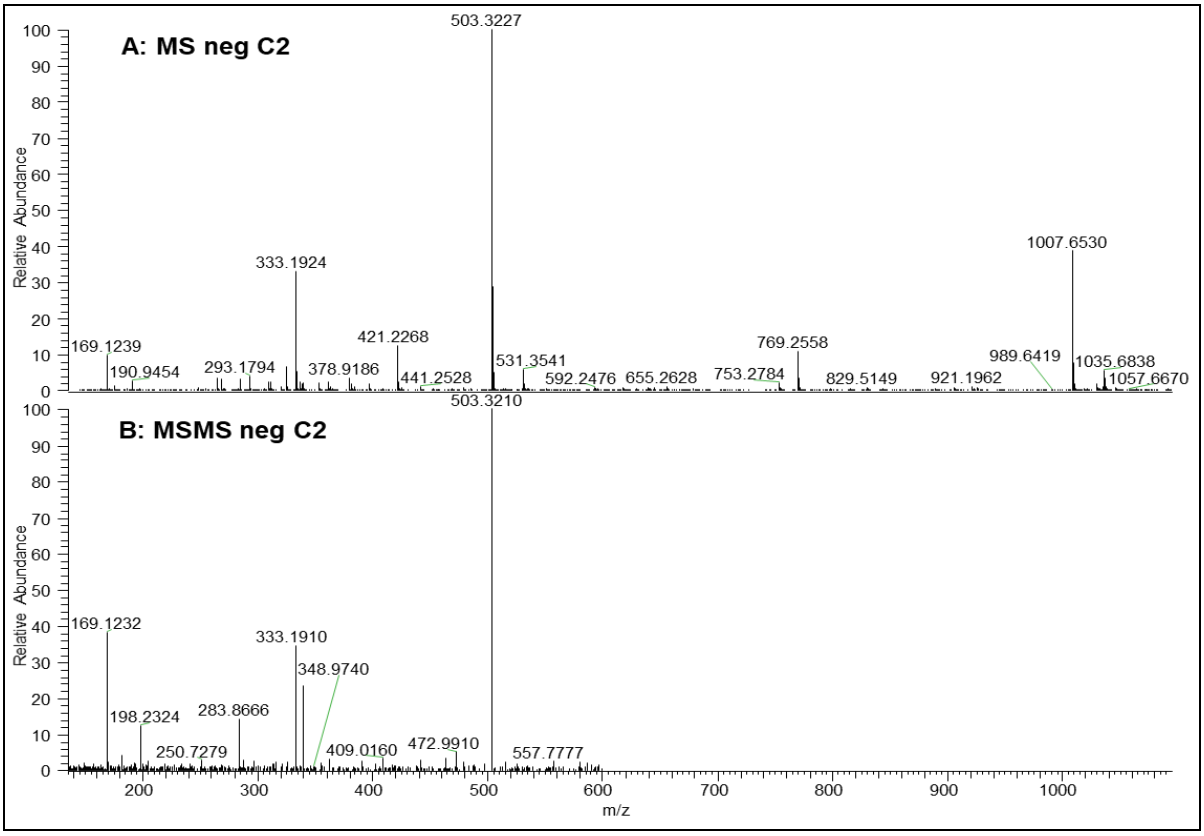



**Figure S2:** A) HRESIMS negative Compound 2. B) HRESIMSMS negative Compound 2.

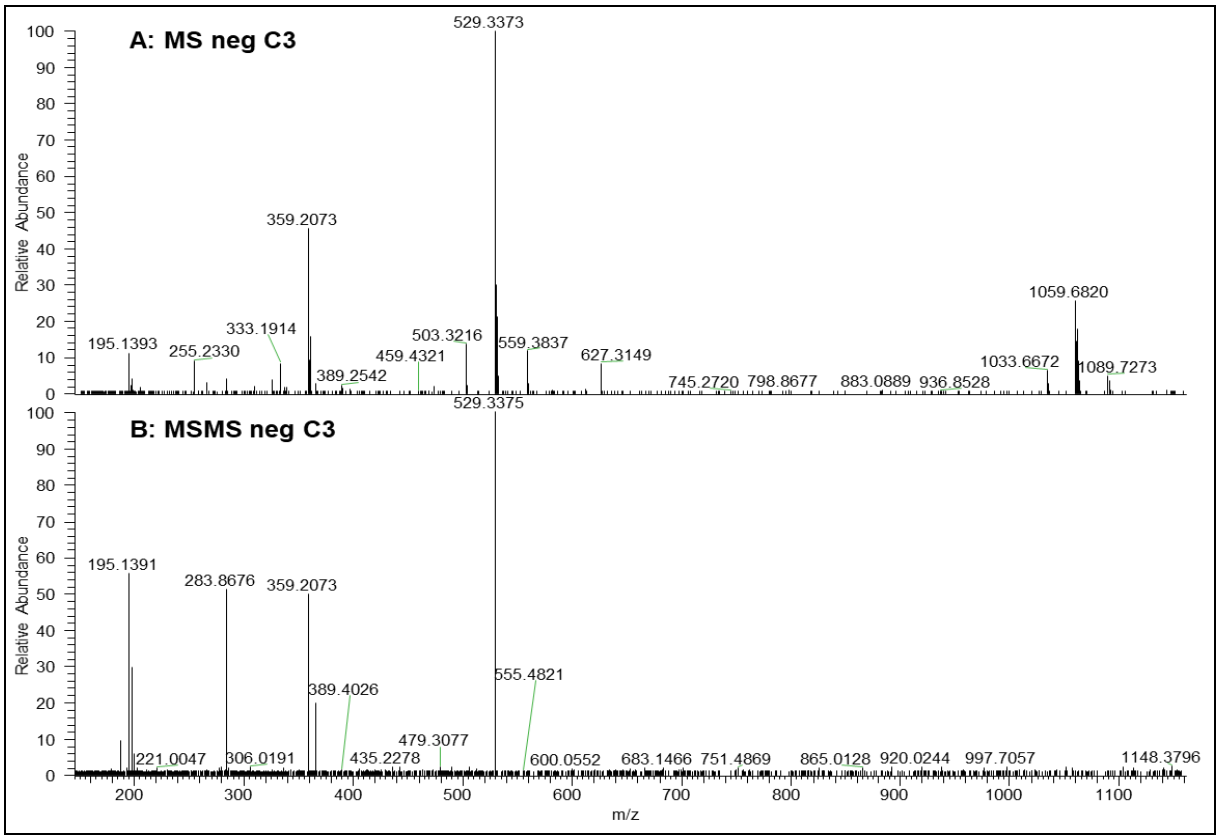



**Figure S3:** A) HRESIMS negative Compound 3. B) HRESIMSMS negative Compound 3.

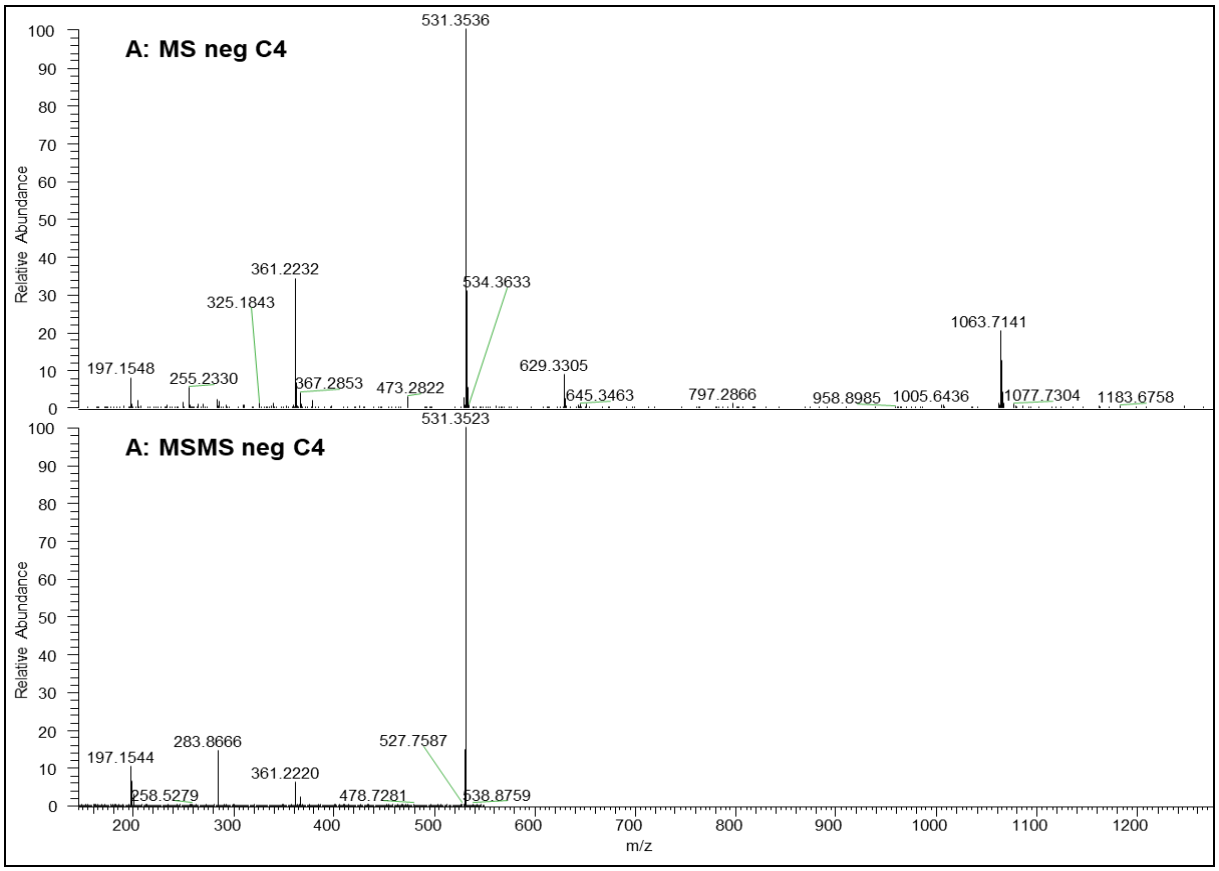



**Figure S4:** A) HRESIMS negative Compound 4. B) HRESIMSMS negative Compound 4.

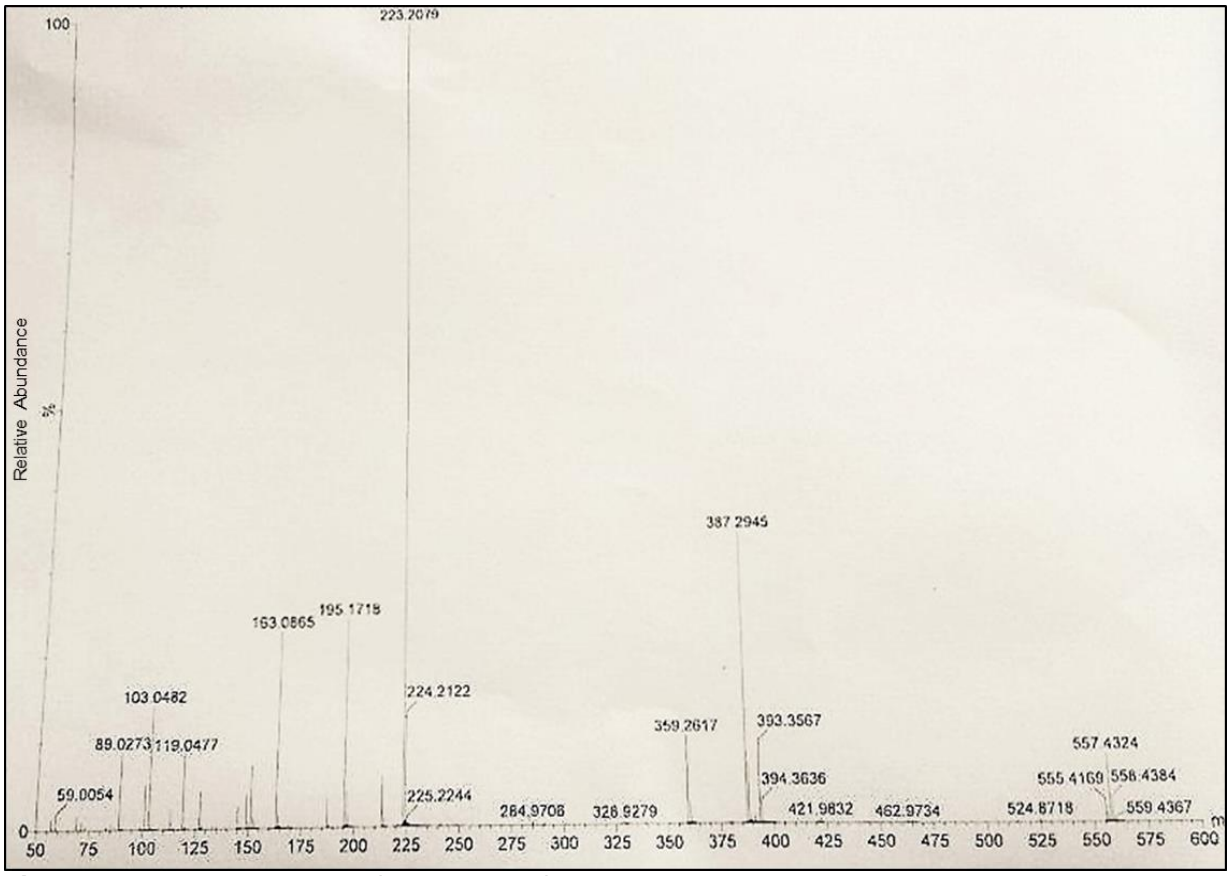



**Figure S5:** HRESIMSMS negative Compound 5.

| | | 3 | | 5 | |
|---|---|---|---|---|---|
| | Position | $\delta_C$/ppm[a], m | $\delta_H$/ppm (m, *J* in Hz)[b] | $\delta_C$/ppm[a], m | $\delta_H$/ppm (m, *J* in Hz)[b] |
| A | 1 | 173.4, C | | 175.5, C | |
| | 2 | 38.9, $CH_2$ | 2.58, m | 40.9, $CH_2$ | 2.54, m |
| | 3 | 71.1, CH | 5.27, pentet, 6.4 | 72.7, CH | 5.29, pentet, 6.5 |
| | 4 | 33.8, $CH_2$ | 1.64, m | 34.9, $CH_2$ | 1.63, bm |
| | 5 | 24.9, $CH_2$ | 1.35, overlap | 26.0, $CH_2$ | 1.35, overlap |
| | 6 | 29.3, $CH_2$ | 1.31, overlap | 30.5, $CH_2$ | 1.31, overlap |
| | 7 | 29.3, $CH_2$ | 1.31, overlap | 30.1, $CH_2$ | 1.31, overlap |
| | 8 | 31.6, $CH_2$ | 1.31, overlap | 30.1, $CH_2$ | 1.31, overlap |
| | 9 | 22.3, $CH_2$ | 1.33, overlap | 30.2, $CH_2$ | 1.36, overlap |
| | 10 | 13.1, $CH_3$ | 0.92, m | 32.7, $CH_2$ | 1.31, overlap |
| B | 1 | 171.4, C | | 172.3, CH | |
| | 2 | 39.5, $CH_2$ | 2.53, m | 41.0, $CH_2$ | A:2.60, m<br>B: 2.50, m |
| | 3 | 72.9, CH | 4.16, pentet, 5.8 | 74.8, CH | 4.10, pentet, 5.9 |
| | 4 | 30.4, $CH_2$ | A:2.39, m<br>B: 2.33, m | 33.5, $CH_2$ | 1.58, bm |
| | 5 | 123.7, CH | 5.40, m | 25.7, $CH_2$ | 1.43, overlap |
| | 6 | 132.8, CH | 5.55, m | 30.1, $CH_2$ | 1.31, overlap |
| | 7 | 27.1, $CH_2$ | 2.08, m | 30.1, $CH_2$ | 1.31, overlap |
| | 8 | 29.3, $CH_2$ | 1.31, overlap | 27.8, $CH_2$ | 2.08, overlap |
| | 9 | 28.9, $CH_2$ | 1.33, overlap | 130.0, CH | 5.37, m |
| | 10 | 31.6, $CH_2$ | 1.31, overlap | 131.2, CH | 5.39, m |
| | 11 | 22.3, $CH_2$ | 1.33, overlap | 32.7, $CH_2$ | 1.31, overlap |
| | 12 | 13.1, $CH_3$ | 0.92, m | 32.7, $CH_2$ | 1.31, overlap |
| | 13 | | | 23.4, $CH_2$ | 1.33, overlap |
| | 14 | | | 14.1, $CH_3$ | 0.92, m |
| C | 1 | 98.5, CH | 4.86, overlap | 100.0, CH | 4.80, d, 1.4 |
| | 2 | 71.2, CH | 3.75, bd, 3.5, | 72.4, CH | 3.76, dd, 3.4, 1.4 |
| | 3 | 70.9, CH | 3.64, dd, 9.5, 3.5 | 71.9, CH | 3.66, dd, 9.7, 3.4 |
| | 4 | 72.7, CH | 3.38, dd, 9.5, 9.8 | 73.8, CH | 3.35, dd, 9.7, 9.8 |
| | 5 | 68.7, CH | 3.67, m | 69.8, CH | 3.68, m |
| | 6 | 16.6, $CH_3$ | 1.27, d, 6.2 | 17.6, $CH_3$ | 1.27, d, 6.3 |

**Table S6:** NMR data of **3** and **5** in $CD_3OD$. [a] 100 MHz; [b] 400 MHz.

# CHAPTER 2

# Isolation of an Antarctic bacterium *Aequorivita* sp. by Miniaturized Culture Chip as a producer of novel bioactive compounds

# Isolation of an Antarctic bacterium *Aequorivita* sp. by Miniaturized Culture Chip as a producer of novel bioactive compounds


## Abstract

The access to microbial diversity is greatly hampered by standard current cultivation techniques. Even today, cultivation is often required to access as-yet-uncultured or rare microorganisms, and novel secondary metabolites from these sources may lead to a “Golden Age” of new drugs with tremendous benefit for human health. In this work, a novel approach, which mimics the natural environment by using a Miniaturized Culture Chip (MCC) method, allowed the isolation of several bacterial strains from Antarctic shallow water sediments. One of the strains, identified as *Aequorivita* sp. was a Gram-negative Antarctic bacterium belonging to the family of Flavobacteriaceae and resulted to be a strain unexplored for the production of antimicrobial compounds. The whole genome of *Aequorivita* sp. was sequenced and a genomics approach was used to identify the presence of biosynthetic gene clusters (BGCs). The newly isolated showed antimicrobial activity towards a panel of Multidrug Resistant (MDR) bacteria and antihelminthic activity towards the nematode *Caenorhabditis elegans*. The purification of active compounds revealed the identification of three new aminolipids showing antimicrobial activity. This is the first multi-approach investigation on the genomics and biotechnological potential of this bacterium with the findings suggesting it as a promising candidate for pharmaceutical applications.


## 2.1 Introduction

The number of bacterial species in the world range from $10^7$ to $10^9$ [1], but most have never been observed or cultivated. Culture-independent methods have demonstrated the enormous richness of biodiversity in the microbial world [2, 3]. The “challenge of uncultivable microorganisms” is a topic that has been concerning scientists for many years. It is well known as the *Great Plate Count Anomaly* [4, 5] based on the observation, that less than 1% of microbes present in an environmental sample is cultivable *in vitro*. Solutions to this problem could facilitate the discovery of new strains holding novel and different features which the biotechnology could exploit. The reasons for the unculturability are diverse and many are probably unknown. It is clear that certain bacteria need specific requirements for the growth, only present in their habitats, such as the presence of certain microbial communities or the need of specific physical or chemical characteristics of the natural environment (light, oxygen, pH, pressure, temperature etc.) [6]. Among the strategies for the isolation and cultivation of new or atypical microorganisms, the simulation of the natural environment is one of the most promising. The use of innovative devices that are able to mimic natural conditions enhances the chance to cultivate different microbial species [7-9]. The isolation of new strains is also aimed at the exploration and exploitation of natural products they biosynthesize, developing novel antimicrobial lead compounds to counteract the spread of multidrug resistant (MDR) bacteria. Recently, the World Health Organization

(http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/) published its first ever list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria that pose the greatest threat to human health. The list includes, among the others, MDR bacteria such as *Acinetobacter baumannii, Pseudomonas aeruginosa* and methicillin resistant *Staphyloccoccus aureus* (MRSA). They can provoke recalcitrant infectious especially in hospitals where they are the major challenge to patient safety resulting in one of the leading cause of death in the US and Europe with a high cost for the public health [10]. The redundancy in the discovery of already known antibiotics produced by well-known species increases the demand of searching novel drugs. Furthermore, when looking for new antibiotics or new molecules of pharmaceutical interest, the screening of microorganisms from extreme environments may lead to the identification of novel strains that can provide new formerly untapped compounds for white or green biotechnology [11]. Antarctica is one of the most extreme places on Earth. The isolated and unique nature of Antarctica, characterised by low temperatures, oligotrophic environment, long periods of light/dark, drawing the attention of scientists. The reasons are obvious; in comparison to many overexplored tropical areas, Antarctica and the ocean that surrounds it is home of a large number of unknown organisms adapted to the "extremely cold temperatures and conditions". These "extremophiles" exhibit physical and chemical adaptations not found elsewhere on the planet [12]. Bacteria belonging to the genus *Aequorivita* have been isolated from terrestrial and marine Antarctic habitats. They are members of the family Flavobacteriaceae and their biotechnological potential has not been investigated. Herein we report the isolation of *Aequorivita* sp. by employing the Miniaturized Culture Chip (MCC) method, the whole genomic sequencing of this strain, the analysis of the biosynthetic gene clusters, the evaluation of its antimicrobial and anthelmintic potential of intracellular extracts and finally the purification and identification of new antimicrobial compounds. To our knowledge, this is the first report aimed at the exploitation of *Aequorivita* genus as a source of bioactive compounds.

## 2.2 Material and Methods

### Collection of Antarctic sediments

Shallow water (50 cm depth) sediments were collected from 3 different sites during an expedition in the framework of National Antarctic Research Program (PNRA) in the area of Edmonson Point, Antarctica, 74° 20' (74.3333°) South, 165° 8' (165.1333°) East, using sterile 50 mL falcon tubes. Samples were transported using dry ice and stored at -80 °C until the beginning of the experiments.

### Design, fabrication and preparation of culture chips for the isolation of microorganisms

Miniaturized culture chips (MCC) were used to screen microorganisms grown to microcolonies [8, 9]. MCC contained arrays of microwells with a Porous Aluminium Oxide (PAO) base (8×36 mm, 60 µm thick, 40% porosity, 20 to 200 µm pore diameter) acting as a sterile filter. MCC was fabricated by patterning the wall material (Ordyl 300 film, Elga, Italy) using photolithography and then applying it to 8×36 mm strips of PAO arrayed on a silicon wafer. Walls were patterned by photolithography of 10 µm thick Ordyl 300 film with development according to the manufacturer's protocols. The resulting perforated and processed material was heat/pressure applied to the PAO. Platinum (10 nm) was used to sputter coat the upper surface of

the MCC. Wells were 180 µm diameter, spaced 160 µm in a hexagonal patterning with 4500 microwells per chip. MCC were sterilized using a high intensity UV ozone cleaner (PSD, Novascan, USA) for 15 min. For growth under near natural conditions were used two different conditions. Condition A, minimal: sediment was packed into a Petri dish and overlaid with a thin layer of agarose 1% mixed with a filtered solution of 0.001% $FeSO_4 \cdot 7H_2O$. Condition B, richer: sediment was packed into a Petri dish and overlaid with a thin layer of agarose 1% mixed with a filtered solution of 0.001% FeSO4 · 7H2O, 3% Sea Salt, 1 g/L Peptone, 0.5 g/L Yeast Extract. Sterile cultivation chips were placed on the solidified agarose and inoculated by spreading bacteria so that the probability of a single cfu/well was < 0.5. After 10 and 45 days of incubation at 7°C, the recovery of microcolonies from wells was carried out using a fine sterile toothpick using a dissection microscope to visualise the target microcolony. Finally, picked microcolonies were dissolved in 50 µL of sterile water. Half of it was added to a PCR mixture to perform the identification while the other half was stored at -80 °C with 20% glycerol.

**Molecular identification and phylogenetic analysis of isolated strains**

The freeze and thaw method was used to obtain bacterial genomic DNA, which was used as a template for the amplification via PCR of 16S rDNA genes. PCR was carried out in a total volume of 50 µL containing DreamTaq PCR Master Mix (a ready-to-use solution containing DreamTaq DNA Polymerase, optimized DreamTaq buffer, $MgCl_2$, and dNTPs) and 1 µM of primer Eub27F (Forward, seq: 5'-AGAGTTTGATCCTGGCTCAG-3') and Univ1492R (Reverse, seq: 5'-GGTTACCTTGTTACGACTT-3') [13]. The reaction conditions used were: one cycle (93 °C for 2 min), 30 cycles (92 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min), with a final extension of 5 min at 72 °C. PCR products were then purified, sequenced and submitted to BLAST for the phylogenetic analysis.

**Genome sequencing and assembling of *Aequorivita* sp.**

The DNA of *Aequorivita* sp., *Aequorivita lipolytica* CIP 107455$^T$, and *Aequorivita antarctica* CIP 107457$^T$ strains was obtained using the Wizard® Genomic DNA Purification Kit, PROMEGA. The whole genome sequencing was carried out at the Mutualized Microbiology Platform (P2M) of the Pasteur International Bioresources network (PIBnet) of the Institut Pasteur, Paris, France, using the Nextera XT DNA sample preparation kit (Illumina, USA) for 2x150-bp paired-end sequencing as per the manufacturer's instructions. All sequenced paired-ends reads were clipped and trimmed with AlienTrimme [14], corrected with Musket [15], merged (if needed) with FLASH [16], and subjected to a digital normalisation procedure with khmer [17]. For each sample, remaining processed reads were assembled and scaffolded with SPAdes [18]. Genomes were analysed using the anti-SMASH 3.0 web server (http://antismash.secondarymetabolites.org) and the Biosynthetic gene clusters were identified.

**Cultivation and extract preparation**

A single colony of a bacterial isolate was used to inoculate 3 mL of liquid Marine Broth (MB, Difco™) medium in sterile bacteriological tubes. After 48 h of incubation at 20 °C at 200 rpm, the culture was used to inoculate 125 mL of MB medium in a 500 mL flask, at an initial cell concentration of 0.01 $OD_{600}$/mL. The flasks were incubated up to five days at 20 °C at 200 rpm. The cultures were then centrifuged at 6000 rpm at 4 °C for 30 min. Then, the exhausted culture broth and the pellet were

collected and subjected to extraction with ethyl acetate separately, yielding the extracellular and intracellular extracts, respectively. Briefly, the exhausted culture broths (125 mL) were subjected to organic extraction using ethyl acetate (3x 125 mL ratio 1:1) in a 500 mL separatory funnel. The organic phase collected was evaporated to dryness using a Laborota 4000 rotary evaporator (Heidolph, Schwabach, Germany) affording the extracellular extract. The pellet was mechanically disrupted using an Ultraturrax for 5 min at 10000 rpm and then extracted by ethyl acetate as described for the exhausted broth, providing the intracellular extract. The dried extracts were weighed, dissolved in 100% DMSO at the final concentration of 50 mg/mL and stored at -20 °C. The same procedure was applied for the scale-up of the culture cultivating the bacterium in 10L of growth medium.

**Pathogen bacteria growth conditions**

The following MDR strains of human pathogens were used in this work: *Pseudomonas aeruginosa* DSM 50071, *Staphylococcus aureus* DSM 346, methicillin-resistant *Staphylococcus aureus* (*MRSA*) DSM 18827, *Klebsiella pneumonia* DSM 30104. Every strain was bought at Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The strains were routinely grown at 37°C in Luria Bertani medium (10 g/L Tryptone, 5 g/L Yeast extract, 10 g/L NaCl) at 200 rpm.

**Antimicrobial activity**

In order to evaluate the antimicrobial potential of the *Aequorivita* sp. strain extracts, samples were placed into each well of a 96-well microtiter plate at an initial concentration of 2% (*v/v*) and serially diluted using LB medium. A single colony of selected pathogen strains, *P. aeruginosa* DSM 50071, *S. aureus* DSM 346, *MRSA* DSM 18827 and *K. pneumonia*e DSM 30104 was used to inoculate 3 mL of liquid LB medium in sterile bacteriological tubes. After 6–8 h of incubation, growth was measured by monitoring the absorbance at 600 nm and about 40,000 CFU were dispensed in each well of the prepared plate. Plates were incubated at 37 °C for 24 h and growth was measured with a Cytation3 Plate Reader (Biotek, Winoosky, VT, USA) by monitoring the absorbance at 600 nm.

**Antihelminthic activity**

In order to test the effect of crude extracts on *Caenorhabtidis elegans* Wild-type (WT) Bristol N2 obtained from the *Caenorhabditis* Genetic Centre (CGC) a liquid toxicity assay has been set-up. The assay has been performed in 24-well plates. Each well contained a 400 µL solution of M9 buffer, 5 µg/mL cholesterol, and *Escherichia coli* OP50 as a food source [19], because these worms feed on bacteria, at the concentration of 0.5 OD/mL as nutrient source. The intracellular and extracellular extracts of *Aequorivita* sp. were then added at different concentrations to each well. *C. elegans* was synchronized by bleaching treatment [20], and 30-40 L4 worms were transferred to each well and incubated at 20 °C up to seven days. The wells were scored for living worms every 24 h. A worm was considered dead when it no longer responded to touch. For statistical purposes, 3 replicates per trial were carried out with a unique egg preparation. 1% DMSO was used as negative control.

**Cell viability assay (MTS assay)**

The assay to determine cell survival was conducted by using HFFF2 (ECACC 86031405) cells as target and the MTS as tetrazolium compound to determine the number of viable cells. Cells were seeded at 7.5 x $10^3$ cells/well in a sterile 96-well microtiter plate and allowed to adhere overnight by growing at 37 °C and 5% $CO_2$. The various concentrations of *Aequorivita* sp. intracellular extracts (30, 62, 125, 250, 500 µg/mL) were added. DMSO, the solvent used to dissolve the extracts served as negative control. Three replicate wells were used per concentration. The treated cells were then incubated for 24 h at 37 °C and 5% $CO_2$. After incubation, the medium was removed and a mixture consisting of 100 µl of fresh medium and 20 µl of reagent (MTS:PMS 19:1) was added to each well. The plate was again incubated for 1 h at 37 °C and 5% $CO_2$ in the dark to allow the reduction of MTS into a coloured formazan product by living cells. In order to quantify the amount of formazan produced, directly proportional to the number of living cells after extracts treatment, absorbance was measured at 490 nm, using an ELISA plate reader.

**Identification of the antimicrobial compounds**

Isolated molecules were chemically characterized by NMR spectroscopy and HRESIMS analysis in collaboration with the GEOMAR-Biotech of Kiel (Germany).

## 2.3 Results

**Isolation of Antarctic microorganisms using MCC**

An innovative device, the Miniaturized Culture Chip (MCC), was used to isolate Antarctic bacteria from shallow water sediments. Two different conditions, one minimal, containing the Antarctic sediments as only nutrient source and the other richer, composed by sediments, Peptone, Yeast extract and Sea Salt were tested, as described in MM. The MCC180.10 culture chip was directly placed on mixture agarose-sediments (Supplementary materials **Fig. S1**) and inoculated with serial dilutions of the same sediments present into the plates. After 10 days at 7 °C, the first colonies appeared, but the chip was kept in incubation up to 45 days, allowing the formation of slow-growing strains (**Fig. 1**). The minimal condition showed the same number of colonies (9 colonies) compared with rich medium condition (10 colonies) highlighting the importance of the sediments into the growth medium. A total of 19 colonies were visualized by microscope and collected. This system simulates the natural habitat, in which microorganisms live in communities, and allows the communication among the microorganisms present on the top and under the surface of the chip. Another advantage of the MCC is the possibility to grow microorganisms using as substrate their own natural environment that could contain all the nutrients necessary for the growth and also avoid slow-growing strains being overgrown by fast-growing strains.

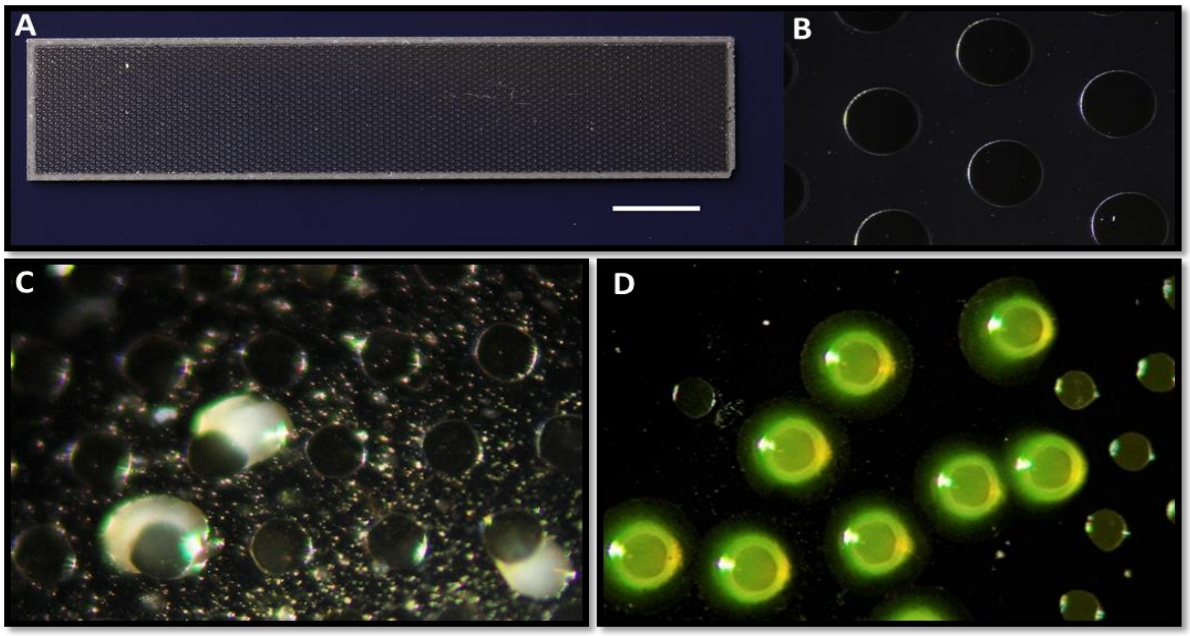



**Figure 1:** MCC180.10 culture chip picture. (**A**). Microscope view of the MCC180.10 (**B**). Bacterial colonies visible on the chip after 7 days of incubation (10X) (**C**). Example of GFP-transformed *Escherichia coli* grown on the chip (**D**). The scale bar indicates 4 mm when applied to (A) 200 µm when applied to (B) and 360 µm when applied to (C) and (D).

## Identification of bacterial isolates

19 microorganisms were isolated from the chip and used for the phylogenetic analysis. The phylogenetic affiliation of bacterial isolates was performed through the 16S rRNA genes amplification and analysis. For this purpose, the 16S rRNA genes were PCR amplified and the nucleotide sequence of the amplicons determined. Each sequence was used as a query in a BLAST search to retrieve the most similar ones. MEGA7 software [21] was used to align the sequences and to construct the phylogenetic tree shown in **Figure 2**. The molecular analysis showed a prevalence of *Pseudomonas* sp. and *Shewanella* sp. strains. Other strains such as *Psychrobacter* sp., one *Algoriphagus* sp. and one *Aequorivita* sp. were identified. This last strain, named, showing 96% of identity with *Aequorivita sublithincola*, was selected for further experiments because of the lack of previous studies on antimicrobial and antihelminthic activities.

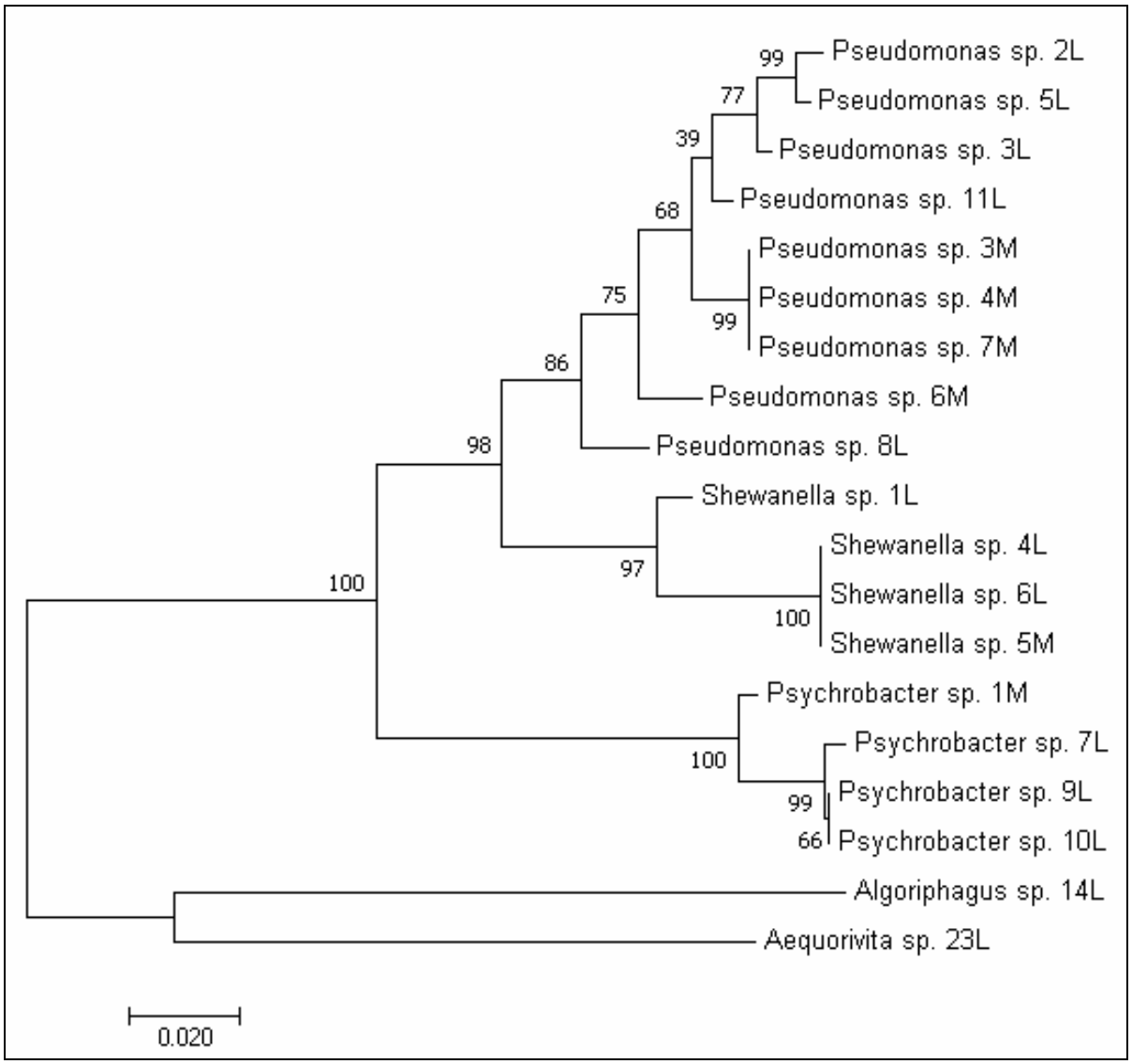



**Figure 2:** Evolutionary relationships of taxa and showing the placement of *Aequorivita* sp. strain. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.58717318 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences.

## Genome mining for *Aequorivita* sp.

The newly isolated strain *Aequorivita* sp. was deposited in the Collection of Institut Pasteur (CIP), France, under accession number CIP 111184$^T$. Due to the lack of information about the *Aequorivita* genus, the whole genomes of *Aequorivita* sp*., A. lipolytica* CIP 107455$^T$ and *A. antarctica* CIP 107457$^T$ strains were sequenced and subjected to a genome mining approach in order to identify the presence of biosynthetic gene clusters (BGCs). The antibiotics and Secondary Metabolite Analysis Shell (anti-SMASH) has served as a comprehensive web server and a stand-alone tool for the automatic genomic identification and analysis of BGCs, facilitating rapid genome mining of a wide range of bacterial and fungal strains. Using the anti-SMASH 3.0 web server is possible to compare identified BGCs with those encoding the biosynthetic pathways for known compounds from the “Minimum

Information about a Biosynthetic Gene cluster" (MIBiG) community project http://mibig.secondarymetabolites.org/ [22]. The gene cluster identification algorithms in tools such as anti-SMASH is based on the detection of one or more conserved protein domains using curated models; certain protein domains or domain combinations are indicative of biochemical functions that are specific to certain biosynthetic pathways; hence they can be used as "signatures" to identify certain classes of BGCs (Tracanna *et al*, 2017). The cluster arylpolyene-resorcinol, similar to the flexirubin biosynthetic gene cluster and the aryl polyenes (APE) biosynthetic gene cluster, were found in the genome of *Aequorivita* sp. as well as in the genome of the *A. antarctica* CIP 107457$^T$ and *A. lipolytica* CIP 107455$^T$ (Table 1). Different saccharide clusters were detected in all *Aequorivita* strains, mainly a cluster similar to the capsular polysaccharide biosynthetic gene. Furthermore, in the genome of *Aequorivita* sp. strain a gene with 69% of identity with the Type III polyketide synthase of *Zobellia galactanivorans* was detected in the gene cluster T3pks-arylpolyene (data not shown). An NRP/Polyketide cluster was detected in the *A. lipolytica* CIP 107455$^T$ strain (**Table 1**).

| Gene Cluster | Type | MIBiG BGC-ID | Most similar known cluster | *Aequorivita* sp. | *Aequorivita antarctica* CIP 107457T | *Aequorivita lipolytica* CIP 107455T |
|---|---|---|---|---|---|---|
| Cf_saccharide | Saccharide | BGC0000733_c1 | Capsular polysaccharide biosynthetic gene cluster | X | X | X |
| Cf_saccharide | Saccharide | BGC0000773_c1 | Lipopolysaccharide biosynthetic gene cluster; O-antigen biosynthetic gene cluster | X | - | - |
| Arylpolyene-Resorcinol | Polyketide | BGC0000838_c1 | Flexirubin biosynthetic gene cluster; Aryl polyene (APE) biosynthetic gene cluster | X | X | X |
| Cf_saccharide | Saccharide | BGC0000766_c1 | Exopolysaccharide biosynthetic gene cluster | - | X | X |
| Cf_saccharide | Saccharide | BGC0000781_c1 | O-antigen biosynthetic gene cluster | X | - | - |
| Cf_saccharide | Saccharide | BGC0000791_c1 | O-antigen biosynthetic gene cluster; Lipopolysaccharide biosynthetic gene cluster; Exopolysaccharide biosynthetic gene cluster | X | - | - |
| Cf_saccharide | Saccharide | BGC0000799_c1 | Colanic acid biosynthetic gene cluster | - | X | - |
| Cf_saccharide | NRP / Polyketide | BGC0000960_c1 | Azinomycin B biosynthetic gene cluster | - | - | X |

**Table 1:** Biosynthetic gene clusters (BGCs) in the *Aequorivita* sp. whole genome compared with two *Aequorivita* type strains: *A. antarctica* CIP 107457$^T$ and *A. lypolitica* CIP 107455$^T$ using the anti-SMASH 3.0 web server (http://antismash.secondarymetabolites.org).

## Evaluation of the antimicrobial activity of *Aequorivita* sp.

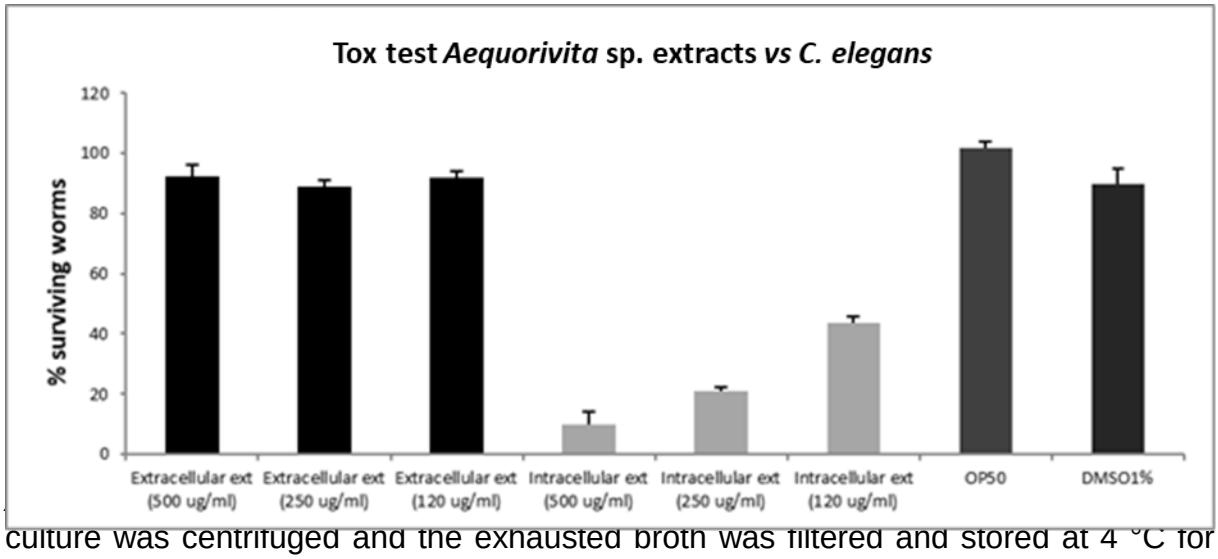
In order to evaluate the capability of the new isolate to produce antimicrobial compounds, intracellular and extracellular extracts from an *Aequorivita* sp. culture were generated. The bacterium was grown for five days in MB, producing a very strong yellow pigment, as described in MM. The $IC_{50}$ (half maximal inhibitory concentration) of the intracellular and extracellular extracts was calculated towards a panel of MDR pathogenic bacteria composed by *P. aeruginosa*, methicillin-resistant *S. aureus* (MRSA), *K. pneumoniae* and *A. baumannii*. Results summarised in **Table 2** show the absence of growth inhibition of the extracellular extract, while the intracellular extract shows an antimicrobial effect against three out four-target pathogens with a promising activity against MRSA, $IC_{50}$ value of 120 µg/mL (this concentration is referred to the crude extract).

| | $IC_{50}$ value | |
|---|---|---|
| **Strain** | **Intracellular** | **Extracellular** |
| ***P. aeruginosa*** | ***500 µg/mL*** | **No inhibition** |
| ***MRSA*** | ***120 µg/mL*** | **No inhibition** |
| ***A. baumannii*** | ***250 µg/mL*** | **No inhibition** |
| ***K. pneumoniae*** | **No inhibition** | **No inhibition** |

**Table 2:** Antimicrobial activity of *Aequorivita* sp. intracellular and extracellular extracts against a panel of MDR bacteria. Bacterial growth was measured in $OD_{600}$/mL. Values are reported in $IC_{50}$.

### Antihelminthic activity of *Aequorivita sp.* crude extracts

Encouraged by the antimicrobial potential showed by *Aequorivita sp.* towards the human pathogens, both intra and extracellular crude extracts were also tested for their ability to kill nematodes using *C. elegans* as a model system. Again, results demonstrated that the intracellular extract has significant *in vitro* antihelminthic effect after 3 days of incubation. As showed in **Figure 3**, it was able to kill *C. elegans* starting from a concentration of 500 µg/mL, where less than 20% of worms survived, until 120 µg/mL that correspond to the $IC_{50}$ value where the percentage of survival was about 50% suggesting a dose-response effect.




culture was centrifuged and the exhausted broth was filtered and stored at 4 °C for further analysis, while the intracellular material was extracted by ultraturrax and ethyl acetate. Subsequently, the crude extract (1 g) was fractionated with a SPE C18 Cartridge. Elution was performed stepwise with an increasing methanol concentration. The 4 eluted fractions were collected, dried and dissolved in DMSO to perform bioassay at a stock concentration of 50 mg/mL. The fraction eluted at 100% methanol was shown to be the most active one exhibiting, an increased antimicrobial activity against *MRSA* with a $IC_{50}$ of 15 µg/mL (**Table 3**), almost tenfold compared with crude extract and anthelmintic activity against *C. elegans* (Data not shown).

| Strain | $IC_{50}$ value of 100% MeOH fraction |
|---|---|
| ***P. aeruginosa*** | ***120 µg/mL*** |
| ***MRSA*** | ***15 µg/mL*** |
| ***A. baumannii*** | ***120 µg/mL*** |
| ***K. pneumoniae*** | ***500 µg/mL*** |

**Table 3:** Antimicrobial activity of the fraction eluted at 100% methanol against a panel of MDR bacteria. Bacterial growth was measured in $OD_{600}$/mL. Values are reported in $IC_{50}$.

For this reason, the 100% methanol fraction (120mg) obtained from SPE purification was purified by semiprep RP-HPLC/ELSD affording 21 peaks which were dried and dissolved in DMSO to perform the activity assays. Data obtained revealed the inhibitory activity against *MRSA* of eight compounds (**Table 4**)

| | ***$IC_{50}$ value of isolated compounds*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Strain** | **C-1** | **C-2** | **C-3** | **C-4** | **C-5** | **C-6** | **C-7** | **C-8** |
| ***MRSA*** | ***>200 µg/mL*** | ***>200 µg/mL*** | ***>200 µg/mL*** | ***90 µg/mL*** | ***22 µg/mL*** | ***145 µg/mL*** | ***>200 µg/mL*** | ***58 µg/mL*** |

**Table 4:** Antimicrobial activity of pure compounds against MRSA. Values are reported in $IC_{50}$

## Identification of antimicrobial compounds

The analyses based on the $^{1}$H-NMR and HRMS spectra of active peaks allowed identifying the chemical family of the compounds. They are aminolipids, N-(β-acyloxyacil)-amino acids including glycine and serine residue, saturated and unsaturated lipidic chains with different numbers of carbons (**Fig. 4**). The calculation of molecular formula and the comparison of the NMR data with the literature afforded the structure identification. The elucidated compounds are: **compound 1,** N- [15-Methyl-3-(13-methyl-tetradecanoyloxy)-hexadecanoyl]glycine, **compound 2,** N- [15-Methyl-3-(13-methyl-tetradecanoyloxy)-hexadecanoyl]glycine methylester, **compound 3,** N- [15-Methyl-3-(13-methyl-4Z-tetradecanoyloxy)-hexadecanoyl]glycine, **compound 4,** N- [15-Methyl-3-(13-methyl-4Z-tetradecanoyloxy)-hexadecanoyl]glycine methylester, **compound 5,** N- [15-Methyl-3-(12-methyl-tridecanoyloxy)-hexadecanoyl]glycine, **compound 6,** WB-3559 A, **compound 7,** WB-3559 B, both isolated by Uchida et. al. from *Flavobacterium sp*. [23] and **compound 8,** methylester of WB-35559B. Dereplication of isolated compounds, based on 1D, 2D NMR and HRMS indicated that **compounds 2**, **4** and **8** are new aminolipids. (The structure elucidation of the compounds was performed in collaboration with GEOMAR-Biotech of Kiel, Germany).

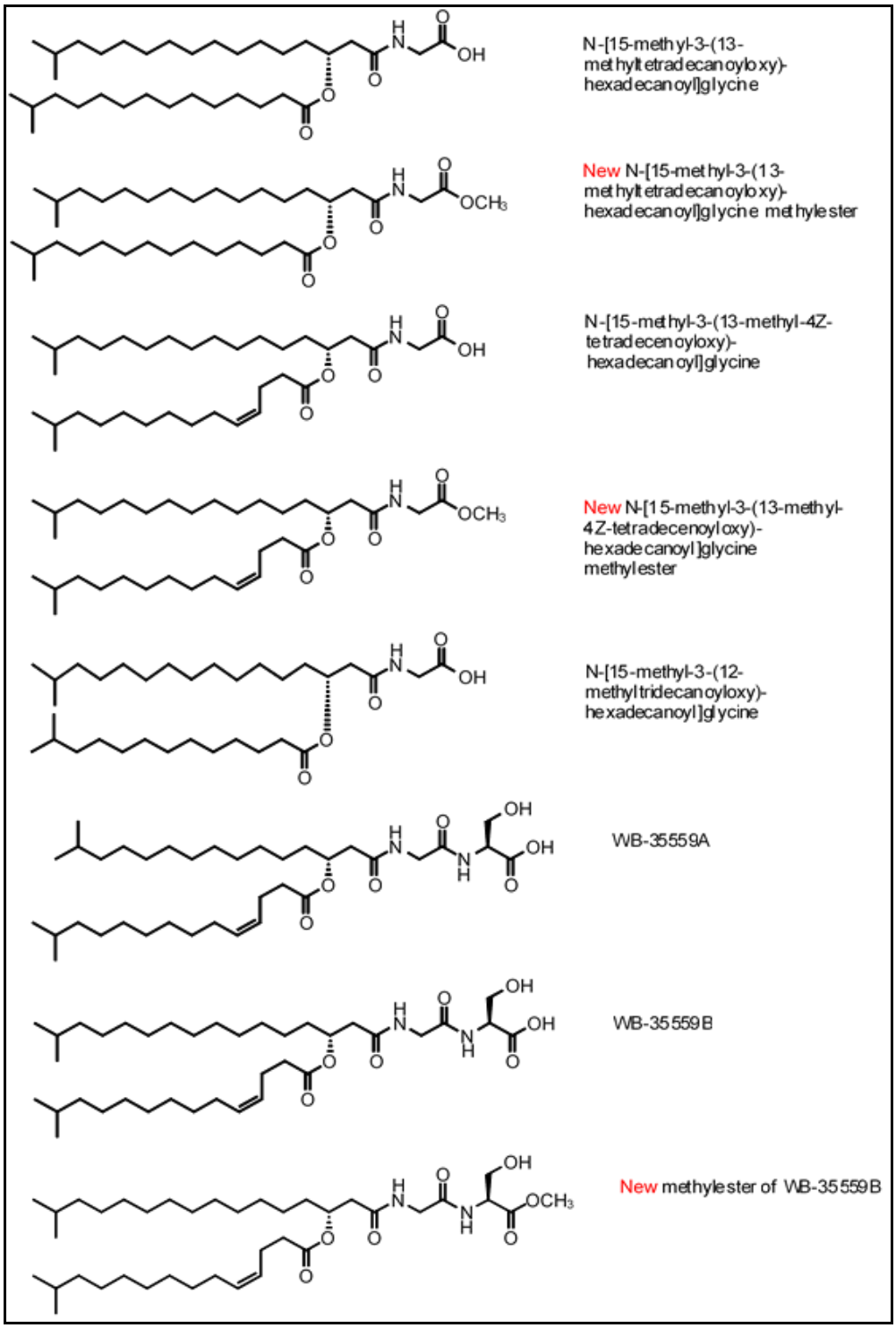



**Figure 4:** Molecular structures of antimicrobial compounds isolated from *Aequorivita* sp.

**Preliminary toxicity evaluation of 100% MeOH fraction**

The active 100% methanol fraction containing the mixture of aminolipids was assayed towards human cells to check the toxicity (In this case the fraction was used instead of the purified compounds for a preliminary toxicity evaluation. Further studies will allow the characterization of compounds). Cell viability assay was carried out by using human fibroblastic cells HFFF2 (ECACC 86031405), that represent a model system of skin cells. The value of the percentage of viable cells after the

treatment was obtained by normalising the value of absorbance in each well with the correspondent value of absorbance of DMSO, at the same concentration. Results demonstrated that the 100% MeOH fraction has no toxic effect against HFFF2 cells at concentration tested for the antimicrobial and anthelmintic activity (**Fig. 5**). Results are shown as percentage of viable cells after the treatment at different sample concentration.

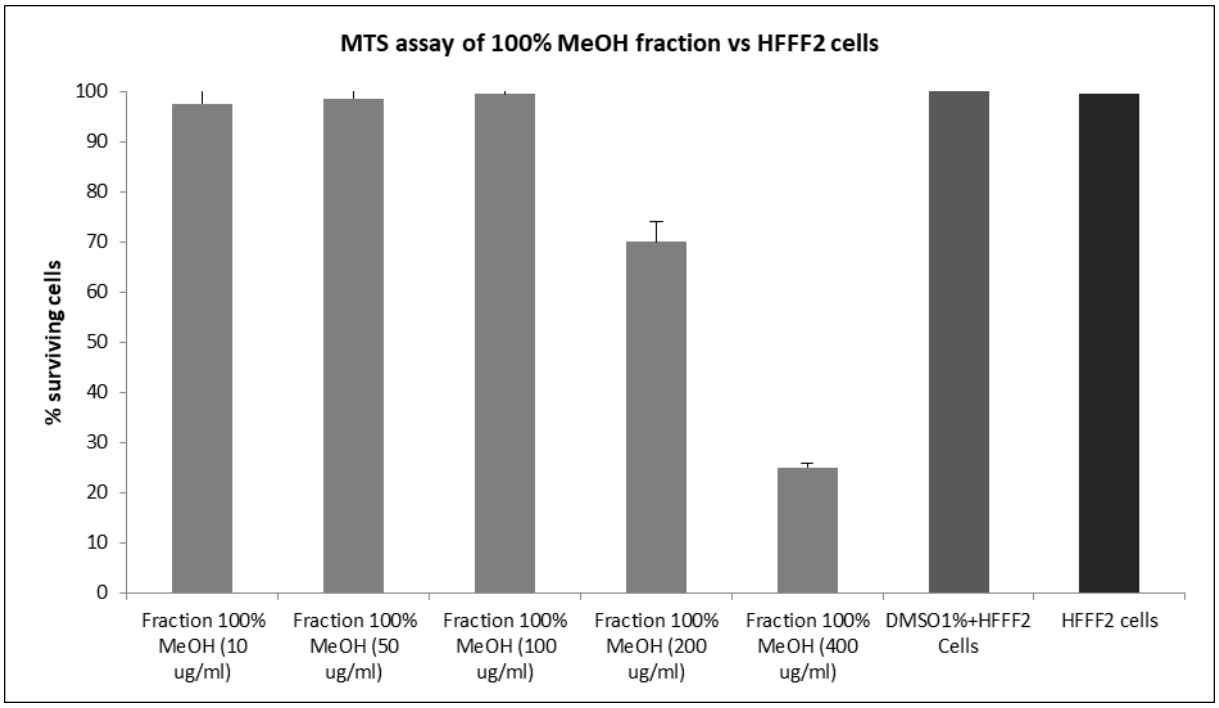



**Figure 5:** Cell viability assay using HFFF2 cells in the presence of different concentration of 100% MeOH *fraction of Aequorivita* sp. Values are reported as percentage of surviving cells.

## Preliminary analysis for the sustainable production of bioactive compounds exploiting *Aequorivita* sp. wastes

In order to make competitive the aminolipids production, a preliminary system for the valorisation of wastes was set-up. For each gram of intracellular extract from *Aequorivita* sp. many litres of MB are wasted. To overcome this problem, the exhausted broth generated from the bacterial cultivation was filtered using a 0.22 µm stericup (autoclaving the liquid broth is energy consuming and furthermore could denaturate many substances present in the medium) and used as a liquid substrate for other microorganisms. For this "second cultivation step" microorganisms isolated from the same sediments of *Aequorivita* sp. were used, such as *Pseudomonas sp*. 11L, *Psychrobacter* sp. 1M, *Shewanella* sp. 1L and *Algoriphagus* sp. 14L. First, the capability of the selected microorganisms to grow in an "exhausted broth" was checked evaluating the turbidity of the medium. As a fact, after only 24 h all the selected bacteria were grown (**Fig. 6**) suggesting that in the medium are still present nutrients or molecules produced during the "first cultivation step".

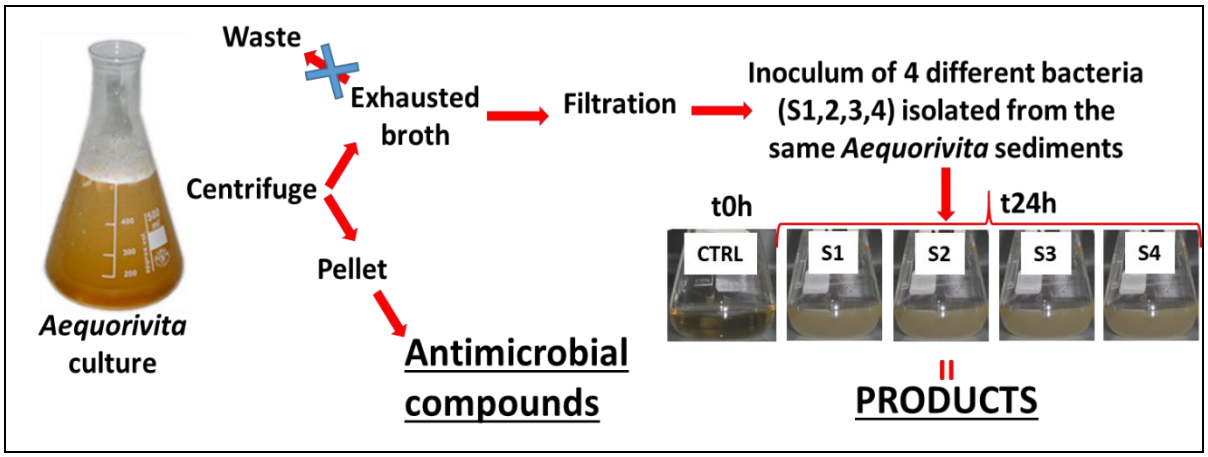



**Figure 6:** In the figure is schematized the process of exhausted broth recycling of *Aequorivita* sp. The first step is the separation of the pellet from the supernatant. Then, while the pellet is used for the production of antimicrobial compounds, the supernatant is filtered (0,22 µm filter) and used as liquid substrate for other strains.

After five days of incubation, the selected bacteria were analysed for their antimicrobial potential extracting the supernatant of the new cultivations by ethyl acetate and performing a liquid inhibition assay against MDR bacteria. Results revealed that crude extracts obtained by *Pseudomonas* sp. 11L and *Algoriphagus* sp. 14L inhibited *S. aureus* and *B. metallica* respectively (**Table 5-6**). Further studies are needed for the optimisation of this method.

| Extract concentration | 1 mg/mL | 0,5 mg/mL | 0,25 mg/mL | 0,13 mg/mL | 0,06 mg/mL | 0,03 mg/mL | 0,015 mg/mL | 0,007 mg/mL | 0,003 mg/mL | DMSO + Cells | Cells only |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pseudomonas* sp. 11L | 100 | 100 | 90 | 80 | 50 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Psychrobacter* sp. 1M | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Shewanella* sp. 1L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Algoriphagus* sp. 14L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 5:** Antimicrobial activity of crude extracts obtained from four strains grown in *Aequorivita* sp. exhausted broth against *S. aureus*. Bacterial growth is measured in $OD_{600}$/mL. Red colour indicates the strong inhibition of the extract at a certain concentration while green colour indicates no inhibition.

| Extract concentration | 1 mg/mL | 0,5 mg/mL | 0,25 mg/mL | 0,13 mg/mL | 0,06 mg/mL | 0,03 mg/mL | 0,015 mg/mL | 0,007 mg/mL | 0,003 mg/mL | DMSO + Cells | Cells only |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pseudomonas* sp. 11L | 40 | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Psychrobacter* sp. 1M | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Shewanella* sp. 1L | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Algoriphagus* sp. 14L | 100 | 96 | 75 | 53 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 6:** Antimicrobial activity of crude extracts obtained from four strains grown in *Aequorivita* sp. exhausted broth against *B. metallica*. Bacterial growth is measured in $OD_{600}$/mL. Red colour indicates the strong inhibition of the extract at a certain concentration while green colour indicates no inhibition.

## 2.4 Discussion

The lack of new antibiotic molecules entering the market and the overuse/misuse of the existing antibiotics are generating an alarming situation for human health. The spread of MDR bacteria underlines the urgent search for new antimicrobial lead compounds. Microbes have been a major source of natural antibiotics and playing a very significant role in historical and modern antibiotic drug discovery. However,

these efforts are seriously hampered by the culturability of microbes in artificial laboratory conditions. Furthermore, genetic (or genomic) studies show that the majority of the BGCs remain silent due to lack of natural cues, ecological challenges in such standard growth conditions and need to be induced using alternative methods [24]. Hence different strategies are mandatory for successful cultivation and the isolation of underexplored strains from new, untapped environments. In this work, we have isolated microorganisms from an extreme environment, Antarctica, using a Miniaturized Culture Chip (MCC). Antarctica is one of the most hostile places on Earth, and a survey conducted by the international Census of Marine Life demonstrated the extraordinary biodiversity of this extreme habitat and the marine environment around it [25]. This habitat has incredibly low temperatures and other extreme traits, such as long light/dark period and low availability of nutrients. These extreme conditions would be expected to shape the evolution and adaptation of both primary and secondary metabolism of the Antarctic bacteria, to lead to the production of unprecedented chemical molecules with unique functions [26]. In this research, the application of the MCC to Antarctic sediments led to the isolation of different genera of bacteria. This method allowed the isolation of microorganisms growing them directly on environmental sediments. The main idea of MCC is the simulation of the natural environment, laying the chip directly on natural sediments that become the only substrate for the growth of microorganisms. The same idea is the base of iCHIP, a new system composed of microwells and semipermeable membranes, which allowed the isolation of a new β-proteobacteria provisionally named *Eleftheria terrae* able to produce a new antibiotic, the teixobactin [27]. By the diffusion of nutrients obtained from sediments and by stimuli received from the bacterial community present in it, microbes can grow on the top of the chip as microcolonies. They are partially protected by the overgrowth of neighbouring microbes but available for imaging and recovery. One strain in particular, identified as *Aequorivita* sp. and deposited in the Collection of Institut Pasteur, France, attracted the attention for the almost complete absence of previous works especially about the production of bioactive compounds. Few strains belonging to the genus *Aequorivita* have been isolated from Antarctica (sea water, ice, algae, stones) [28] and even fewer studies about these bacteria were performed. The lack of previous works addressed to evaluate the bioactive potential of this genus makes this strain a good candidate for further analysis. One strain of this genus was isolated from the chip, together with an *Algoriphagus* sp. and other bacteria belonging to the genus *Pseudomonas* sp., *Shewanella* sp. and *Psychrobacter* sp. For this experiment, two nutritional conditions (A and B) were applied. In the condition A (minimal medium) sediments were covered by a thin layer of agarose mixed with $FeSO_4$. This element was proved to be a key factor for the isolation of previously uncultivated bacteria [29]. The condition B (rich medium) had the same composition of condition A but supplemented with sea salt, peptone and yeast extract. This differentiation was made to see some changes in the microbial community, but surprisingly, a similar number of colonies were observed without any significant variation in the strains distribution, with only exception of *Aequorivita* sp. and *Algoriphagus* sp. which were retrieved exclusively from condition A. This could indicate that bacteria used natural sediments as primary nutrient source for the growth, highlighting the importance of the reproduced microenvironment for bacterial survival. The diffusion of nutrients from sediments, the communication among microorganisms up and down the MCC, the protection of slower growing strains by an overgrowth of more aggressive strains by the MCC wells could be all important factors that allowed the cultivation of microorganisms.

The whole genome sequencing of *Aequorivita* sp. and its analysis by anti-SMASH indicated the presence of several BGCs. BGCs have been described for hundreds of bacterial metabolites and even though they can be accurately identified and quantified, the question still remains, which of these are most likely to encode the production of potent antimicrobials. The most prominent family of BGCs already described includes two subfamilies distributed throughout the Proteobacteria; their products are aryl polyenes (APEs) [30] also found in *Aequorivita* sp. and in other strains. Although these clusters are widely divergent in sequence, their small molecules are remarkably conserved, suggesting the important role that these compounds play in Gram-negative cell biology. The APEs are structurally similar to flexirubin, a pigment that was previously isolated from *Flexibacter elegans*. A role for APEs in protecting Gram-negative bacteria against oxidative stress make them analogous to the chemically similar Gram-positive carotenoids [30] although biosynthetically distinct. The strong yellow/orange *Aequorivita* sp. pigmentation could support the production of these pigments, and antioxidant assays are needed to confirm the expression of these genes. Numerous gene clusters encoding genes for saccharides were found. Cell-associated saccharides such as lipopolysaccharides, capsular polysaccharides and polysaccharides are known to play key roles in microbe-host and microbe-microbe interactions, while diffusible saccharides have a range of biological activities, most notably antibacterial [31, 32]. Non-ribosomal peptides (NRPs) are the major multi-modular enzyme complex which synthesizes secondary metabolites in bacteria and fungi and some genes were found in *A. lypolitica* CIP 107455$^T$, while in the genome of *Aequorivita* sp. a gene with 69% of identity with the Type III polyketide synthase of *Zobellia galactanivorans* was detected in the gene cluster T3pks-arylpolyene (data not shown), indicating a possible role of this cluster in the production of polyketides. The analysis of the antimicrobial potential of this bacterium revealed a promising inhibitory activity against some MDR pathogenic bacteria. In particular, the intracellular crude extract of *Aequorivita* sp. (obtained by mechanical disruption of bacterial cells followed by organic extraction) showed promising antimicrobial effect against MRSA which is the causative agents of serious hospital infections representing today one of the most dangerous antibiotic-resistant bacteria. vancomycin and linezolid have become the only drugs to counteract the infection but, treatment failures, adverse side effects and the early developed resistance make necessary alternative therapies [33]. Even if most of the antimicrobials are expressed outside the cell, the intracellular nature of some antimicrobial compounds is not unusual, although their ecological role is still not clear [34, 35]. They could be involved in intracellular signalling or have a defensive role [36]. Moreover, we have shown *Aequorivita* sp. intracellular extract to be active towards the nematode *C. elegans* used as model system for studies of anthelmintic drugs which are urgently needed to treat and control diseases which affect millions of people each year [37]. A preliminary purification step allowed the isolation of a fraction with an increased bioactivity against the MDR bacteria, especially MRSA without toxic effects against human cell lines. The final purification and identification of pure molecules revealed the presence of 8 active aminolipids, 5 known compounds and 3 never isolated before. Some bacteria species are known to contain in their inner and outer membranes amphipathic lipids based on one or two amino acids linked to a fatty acid through an amide bond and sometimes another through an ester bond. New forms of these compounds are frequently described in marine organisms. Among them, unusual structures formed of fatty acids and amino acid derivatives (bromotyrosine) were isolated from sponges, they were named

mololipids and showed anti-HIV activity [38]. Numerous examples of bacterial lipids containing amino acids or peptides are known [39] and many of them display interesting properties as antibiotics [40] and biosurfactants [41] attracting the interest of pharmaceutical and biotechnological industries. Compound 1 produced by *Aequorivita* sp. was previously isolated from a marine bacterium *Cytophaga sp*. SANK 71996 as N-type calcium channel blockers [42]. *Cytophaga* strains are Gram-negative, rod-shaped or filamentous gliding bacteria. Some of these bacteria belong to 'Cytophaga-Flavobacterium-Bacteroides' group. The genus *Aequorivita*, belongs to the family Flavobacteriaceae and contains five recognized species: *Aequorivita antarctica*, *A. lipolytica*, *A. crocea*, *A. sublithincola* [28] isolated from Antarctic terrestrial and marine habitats and *A. capsosiphonis* [43] isolated from a green alga collected from the South Sea, Republic of Korea. Previous analysis of fatty acids revealed the presence of aminolipids but they were never isolated, characterized or used as antimicrobial molecules. To our knowledge, this is the first report describing the potential of *Aequorivita spp*. as producers of antimicrobial compounds. The multi-approach applied in this work involving the isolation by MCC, the analysis of BGCs and the final identification of new antimicrobial compounds emphasize the importance of unexplored microorganisms as a source of new bioactive compounds for biotechnological and pharmaceutical applications.

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## 2.6 Supplementary materials

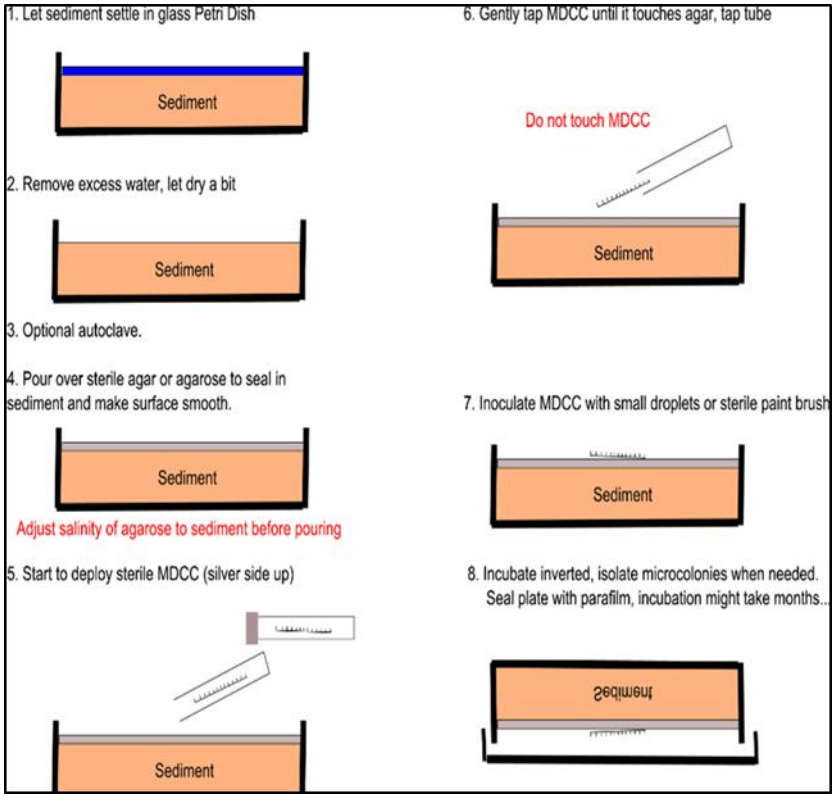


**Figure S1.** Miniaturized Culture Chip (MCC) is basically, a large number of miniaturized “Petri Dishes on-a-chip” made out of porous aluminium oxide (PAO). The sediments in the Petri dish are covered with a thin layer of agarose. The chip is placed directly on the agarose layer and then is inoculated with a solution obtained from the same sediments. Nutrients from beneath the chip diffuse through the pores to the surface and consequently supply microorganisms with nutrients that are growing on top of the surface as microcolonies.

# CHAPTER 3

# The antimicrobial potential of algicolous marine fungi for counteracting multidrug-resistant bacteria: phylogenetic diversity and chemical profiling

# The antimicrobial potential of algicolous marine fungi for counteracting multidrug-resistant bacteria: phylogenetic diversity and chemical profiling

## Abstract

Marine fungi represent an important but still largely unexplored source of novel and potentially bioactive secondary metabolites. The antimicrobial activity of nine sterile mycelia isolated from the green alga *Flabellia petiolata* collected from the Mediterranean Sea was tested on four antibiotic-resistant bacterial strains using extracellular and intracellular extracts obtained from each fungal strain. The isolated fungi were identified at the molecular level and assigned to one of the Dothideomycetes, Sordariomycetes or Eurotiomycetes classes. Following assessment of inhibition of bacterial growth ($IC_{50}$), all crude extracts were subjected to preliminary $^{1}H$ NMR and TLC analysis. According to preliminary pharmacologic and spectroscopic/chromatographic results, extracts of fungal strains MUT 4865, classified as *Beauveria bassiana*, and MUT 4861, classified as Microascacea sp.2, were selected for LC-HRMS analysis. Chemical profiling of antibacterial extracts from MUT 4861 and MUT 4865 by LC-HRMS allowed identification of the main components of the crude extracts. Several sphingosine bases were identified, including a compound previously unreported from natural sources, which gave a rationale to the broad spectrum of antibacterial activity exhibited.

## 3.1 Introduction

The worldwide diffusion of antibiotic-resistant microorganisms requires the development of new efficient antimicrobial molecules. For more than half a century, the main strategy for obtaining new antimicrobial agents has consisted of semisynthetic remodelling of natural products. However, drugs obtained in this way are only temporarily effective against pathogenic microorganisms, which develop antibiotic resistance [1]. The problem regarding microbial resistance to antibiotics may be overcome by the discovery of new natural products which, due to their chemical novelty, could inhibit unknown single or multiple microbial targets. The search for natural products of pharmaceutical interest in the marine environment has been progressing at an unprecedented rate, resulting in the discovery of a number of molecules, many of which have new carbon skeletons and interesting biological activities [2,3]. Among marine microorganisms, fungi play a crucial role, as they are a reservoir of biologically active secondary metabolites [4-6]. Recently, several new metabolites from marine fungi have been reported to display notable antibacterial activities [7-9]. Despite their proven biosynthetic potential, scientific research has not intensively focused on marine fungi for seeking new drugs [10]. However, promising fungi are equipped with gene clusters potentially involved in the biosynthesis of secondary metabolites [11]. Therefore, research into the isolation, identification and characterization of new fungal strains capable of producing useful bioactive natural

compounds should be carried out. Hence, the aim of this work was to assess the antibacterial potential of nine sterile mycelia isolated from the green alga *Flabellia petiolata* collected from the Mediterranean Sea, against some representative multidrug-resistant (MDR) bacteria, relevant in cystic fibrosis and nosocomial infections, and to analyse the chemical profiles of the most active fungal crude extracts.

## 3.2 Material and methods

### Fungal strains

Fungi were isolated and roughly identified from the green alga *F. petiolata* collected in March 2010 near Elba Island in the Mediterranean Sea [12], and are preserved at the Mycotheca Universitatis Taurinensis - MUT (DBIOS - University of Turin). All selected fungi were revealed to be sterile mycelia and were identified by molecular analysis (**Table 1**).

| MUT code | Fungal taxa | GenBank accession number ITS and LSU |
|---|---|---|
| 4883 | Biatriospora sp. | KR014352<br>KP671728 |
| 4865 | *Beauveria bassiana* | KR014380<br>KP671729 |
| 4860 | Massarina sp. | KR014362<br>KP671730 |
| 4885 | Microascacea sp.1 | KR014356<br>KP671717 |
| 4861 | Microascacea sp.2 | KR014360<br>KP671746 |
| 4859 | Roussoellacea sp.1 | KR014355<br>KP671716 |
| 4886 | Roussoellacea sp.2 | KR014358<br>KP671720 |
| 4966 | Roussoellacea sp.3 | KR014366<br>KP671740 |
| 4979 | *Knufia petricola* | KR014376 |

**Table 1:** MUT code, taxonomic assessment of sterile mycelia isolated from *F. petiolata* and GenBank accession numbers.

### Molecular, bioinformatics and phylogenetic analyses

Genomic DNA was extracted using cetyl trimethyl ammonium bromide (CTAB, Sigma-Aldrich St. Louis, USA) according to the protocol of Graham et al. [13]. The nrDNA internal transcribed spacer (ITS) and large ribosomal subunit (LSU) partial regions were amplified using universal primers ITS1F/ITS4 (Sigma-Aldrich St. Louis, USA) and LR0R/LR7, as previously described [14]. Amplification products were sequenced at Macrogen Europe (The Netherlands). Sequences were checked and assembled using Sequencher 4.9 software and compared to those available in the GenBank database using the BLASTn option of the BLAST program (www.blast.ncbi.nlm.nih.gov) and CBS Mycobank pairwise sequence alignment (www.mycobank.org). Newly generated sequences were deposited in the GenBank database and were assigned accession numbers reported in Table 1. Phylogenetic analysis was performed only on LSU sequences, as comparable ITS sequences of fungi studied in this article are rarely found in public databases and/or are poorly informative. LSU sequences were selected for phylogenetic analysis on the basis of BLASTn and CBS results. Two sequence datasets were composed, following reference [14] for Pleosporales and reference [15] for Sordariomycetes. Alignments

were generated using MEGA 5.10 [16] and manually refined. Phylogenetic analyses were performed using both Bayesian inference (BI; MrBayse3.2.2) [17] and maximum likelihood (ML; RAxML v.7.3.2) [18] approaches, as previously described [14]. Bayesian posterior probability (BPP) values over 0.6 (with MLB over 50%) are reported in the resulting trees.

**Fungal growth conditions**

Preliminary growth condition tests were performed in order to define the most effective and appropriate medium for inducing production of bioactive secondary metabolites in the selected fungal strains. Each fungal strain was inoculated in duplicate by 10 agar plugs of 5 mm diameter cut from the edge of actively growing culture onto malt extract agar in 150 mL flasks containing 100 mL of three different media: PCB (10 g of crushed potatoes and 10 g of crushed carrots in 1 L of dd$H_2O$), MeCl (20 g malt extract, 17 g NaCl in 1 L of dd$H_2O$) and WST30 (10 g glucose monohydrate, 5 g soya peptone, 3 g
malt extract, 3 g yeast extract, 30 g NaCl). Flasks were incubated in the dark at 24 °C and rotated at 150 rpm. The broth and mycelium of each strain were collected after 2 and 4 weeks and submitted to an extraction procedure for preliminary biochemical analysis (see below). MeCl medium and 4-week incubation were selected as the best conditions (24 °C in the dark). Hence, each fungus was inoculated (100 agar plugs of 5 mm diameter) in 2 L flasks containing 1.5 L of MeCl, which was incubated in the dark at 24 °C at 180 rpm for 4 weeks.

**Extract preparation**

Samples were centrifuged at 11,200 x rcf for 30 min at 4 °C and filtrated in order to separate mycelium from culture broth. Supernatants were extracted with ethyl acetate (EtOAc) and the resulting extracts were dried out using a Rotavapor, weighed, solubilized in dimethyl sulfoxide (DMSO, 100%) at a final concentration of 100 mg/mL and stored at -20 °C. The presence of antimicrobial compounds in the mycelia was also evaluated. In order to efficiently lyse the cells, different mechanical disruption methods were used in a sequential manner. The first step consisted of homogenization with Ultra Turrax T25 (IKA-Werke, Staufen, Germany). The homogenate was then washed twice with 20 mL of EtOAc to recover intracellular extract; in addition, to improve fungal lysis, mycelia were treated with liquid nitrogen (15 mL $N_2$/g mycelium). Samples were transferred to precooled mortar and minced under liquid nitrogen with a pestle and washed twice with 20 mL of EtOAc. At the last step, to completely destroy the membrane, all mycelium was transferred and processed in a Potter-Elvehjem homogenizer (Sigma-Aldrich, Saint Louis, MO) in the presence of EtOAc. Subsequently, the powdered mycelium was transferred to a separator funnel and mixed five times with two volumes of EtOAc. In order to increase the yield of some extracts, mycelia were further soaked in acetone for 18 h under agitation. The whole EtOAc and acetone fractions were collected and dried out using a Rotavapor. Final extracts were weighed, solubilized in DMSO (100%) at a final concentration of 100 mg/mL and stored at -20 °C.

**Antimicrobial assay**

The extracts produced as such were checked for the ability to inhibit growth of a selected panel of human pathogens. An $IC_{50}$ assay was used to evaluate the concentration of extracts at which bacterial target growth was inhibited by 50%. The following multidrug-resistant bacteria were used for antimicrobial screening:

*Burkholderia metallica* LMG 24068 [19], *Pseudomonas aeruginosa* PA01 [20], *Klebsiella pneumoniae* DF12SA [21] and *Staphylococcus aureus* 6538P [22]. All bacteria were routinely grown at 37 °C in lysogeny broth (5 g yeast extract, 10 g sodium chloride, 10 g tryptone in 1 L of dd$H_2O$), with the exception of *S. aureus*, which was grown in Mueller Hinton broth (Applichem, Darmstadt, Germany). Extracts were placed into each well of a 96-well microtiter plate at an initial concentration of 2 mg/mL and serially 2-fold-diluted using the appropriate medium. Wells containing only DMSO (2% v/v) were used as a control to determine the effect of this solvent on bacterial growth. Cells were prepared as follows: a single colony of each pathogenic strain was used to inoculate 3 mL of liquid medium in a sterile bacteriological tube. After 5-8 h of incubation, growth was measured by monitoring the absorbance at 600 nm and about 40,000 colony-forming units were dispensed into each well of the prepared plate. Plates were incubated at 37 °C for 20 h and growth was measured using a VICTOR X Multilabel Plate Reader (PerkinElmer, Waltham, MA) by monitoring the absorbance at 600 nm.

**Metabolic profiling of crude extracts**

All crude extracts were subjected to thin layer chromatography (TLC) analysis and $^1H$ nuclear magnetic resonance (NMR). TLC analysis was carried out on Alugram silica gel G/UV254 plates with a solvent mixture of different polarity using vanillin reagent as the revelation system; $^1H$ NMR analysis were performed with a Varian INOVA 400 MHz instrument in $CDCl_3$ solvent at room temperature with tetramethylsilane (TMS) as internal reference. Selected extracts were analyzed using an LTQ XL liquid chromatography high resolution mass spectrometry system (LC-HRMS) (ThermoScientific) equipped with an Accelera 600 pump and Accelera auto sampler system. A volume of 10 µL of sample was injected at a concentration of 10 mg/mL in methanol. The mixture was separated on a Phenomenex LUNA C8 (150 x 2.1 mm, 5 mm particle size) column at a flow rate of 200 mL/min using an acetonitrile-water gradient. Mobile phase A was 90% $H_2O$ 10% acetonitrile (ACN) 0.1% formic acid (FA) and mobile phase B was 10% $H_2O$ 90% ACN 0.1% FA; the gradient started at 10% B up to 90% B in 70 min and was kept at 90% of B for 10 min before the reequilibration step. The mass spectrometer operated in positive electrospray ionization (ESI) mode at 4 kV capillary voltage and 280 °C. The calibration procedure was carried out using a ThermoScientific positive calibration solution composed of caffeine, MRFA and Ultramark. All spectra were acquired in the m/z range from 280 to 700 u.m.a., setting resolution at 30,000; MSMS spectra were acquired in an opportune m/z range using 35 collision energy. Thermo-Scientific software Xcalibur was used to obtain molecular formulas (MFs). MFs deduced by high-resolution electrospray ionization mass spectrometry (HRESIMS) were checked by available data banks [23-25] and, in the case of alternative structures, they were discriminated by $MS^n$ analysis using data available in the literature [26] or ex novo analysis, and then by checking diagnostic signals in the $^1H$ NMR spectrum of the crude extracts.

## 3.3 Results

**Phylogeny and taxonomic identification of fungal isolates**

Molecular and phylogenetic analyses revealed that strains MUT 4859, MUT 4860, MUT 4883, MUT 4886 and MUT 4966 belong to the order Pleosporales (Dothideomycetes class). In particular, MUT 4860 was identified as *Massarina sp*. and MUT 4883 as *Biatriospora sp*., both clustering in the Biatriosporaceae family, while MUT 4859, MUT 4886 and MUT 4966 were identified at the family level (Roussoellaceae) [27]. MUT 4861, MUT 4865, and MUT 4885 belonged to the Sordariomycetes class; specifically, MUT 4865 belonged to *Beauveria bassiana*, while MUT 4861 and MUT 4885 clustered within the Microascaceae family. Finally, MUT 4979 was identified as *Knufia petricola* (syn. *Sarcinomyces petricola*, Incertae sedis, Chaetothyriales, Eurotiomycetes) by both ITS and LSU sequences (homology percentage = 99%).

**Antimicrobial activity**

In order to select the best growth medium for producing the antimicrobial compounds, preliminary extractions and antimicrobial assays were performed on small-scale cultures of fungi grown in MeCl, PCB and WST30. These analyses demonstrated that fungi grown in MeCl exhibited the highest degree of antimicrobial activity (Supplementary materials **Table S1**). This medium was therefore selected for further experiments. Moreover, the antimicrobial potentials of the extracellular and intracellular extracts were compared; results revealed that the latter exhibited the highest yield and activity (Supplementary materials **Table S2**). Starting from these preliminary results, extracts obtained from mycelium lysates were used for antimicrobial screening, targeting a panel of MDR human pathogens. The antimicrobial activity displayed by the different fungal strains against the four MDR bacteria is reported in **Table 2** as $IC_{50}$ values. The resistance of each strain to ampicillin, chloramphenicol, kanamycin, tetracycline and trimethoprim was confirmed by liquid inhibition assays. Extracts produced from strains MUT 4861, MUT 4865 and MUT 4979 were shown to be the most active and promising ones. In particular, MUT 4861 was able to strongly inhibit *B. metallica* ($IC_{50}$ 0.5-0.25 mg/mL) and *S. aureus*, and was the only one to show, by both EtOAc and acetone extracts, an inhibitory effect against *P. aeruginosa*. Both extracts from MUT 4865 were able to inhibit *B. metallica* and *S. aureus* ($IC_{50}$ 0.5-0.25), and the EtOAc extracts also showed inhibition against *K. pneumoniae*. No effects were observed against *P. aeruginosa*. The extract from MUT 4979 showed antimicrobial activity against three out of the four pathogens ($IC_{50}$ 1.0-0.25), with the exception of *K. pneumoniae*. Extracts of MUT 4859, 4860, and 4966 only showed significant activity against *B. metallica* and *S. aureus*, which were the bacterial strains most sensitive to the fungal extracts. MUT 4883, 4885 and 4886 extracts were the weakest strains, showing no significant effects against the target bacteria. Acetone extracts showed similar antimicrobial activity compared to EtOAc extracts. The only exception was MUT 4861, of which the acetone extract was more active than the EtOAc extract. Overall, the most promising strains were MUT 4865, 4979 and 4861, which exhibited the highest degree of antibacterial activity.

| Fungi | $IC_{50}$ (mg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| MUT code | *B. metallica* LMG 24068 | | *P. aeruginosa* PA01 | | *K. pneumoniae* DF12SA | | *S. aureus* 6538P | |
| | Ethyl acetate | Acetone | Ethyl acetate | Acetone | Ethyl acetate | Acetone | Ethyl acetate | Acetone |
| 4859 | 0.5-0.25 | >2.0 | >2.0 | >2.0 | >2.0 | >2.0 | 1.0-0.5 | >2.0 |
| 4860 | 0.5-0.25 | 0.5-0.25 | >2.0 | >2.0 | >2.0 | >2.0 | 2.0-1.0 | >2.0 |
| 4861 | 0.5-0.25 | 0.5-0.25 | 2.0-1.0 | 1.0-0.5 | >2.0 | 2.0-1.0 | 1.0-0.5 | ND |
| 4865 | 0.5-0.25 | 0.5-0.25 | >2.0 | >2.0 | 1.0-0.5 | >2.0 | 0.5-0.25 | 0.5-0.25 |
| 4979 | 1.0-0.5 | ND | 1.0-0.5 | ND | >2.0 | ND | 0.5-0.25 | ND |
| 4966 | 1.0-0.5 | ND | >2.0 | ND | >2.0 | ND | 1.0-0.5 | ND |
| 4885 | 2.0-1.0 | ND | >2.0 | ND | >2.0 | ND | 2.0-1.0 | ND |
| 4886 | 2.0-1.0 | ND | >2.0 | ND | >2.0 | ND | 2.0-1.0 | ND |
| 4883 | 2.0-1.0 | ND | 2.0-1.0 | ND | >2.0 | ND | 2.0-1.0 | ND |

**Table 2:** Antimicrobial activity of the fungal intracellular extracts vs four bacterial strains belonging to different species. The data are reported as capacity to inhibit the microorganism growth in more than 50% ($IC_{50}$). Growth in the presence of 2% DMSO was considered as 100% growth. ND: Not detected.

## Secondary metabolite analyses

Based on results of preliminary pharmacologic, spectroscopic and chromatographic screening, extracts of MUT 4865 and MUT 4861 were selected for chemical profiling and were analysed by LC-HRMS. Other strains did not produce detectable amounts of secondary metabolites under culture conditions; therefore, their potential for secondary metabolite production will require further investigation.

## *Beauveria bassiana* MUT 4865

Both acetone and EtOAc extracts were subjected to HRESIMS analysis (**Fig. 1A**). Compound 1 was analysed for $C_{22}H_{43}O_2N$ by HRMS analysis (calculated for $C_{22}H_{43}NO_2$ Na:376.3192, found $[M+Na]^+$: 376.3195). In the $MS^2$ spectrum, the sequential loss of one ammonia and two neutral water molecules indicated the presence of one amino and two hydroxyl groups. The planar structure of this compound was deduced from analysis of the $MS^3$ spectrum, which showed a fragmentation pattern compatible with localization of the two double bonds at the unusual positions of 6 and 17, revealing that it corresponded to the long chain sphingadienine (**Fig. 2**). Therefore, a 1,3-dihydroxy-2-amino-6,17-docosadiene structure was tentatively proposed. Assignment of the relative configuration of the two contiguous stereogenic centers as well as of the two double bonds would require isolation of the compound from a large-scale cultivation batch of the fungal strain (see **Fig. 3**). As shown in **Fig. 1B**, the acetone extract did not contain a detectable amount of compound 1, whereas some sphingosine compounds were detected, such as phytosphingosine (2), dihydrosphingosine (3) and phytoceramide C2 (4).

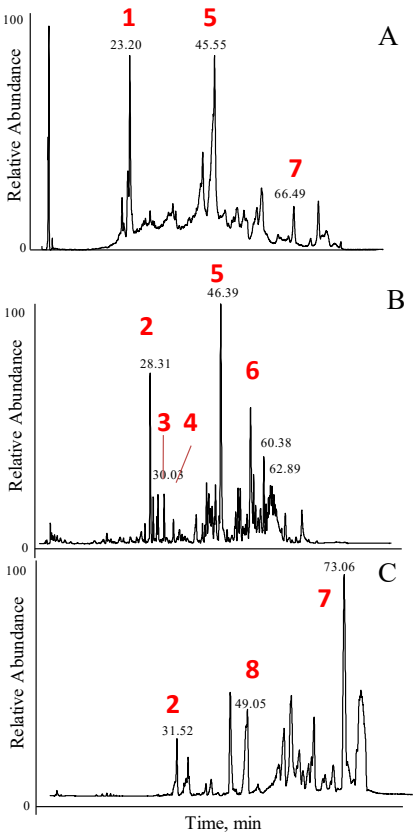


**Fig. 1**. ESI positive mode base peak chromatograms of the active samples MUT 4865 EtOAc extract (panel A), acetone extract (panel B) and MUT 4861 acetone extract (panel C). Numbers above the peaks identify the metabolites listed in Tables 2 and 3.

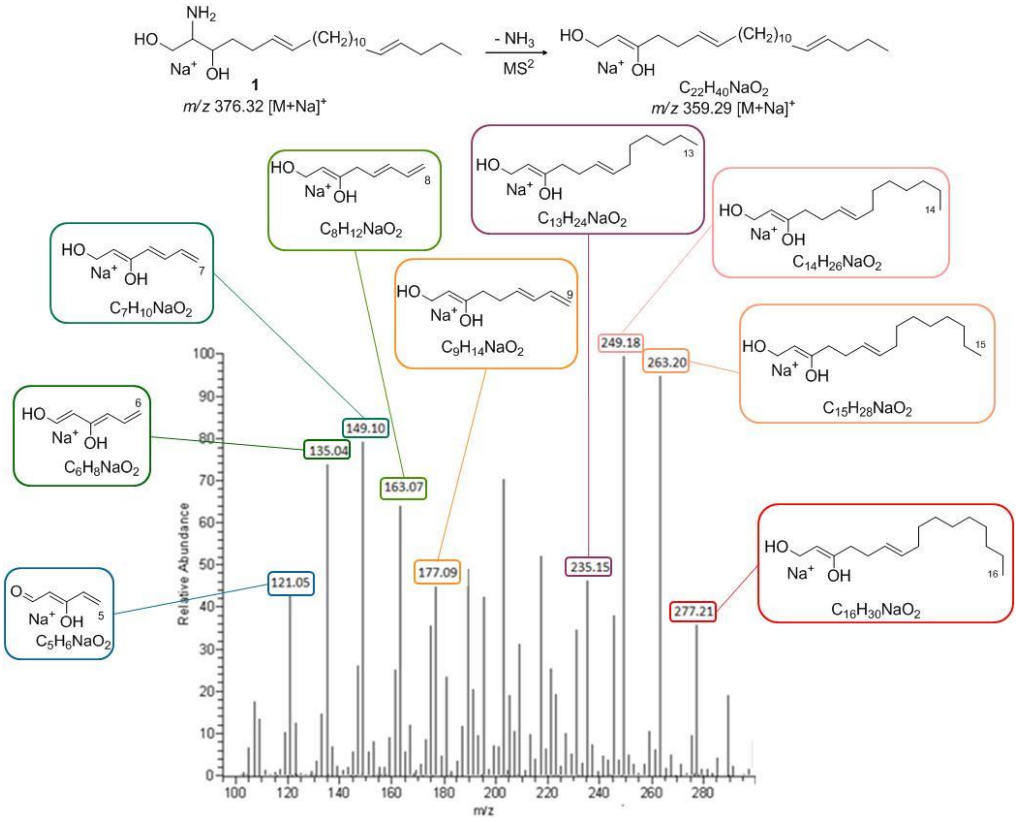


**Fig. 2**. $MS^3$ ESI positive mode spectrum of the precursor ion at m/z 359.30 derived from MSMS at m/z 376.31 and its proposed fragmentation.

$MS^2$ pattern analysis (**Table 3**) led to a straightforward assignment of a planar structure for these compounds. Compound 5, which was present in both EtOAc and acetone extracts, was tentatively identified as aphidicolin; compound 6 was tentatively identified as fusoxysporone and compound 7, a minor component of the EtOAc extract, was identified as bis (2-ethylhexyl) hexanedioic acid.

| Strain | RT (min) | MS and MS/MS | Suggested MF | Proposed structure |
|---|---|---|---|---|
| MUT4865 | 23.20 | 376.3195 $[M+Na]^+$ (Δppm: 1.049) $MS^2$ (**S5**): 359.29, 341.28; $MS^3$ see **Figure 2** | $C_{22}H_{43}NO_2$ | 1,3-dihydroxy-2-amino-6,17-docosadiene (**1**) |
| | 28.32 | 318.30015 (Δppm: -0.379) $MS^2$ (**S5**) :300.29, 282.29, 265.33 | $C_{18}H_{39}NO_3$ | 2-aminooctadecan-1,3,4-triol (4-hydroxysphiganine or phytosphingosine) (**2**) |
| | 29.11 | 302.30543 (Δppm: 0.245) $MS^2$ (**S6**): 284.29, 266.31, 249.26 | $C_{18}H_{39}NO_2$ | 1,3-dihydroxy-2-aminooctadecane (dihydrosphingosine) (**3**) |
| | 30.03 | 360.31079 (Δppm: -0,126) $MS^2$ (**S6**) :342.31,324.32, 300.31, 264.30, 212.19 | $C_{20}H_{42}NO_4$ | N- Acetyl-1,3,4-trihydroxy-2-aminooctadecane (phytoceramide C2) (**4**) |
| | 45.65 | 339.25320 (Δppm: - 0.876) | $C_{20}H_{34}O_4$ | Aphidicolin (**5**) |
| | 54.04 | 287.23634 (Δppm: 0.584) $MS^2$ (**S7**): 269.23, 203.14, 175.11 [25] | $C_{20}H_{30}O$ | Fusoxysporone (**6**) |
| | 60.38 | 395.3309 (Δppm: 0.145) | $C_{28}H_{42}O$ | Ergosta-5,7,22-trien-3-β-ol (ergosterol) |
| | 62.89 | 393.3153 (Δppm: 0.401) | $C_{28}H_{40}O$ | Ergosta-4,6,8(14),22-tetraen-3-one |
| | 66.49 | 371.31453 (Δppm: -1.056) $MS^2$ (**S7**): 259.01, 240.70, 146.9, 128.9, 110.99 [36] | $C_{22}H_{42}O_4$ | Bis(2-ethylhexyl) hexanedioic acid (**7**) |

**Table 3**: Annotated peaks observed in the chromatograms of the EtOAc and Acetone extracts of *Beauveria bassiana* MUT 4865.

**Microascacea sp.2 MUT 4861**

The EtOAc extract contained a very complex mixture of lipid and polysaccharide components evidenced by $^{1}H$ NMR analysis which, however, did not allow its de-replication by HRESIMS. Conversely, the main components of the acetone extract were identified. For this fungal strain, two polar components were revealed to be sphingoid bases. In addition to phytosphingosine (2), an “unusual” sphingoid base with a molecular formula $C_{19}H_{39}NO_3$ was detected. The $MS^2$ spectrum showed fragmentation peaks resulting in sequential loss of three water molecules, whereas no ammonia elimination was measured. This finding could suggest involvement of a nitrogen atom in an azetidine ring, as in isomeric penaresidins A and B. Although the fragmentation pattern observed in the $MS^3$ spectrum is compatible with these structures, no ambiguous information relative to the position of the hydroxyl groups, methyl branching, or even the nature of unsaturation could be obtained. Finally, Scopularide A (8) [28] was identified by MF analysis and by diagnostic MS2 fragmentations (**Fig. 3**, **Table 4**).

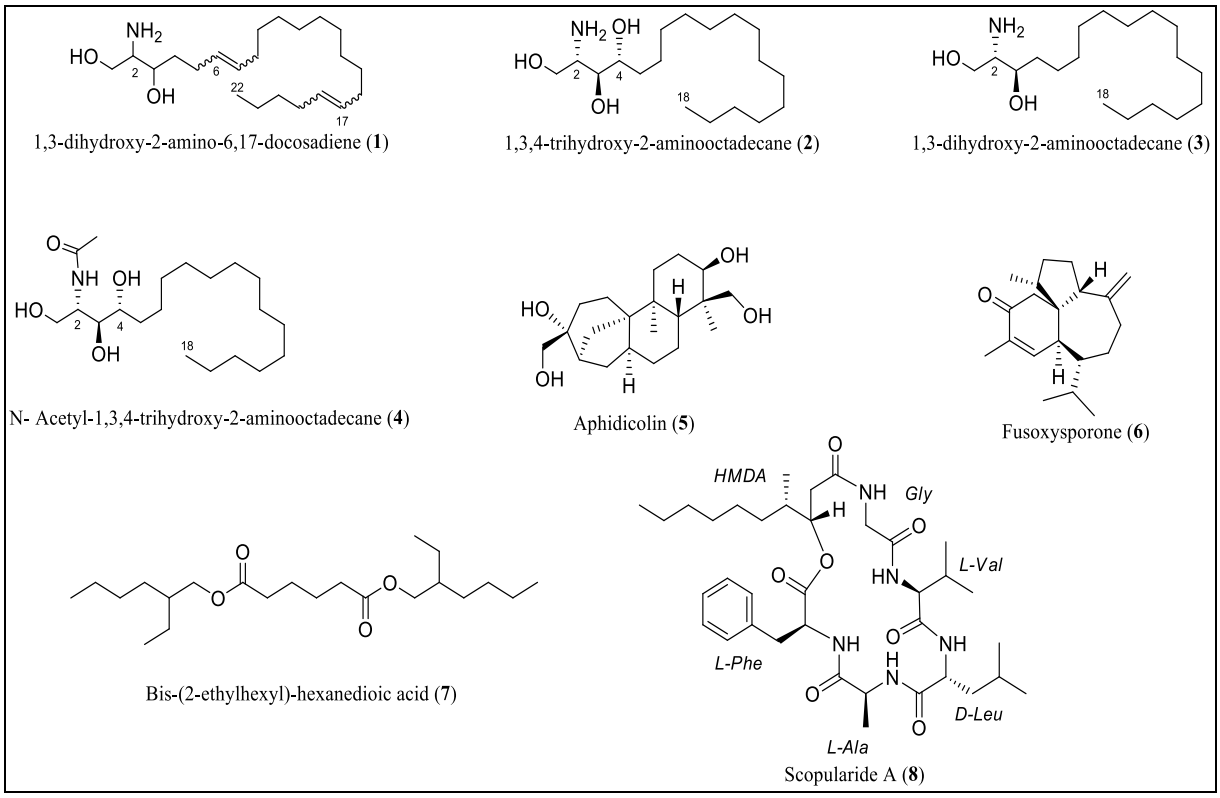


**Fig. 3**. Chemical structures of secondary metabolites (1e8) identified by LC-HRMS in bioactive extracts of *B. bassiana* MUT 4865 and MUT 4861.

| Strain | RT (min) | MS and $MS^n$ | Suggested MF | Proposed structure |
|---|---|---|---|---|
| MUT 4861 | 31.52 | 318.30002 (Δppm -0.756) $MS^2$ (**S5**): 300.29, 282.29, 265.33 | $C_{18}H_{39}NO_3$ | 2-amino-octadecane-1,3,4 triol (4-hydroxysphiganine or phytosphingosine) (**2**) |
| | 34.29 | 330.30024 (Δppm -0.031) $MS^2$ (**S8**): 312.26, 294.33, 282.32, 256.32 $MS^3$[@ 294.33)] (**S9**): 266.33, 168.18, 154.07, 140.11, 133.01, 126.0, 111.96, 97.94) | $C_{19}H_{39}NO_3$ | |
| | 49.05 | 672.43291 (Δppm-0.166) $MS^2$ (**S8**) 654.5, 525.3, 507.2, 454.2, 436.2, 323.1 [38] | $C_{36}H_{57}N_5O_7$ | Scopularide A (**8**) |
| | 58.14 | 409.3101 (Δppm 0) | $C_{28}H_{40}O_2$ | ergostane |
| | 59.94 | 393.3154 (Δppm 0) | $C_{28}H_{40}O$ | ergostane |
| | 65.6 | 395.3307 (Δppm 0) | $C_{28}H_{42}O$ | ergosterol |
| | 73.06 | 371.31576 (Δppm 0) $MS^2$ (**S7**): 259.01, 240.70, 146.9, 128.9, 110.99 | $C_{22}H_{42}O_4$ | Bis(2-ethylhexyl) hexanedioic acid (**7**) |
| | 77.20 | 377.32019 (Δppm 0) | $C_{28}H_{40}$ | Ergosta-3,5,7,9(11),22-pentaene |

**Table 4**: Annotated peaks observed in the chromatograms of the acetone extract of Microascacea sp.2 MUT 4861.

## 3.4 Discussion

In this study, the green marine alga *F. petiolata* was chosen as a source of promising marine fungi, since it had been previously demonstrated that fungi isolated from marine algae showed strong antimicrobial activity against several human pathogenic bacteria [29], probably deriving from the ability to protect their algal host from external threats [30]. Identifying new fungal strains could lead to the discovery of new and unusual compounds with biotechnological and pharmaceutical applications. The first step of this work was the phylogenetic affiliation of fungal strains, which was carried out according to molecular and phylogenetic analysis. *Massarina sp*. (MUT 4860) and *Biatriospora sp*. (MUT 4883) clustered in the Biatriosporaceae family, which accommodates genera that have often been collected from a range of both terrestrial and aquatic hosts, and are commonly found in decaying submerged intertidal mangrove wood [27]. Recently, it has been demonstrated that a strain identified as *Biatriospora sp*. is an efficient producer of secondary metabolites, in particular, naphthoquinone derivatives [31]. MUT 4859, MUT 4886 and MUT 4966 clustered in the Roussoellaceae family, which includes species of saprobic fungi isolated from decaying bamboo culms or palm fronds [32]. *B. bassiana* (MUT 4865)

is a marine isolate of well-known enthomopathogenic fungus, commonly isolated from decaying arthropods or from plant tissue as an endophyte [33]. On the basis of molecular and phylogenetic data, MUT 4861 and MUT 4885 could be considered putative new species and even new genera of the Microascales, a small order of primarily saprobic fungi in soil, rotting vegetation and dung. Some species of this order are responsible for plant diseases, while other members cause human diseases [34]. *K. petricola* (MUT 4979) is an algicolous strain of microcolonial fungus with a meristematic-black yeast morphology that has only been previously found on stone substrates, such as unlichenized fungus with its natural ecological niche [35]. To the best of our knowledge, this is the first report of the presence of this species in a marine environment. As the antimicrobial activity of these algicolous fungi on MDR bacteria (according to the results of the bioassay tests) was in agreement with the known antimicrobial potential of marine fungi, further investigations are recommended, also taking into account the value of producing antimicrobial compounds from new taxonomic entities that have never been previously explored. The most promising fungal strains were MUT 4865, 4979 and 4861, which exhibited the highest degree of antibacterial activity. MUT 4865, identified as *B. bassiana*, representatives of which are well known producers of insecticides and antimicrobials [36], showed strong activity against all pathogens tested. For *K. petricola* (MUT 4979), this is the first report of antimicrobial activity exhibited by fungal extracts from this species. Further studies are necessary, considering that the class this organism belongs to (Eurotiomycetes) includes several species (e.g. *Aspergillus spp*., *Paecilomyces spp*., *Penicillium spp*.) that have been reported to be a source of many antimicrobial metabolites [37,38]. Finally, MUT 4861 is of special interest due to the fact that it is presumed to belong to a new species of Microascaceae, a family that includes a number of fungi capable of producing several antimicrobial secondary metabolites [37,38]. Chemical profiling of the most active crude extracts highlighted the presence of chemically diverse metabolites. In particular, both strains were found to contain sphingoid bases. Diverse variants of the long-chain bases sphingosine and phytosphingosine have been reported from marine organisms, especially sponges and tunicates [39,40], but to the best of our knowledge, this is the first report of sphingosine-free bases from marine fungi. In particular, long-chain sphingadienine 2-aminodocosa- 6,17-dien-1,3-diol has never been described as a free base or as a component of polar lipids from natural sources. Related docosa-4,15-sphingadienine and 4-hydroxy-docosa-15-sphingenine have been reported as components in sphingophosphonolipids from the marine gastropod *Turbo cornutus* [41]. It is noteworthy that recent years have witnessed an everincreasing interest in the so-called “sphingoid bases” for their role in regulation of physiological and pathological conditions [42]. In particular, a recent study [43] revealed that sphingoid long-chain bases displayed antibacterial activity against a broad spectrum of pathogenic bacteria, including *P. aeruginosa, Acinetobacter baumannii, Haemophilus influenzae*, *Moraxella catarrhalis* and even *Burkholderia cepacia*, at nanomolar to low micromolar concentrations. Therefore, although we cannot exclude a priori the possibility that the antimicrobial activity could rely on a combination of different molecules, compound 1 and co-occurring sphingosines 2, 3 and 4, previously reported to be common components of fungal membrane sphingolipids [44], may be responsible for the antimicrobial effects exhibited by MUT 4865 crude extracts towards the pathogenic bacteria investigated thus far. However, tests with the purified compound will be necessary to validate this hypothesis. Regarding the other tentatively identified components of MUT 4865 extracts, aphidicolin is a tetracyclic diterpene with known

antiviral and antimitotic properties, first isolated from the fungus *Cephalosporium aphidicola* [45]. Fusoxysporone, is a viscidane-type diterpene first isolated from *Fusarium oxysporum* [46], and is also found as a component of the cytotoxic extracts of a *Penicillium* strain isolated from bivalve mollusks [47]. To the best of our knowledge, no biological activities have thus far been described for this compound. Compound 7, identified as bis (2-ethylhexyl) hexanedioic acid, is known as a plasticizer [48], and is described as a component of cyanobacteria, Antarctic [49] and terrestrial [50] strains of *Streptomyces*, and of a tropical plant [51]. Sphingosine-related compounds were also detected in the EtOAc extract of Microascacea sp.2 MUT 4861, which also contains a member of the class of so-called anhydrophyto sphingosines; in particular, the detected compound is isomeric with azetidine-derived penaresidins A and B, which were first isolated from the marine sponge *Penares sp*. [52]. Conversely, compound 8 is a cyclodepsipeptide scopularide A, a molecule with antiproliferative activity previously isolated from a marine strain of the fungus *Scopulariopsis brevicaulis* [28], belonging to the same Microascaceae family assigned to MUT 4861. In conclusion, nine selected strains isolated from the green alga *F. petiolata* were chosen as a promising source of antimicrobial compounds. All fungal strains demonstrated interesting antimicrobial activity against four human pathogenic MDR bacteria. Crude extracts of three of the selected fungal strains, preserved at the MUT collection as MUT 4865, MUT 4979 and MUT 4861, were able to strongly inhibit the entire panel of pathogens. Chemical profiling of the antibacterial extracts from *B. bassiana*, MUT 4865 and Microascacea sp.2, MUT 4861, by LC-HRMS allowed identification of the main components of the crude extracts. No detectable amounts of peptide mycotoxins, such as beauvericin or enniatins, known for their antimicrobial and anti-tumor activities [53], were detected. Isolation of several sphingosine bases, including compound 1, previously unreported from natural sources, gave a rationale to the broad spectrum of antibacterial activity exhibited by the crude extract of this fungal strain. Further experiments aimed at isolation of pure compounds and determination of their biological activity are currently under way.

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## 3.6 Supplementary materials

| MUT Code | Growth media | | |
|---|---|---|---|
| | MeCl | WST30 | PCB |
| **4859** | 55 ± 2.4 | 38 ± 1.2 | ND |
| **4860** | 50 ± 1.7 | 48 ± 2.4 | ND |
| **4861** | 65 ± 3.5 | 38 ± 4.5 | 10 ± 0.6 |
| **4865** | 60 ±1.0 | 60 ± 5.7 | ND |
| **4883** | 25 ± 0.7 | ND | 20 ± 1.2 |
| **4885** | 35 ± 1.4 | 33 ±3.2 | 25 ± 0.3 |
| **4886** | 30 ± 0.4 | 40 ± 4.3 | 40 ± 0.9 |
| **4966** | 50 ± 0.8 | 10 ± 0.2 | ND |
| **4979** | 62 ± 1.4 | 45 ± 3.5 | 38 ± 0.9 |

**Table S1. Selection of the best fungi growth media antimicrobial compounds production.** The table reports the antimicrobial activity as the percentage of inhibition of a selected target bacterium (*Burkholderia metallica* LMG 24068) in presence of the fungal extracellular extracts from the three different growth media. MeCl medium showed the best antimicrobial activity. ND: Not detected.

| MUT Code | Intracellular extract | Extracellular extract |
|---|---|---|
| **4859** | 70 ± 3.4 | 40 ± 3.2 |
| **4860** | 67 ± 2.1 | 33 ±1.3 |
| **4861** | 56 ± 0.9 | 30 ± 0.5 |
| **4865** | 60 ± 2.5 | 32 ± 0.7 |
| **4883** | 54 ± 3.1 | 25 ± 0.8 |
| **4885** | 76 ± 4.3 | 33 ± 1.2 |
| **4886** | 60 ± 3.8 | 10 ± 0.6 |
| **4966** | 60 ± 2.1 | 15 ± 1.3 |
| **4979** | 60 ± 6.5 | 30 ± 2.1 |

**Table S2**. **Comparison of the antimicrobial activity between intracellular and extracellular. extracts.** Antimicrobial activity is reported as the percentage of inhibition of the selected target bacterium (*Burkholderia metallica* LMG 24068) in presence of intracellular and extracellular fungal extracts. Intracellular extracts resulted to be the most active.

## Conclusions

The discovery of bioactive marine natural products at the end of the last decades increased the interest in marine bioprospecting, which has today evolved towards a more biotechnological prospection exploiting marine microorganisms as a source of new products. In this work, the bioprospecting of extreme marine environment allowed the isolation and identification of different species of bacteria and fungi which have been exploited for the production of antimicrobial compounds. The application and optimization of a “Drug discovery” pipeline allowed the selection of promising strains able to inhibit the growth of a panel of MDR bacteria. Novel isolation and cultivation methods were applied discovering new antimicrobial molecules highlighting the importance to shift from classic microbiological techniques to more innovative ones. The need to overcome the problem represented by the decreasing of new effective antibiotics led to the exploration of different strategies. The obtained results showed a very promising antimicrobial effect of new molecules structurally related to fatty acids. Over last years, these class of compounds attracted the attention of scientists because of their multiple applications. As antibiotics, they can elude the resistance mechanisms interacting directly with the bacterial membrane. Moreover, they can be used in the bioremediation as biosurfactants. These characteristics make them interesting from a commercial point of view and future experiments will allow increasing the yield of the bioactive molecules to make the process competitive. All this work was highly focused on culture-dependent methods as a strategy for obtaining novel molecules, but the future of the drug discovery needs a synergistically approach. Culture-independent approaches are necessary to guide the isolation of novel promising strains. In particular, thanks to the enormous progress made by the omic techniques (metagenomics, metatranscriptomics and metabolomics) the selection of the “best” strains will be performed following the presence of new/unknown biosynthetic gene clusters in the microbial genome and evaluating their expression by metatranscriptomics and metabolomics. In addition, a different strategy is represented by the application of the synthetic biology through which organisms with new desirable biosynthetic capabilities will be built. The fighting against infectious bacteria is not over, but biotechnology could help to counteract this threat.

## Publications

Evaluation of *Burkholderia cepacia* Complex Bacteria Pathogenicity Using *Caenorhabditis elegans*. Tedesco P, Di Schiavi E, **Esposito FP**, de Pascale D. **Bio Protoc**. 2016 Oct 20; 6(20): e1964.

The antimicrobial potential of algicolous marine fungi to counteract multidrug resistant bacteria: phylogenetic diversity and chemical profiling. Gnavi G, **Palma Esposito F**, Festa C, Polia A, Tedesco P, Fani R, Monti MC, de Pascale D, D'Auria MV, Varese GC. **Res Microbiol**. 2016 May 9.

Antimicrobial activity of monoramnholipids produced by bacterial strains isolated from Ross sea (Antarctica). Tedesco P, Maida I, **Palma Esposito F**, Tortorella E, Subko E, Ezeofor CC, Zhang Y, Tabudravu J, Jaspars M, Fani R, de Pascale D. **Mar Drugs**. 2016 Apr 26;14(5)

Marine metagenomics, a valuable tool for enzymes and bioactive compounds discovery Rosalba Barone*, Concetta De Santi*, **Fortunato Palma Esposito***, PietroTedesco* (* Co-authors), Marco Visone, Federica Galati, Alessia Di Scala, Donatella De Pascale **Frontiers in Marine Science**, 04 September 2014 |doi: 10.3389/fmars.2014.00038

## Submitted Manuscripts

Isolation of an Antarctic bacterium *Aequorivita* sp. by Miniaturized Culture Chip as a producer of novel bioactive compounds. **Fortunato Palma Esposito,** Colin Ingham, Deniz Tasdemir, Donatella de Pascale. **Research in Microbiology**.

Identification of a sorbicillinoid-producing *Aspergillus* strain with antimicrobial activity against *Staphylococcus aureus*: a new potential poliextremophilic marine fungus from Barents Sea. Paulina Corral*, **Fortunato Palma Esposito*,** Pietro Tedesco*, Angela Falco, Emiliana Tortorella, Luciana Tartaglione, Carmen Festa, Maria Valeria D'Auria, Giorgio Gnavi, Giovanna Cristina Varese, Donatella de Pascale. **Marine Biotechnology**.

## Oral communication

**Fortunato Palma Esposito. "**Uncultivable" microorganisms as source of new antimicrobial compounds. **1st TA call of the EMBRIC Transnational Access program. September 20th 2017. Faro, Portugal.**

**Fortunato Palma Esposito**. Exploitation of new bioactive compounds produced by *Beauveria bassiana* to fight MDR bacteria. **2nd International Conference on Marine Fungal Natural Products (MaFNaP_2017). June 27-29th 2017. Kiel, Germany.**

**Fortunato Palma Esposito**. New Antimicrobial compounds from Antarctic bacteria. **PharmaSea final GA meeting. March 14th 2017. Granada, Spain.**

**Fortunato Palma Esposito**. Evaluation of antimicrobial potential of the Antarctic bacterium *Aequorivita sp*. isolated by the Microdish Culture Chip. **MARINE MICROBIOME discovery & innovation. June 27-30th 2016. Berlin, Germany.**

**Fortunato Palma Esposito**. Antimicrobial activity of monoramnholipids produced by bacterial strains isolated from Ross Sea (Antarctica). **47° Congresso della Società Italiana di Biologia Marina. June 13-17th 2016. Turin, Italy.**

## Poster communication

**Fortunato Palma Esposito**, Pietro Tedesco, Emiliana Tortorella, Maura Mirra, Antonio Masino, Angela Falco and Donatella de Pascale. Microbial communities: isolation of antimicrobial compounds by an integrated omic approach. **BIOPROSP_17**: 8th International Conference on Marine Bioprospecting and Biotechnology, **March 8-10th 2017, Tromso, Norwey.**

**F. Palma Esposito**, CJ. Ingham, D. Tasdemir, P. Tedesco, P. Corral Villa, E. Tortorella, A. Mondini and D. de Pascale. Evaluation of antimicrobial potential of the Antarctic bacterium *Aequorivita sp*. isolated by the MicroDish Culture Chip**. Exploitation and Legal Aspects on Marine Genetic and Chemical Resources April 4-5th 2016 Naples, Italy**

**F. Palma Esposito**, CJ. Ingham, G. Brodie, C. Festa, J. Silber, P. Tedesco, P. Corral Villa, E. Tortorella, A. Mondini, M. Jaspars, MV. D'Auria, D. Tasdemir and D. de Pascale. Psychrophilic bacteria as source of novel bioactive compounds. **Challenges and Opportunities in Marine Biotechnology Research and Development in Europe, December 17-18th 2015, Kiel, Germany**

## Experiences in foreign laboratories

During my PhD project, I have spent one month (May 2015) of my first year at MicroDish, a biotechnological company at University of Utrecht (Netherlands) under the supervision of Dr. Colin Ingham. The research activities performed were focused on the use of the MicroDish Culture Chip, a new device for improving microbial cultivability. I used this system to isolate Antarctic bacteria.

I attended MaCuMBA summer school (July 12th-24th, 2015) on Texel island (Netherlands), focused on learning the most advanced techniques to cultivate "not-yet-cultivated" microorganisms by theoretical and practical lessons.

During my second year, I won the German scholarship "DAAD" and I spent 4 months (November 2015 - February 2016) performing my research activities at GEOMAR-Biotech in Kiel (Germany), under the supervision of the Prof. Deniz Tasdemir. The research activities I have performed at the GEOMAR were focused on the purification and identification of bioactive compounds from Antarctic bacteria targeting human pathogens, by using SPE and HPLC, as well as NMR and LC-MS instrumentation.

During my third year, I won the 1st TA call of the EMBRIC Transnational Access program with the proposal entitled "Learning from the metagenome: from culture-independent methods to lab cultivation". The project was financed by EMBRIC

consortium which promotes the exchange of young researchers and transfer of knowledge among several European partners. The goal of the project was combining omics techniques with traditional cultivation methods to isolate uncultivated microorganisms. In the framework of this project, I spent 15 days (September 10$^{th}$-24$^{th}$ 2017) at CCMAR in Faro (Portugal) to collect marine samples from Ria Formosa lagoon. Then I spent other 15 days (October 15$^{th}$-29$^{th}$ 2017) at DSMZ in Braunschweig (Germany) where I applied met-omics approaches on the samples collected from Portugal.