Dottorato in Biotecnologie –XXXII ciclo

Università di Napoli Federico II

ECO FRIENDLY "OCEAN SOLUTIONS" FOR IMPROVEMENT OF HUMAN WELLNESS

Emiliana Tortorella

Dottorando: **Emiliana Tortorella**

Relatore: **Prof. Giovanni Sannia**

Correlatore: **Dott. Donatella de Pascale**

Coordinatore: **Prof. Marco Moracci**

Settore Scientifico Disciplinare: **Biotecnologie**

Somewhere, something incredible
is waiting to be known

Carl Sagan

Summary

Bioprospecting is the process of discovery and commercialization of new products based on biological resources.
One of the most promising nature's treasure is the marine biota. It harbours a huge number of organisms that have developed unique metabolic abilities to ensure their survival in diverse and hostile habitats, resulting in the biosynthesis of an array of metabolites with specific activities, most of which finding application in a wide range of industries. Marine bioprospecting is increasingly becoming the excellence tool to face up the today's societal challenges. Nowadays, marine natural products are unanimously acknowledged as the "blue gold" in the urgent quest for new pharmaceuticals.
Marine bioprospecting is therefore an important field of innovation, but this process is based on biodiversity and, to preserve it, there is an urgent need for a sustainable approach to the use of marine organisms as a source. In this project, three marine sources were explored for their bioactive potentialities, paying attention to analysis of their sustainable supply.
Antarctic sea ice samples were used for isolation of cold-adapted bacteria. A fast and effective screening method, based on the creation of cell-free supernatant collection, allowed the selection of a promising strain, identified as *Shewanella antarctica*, able to produce anthelmintic and antibiofilm activity.
The second work explored the bioactive potential of the edible marine gastropod *Haliotis tuberculata.* The results obtained showed the potential of the marine oil (with antimicrobial, anthelmintic and anticancer activity) derived from this species, suggesting its importance as promising candidate for further investigation aimed at the development of functional foods, food ingredients, as well as pharmaceuticals.
In the third work the bioactive potential of shellfish by-products was investigated and, in particular, the microbial bioconversion of this waste material into valuable products. Two marine bacteria with proteolytic activity, *Pseudoalteromonas issachenkonii* and *Pseudoalteromonas artica* showed the ability to efficiently convert crab shells into anthelmintic and antibiofilm compounds.
The results of this project confirmed the huge potential of marine organisms for bioprospecting.

Riassunto

Da sempre la natura ha contribuito considerevolmente alla scoperta di molecole utili per gli esseri umani. Il processo di scoperta di prodotti naturali da risorse biologiche è definito “bioprospecting”. Uno dei tesori naturali più promettenti è il biota marino e il bioprospecting marino sta diventando sempre più lo strumento d’eccellenza per far fronte alle moderne sfide della società, quali l’antibiotica resistenza, le infezioni elmintiche, il cancro, i biofilm batterici. L’ambiente marino ospita un gran varietà di organismi che hanno sviluppato abilità metaboliche uniche per assicurarsi la sopravvivenza in ambienti peculiari ed ostili, risultanti nella biosintesi di un arsenale di metaboliti con attività specifiche. Molti di questi metaboliti sono prodotti commerciali ad alto valore e trovano applicazione in diverse industrie. Oggigiorno, i prodotti naturali marini sono unanimemente riconosciuti come l’“oro blu” nella urgente richiesta di nuovi farmaci.
Il bioprospecting marino è, pertanto, un importante campo d’innovazione, ma è importante capire che esso, come tutti i processi di bioprospecting, si fonda sulla biodiversità ed è cruciale, per una crescita sostenibile, mantenere questa biodiversità.
E’ opinione comune che seguire un approccio tradizionale al bioprospecting, comporta un grande rischio, non solo per l’economia, ma anche per il nostro pianeta, consumando il capitale naturale marino dal quale la crescita sostenibile dipende. Pertanto, c’è un urgente bisogno di un approccio sostenibile nell’uso delle risorse marine come fonte per il bioprospecting, non solo per raggiungere benefici economici, ma anche per promuovere la protezione e la conservazione della biodiversità dei composti marini.

L’**obiettivo di questo progetto di ricerca** è stata l’esplorazione di risorse marine in termini di attività biologiche. Le risorse selezionate sono state valutate per la loro abilità di produrre composti ad azione antielmintica, antimicrobica, antibiofilm e antitumorale. Le risorse sono state selezionate sulla base della possibilità di ottenere queste fonti in maniera sostenibile. Sono state selezionate tre risorse, presentate in tre diversi capitoli in questa tesi.

Capitolo 1. **Batteri da ambienti estremi: *Shewanella* sp. S3A, un promettente candidato per contrastare le infezioni elmintiche e i biofilm batterici**

In questo lavoro sono stati utilizzati campioni di ghiaccio marino antartici per l'isolamento di microorganismi. L'isolamento è stato condotto con metodi tradizionali, su piastre di Marine Broth, a 15° con tempi di incubazione fino a 30 giorni. Dopo il periodo di incubazione, sono state ottenute 40 colonie in totale. Allo scopo di selezionare potenziali produttori di composti bioattivi tra gli isolati è stata messa a punto una metodologia di screening basata sulla creazione di una collezione di surnatanti di fermentazione. I batteri isolati sono stati utilizzati per ottenere crescite liquide di 10 ml in diversi quattro terreni e dopo 5 giorni di crescita, di queste sono stati ottenuti i surnatanti allontanati dalle cellule, contenenti tutte le molecole prodotte dal batterio e rilasciate nel microambiente esterno. Questi surnatanti sono stati quindi valutati per la presenza di attività biologiche. In particolare, sono stati effettuati screening per l'attività antimicrobica, antielmintica, antibiofilm e antitumorale. Da questo screening preliminare è stato selezionato il surnatante prodotto dal batterio S3A come produttore di attività antielmintica e antibiofilm. Il batterio produttore è stato quindi identificato, attraverso l'analisi dei geni 16S rRNA, come *Shewanella antarctica*. Per confermare le attività riscontrate dallo screening il batterio è stato cresciuto a 4°C e 20°C e il brodo di coltura esausto, separato dalle cellule, è stato usato per l'estrazione dei composti. Dapprima è stato acidificato a pH2 per ottenere la precipitazione di composti biosurfattanti/lipopeptidi. Dopodiché, il mezzo di coltura residuo è stato sottoposto ad ulteriore estrazione liquido-liquido con etile acetato. I due estratti sono quindi stati testati per l'attività antielmintica ed antibiofilm. L'estratto in metanolo ottenuto dalla crescita a 4°C ha mostrato la maggiore attività, con la minima concentrazione antielmintica di 125 µg/mL e la minima concentrazione in grado di inibire la formazione di biofilm di *Staphylococcus epidermidis* di 250 µg/mL. L'estratto attivo è stato caratterizzato in termini di acidi grassi, presentando una distribuzione così composta: 56% acidi grassi saturi, 39% acidi grassi monoinsaturi (MUFa) e 5% acidi grassi poliinsaturi (PUFa). Ulteriori analisi sono necessarie per la completa caratterizzazione dell'estratto e analisi tramite spettrometria di massa ad alta risoluzione sono in corso, in collaborazione con il Dipartimento di Farmacia dell'Università di Napoli Federico II.
Questo lavoro ha mostrato la validità del metodo basato sulla creazione di una collezione di surnatanti, come un veloce ed

efficiente metodo di screening per il bioprospecting di microorganismi, in quanto permette lo screening parallelo per diverse attività e dagli organismi cresciuti in diverse condizioni.
Per quanto riguarda la sostenibilità, i microorganismi costituiscono la fonte di bioprospecting sostenibile per eccellenza, in quanto la coltivazione su larga scala permette la produzione di grandi quantità di metaboliti secondari in maniera economicamente conveniente e senza alcun impatto sull'ecosistema.

Capitolo 2. L'estratto ricco in lipidi del gasteropode marino Haliotis tuberculta: una fonte potenziale di composti bioattivi

Il phylum dei Molluschi rappresenta uno dei gruppi più estesi tra gli invertebrati marini. Oggigiorno, i molluschi appartenenti alle classi Bivalvia e Gastropoda hanno un interesse commerciale nella pesca e nell'acquacoltura, in quanto molti di questi organismi sono ampiamente consumati a livello mondiale. Durante le passate decadi, le proprietà bioattive dei molluschi bivalvi sono state ampiamente investigate e diversi integratori formulati con estratti da molluschi sono stati commercializzati. Un minior numero di lavori riguardante le potenzialità dei gasteropodi marini è invece disponibile. In questo sono state studiate le proprietà bioattive dell'estratto tissutale derivato da *Haliotis tuberculta*, una specie di abalone commestibile distribuita nel Mar Mediterraneo e nell'Oceano Atlantico nord-est.
Il tessuto dell'animale è stato utilizzato per l'estrazione sia di composti idrofilici/polari, utilizzando resine assorbenti come Diaion HP20 e Amberlite XAD-16N, sia di composti organici/idrofobici utilizzando diversi solventi organici. Un estratto bioattivo ricco in lipidi è stato ottenuto con l'etile acetato come solvente estraente. Questo estratto ha mostrato attività antimicrobica contro il ceppo meticillino-resistente *Staphylococcus epidermidis* RP62A, l'emergente ceppo multiresistente *Stenotrophomonas maltophilia* D71 and *Staphylococcus aureus* ATCC 6538P, con valori di MIC (Minima Concentrazione Inibente) di 125 µg/mL, 125 µg/mL e 62.5 µg/mL rispettivamente. L'estratto ha anche mostrato attività antielmintica, valutata attraverso la tossicità contro *Caenorhabditis elegans,* l'elminta target modello. In aggiunta, l'estratto in etile acetato ha mostrato una citotossicità selettiva sulle linee tumorali A375, MBA-MD 231, HeLa e MCF7, alla concentrazione di 250 µg/mL. L'estratto è stato caratterizzato in termini di acidi grassi,

presentando una distribuzione cosi composta: 45% acidi grassi saturi, 22% acidi grassi monoinsaturi (MUFa) e 33% acidi grassi poliinsaturi (PUFa). La presenza nell'estratto di alcuni metaboliti secondari biologicamente importanti anche è stata analizzata, utilizzando reagenti generali di rilevamento (Dragendorff's test; Mayer's test; Hager's test, Salkowski test, Shinoda test; alkaline reagent test) ed è stata riscontrata positività per la presenza di alcaloidi, terpeni e flavonoidi.
Ulteriori studi sono necessari per la completa caratterizzazione della parte lipidica e per identificare i metaboliti secondari tra le classi strutturali rilevate. I risultati ottenuti, comunque, mostrano il potenziale dell'olio marino derivato da *H. tuberculata*, suggerendo l'importanza di questa specie come un candidato promettente per ulteriore investigazione mirata allo sviluppo di cibi funzionali, integratori e farmaci. Ciò è particolarmente interessante se si considera che, contrariamente ad altri invertebrati marini, il bioprospecting da molluschi coltivati e la produzione dei derivati composti bioattivi può essere condotto in maniera sostenibile. L'acquacoltura dei molluschi è considerata la coltura di invertebrati marini a minor impatto ambientale e anzi, grazie all'alimentazione per filtraggio, i molluschi hanno il potenziale di influire positivamente sugli ambienti di coltura, fornendo servizi benefici all'ecosistema in cui crescono, come l'estrazione di nutrienti e la filtrazione dell'acqua.

Capitolo 3. **BlueShell: esplorando i prodotti di scarto dei frutti di mare come fonte per le blu bioattività**

L'industria del pesce genera ogni anno il 50-70% di prodotti di scarto non commestibili, i più abbondanti provenienti da crostacei e molluschi. A parte l'uso per l'estrazione di chitina/chitosano, questa biomassa marine è usata per creare prodotti a basso valore economico, come fertilizzanti o cibo per animali, o viene semplicemente smaltita negli inceneritosi, o peggio ancora, nel mare con conseguente impatto negativo sull'ambiente. Il progetto europeo Blueshell mira a risolvere questo problema esplorando tre tipici prodotti di scarto, quali gusci di gamberetti (*Pandalus borealis*), gusci di granchio (*Cancer pagurus*) e cozze difettate non commerciabili (*Mytilus edulis*), per potenziali composti bioattivi.
Come parte del progetto Blueshell, lo scopo di questo lavoro era l'analisi delle attività biologiche di questo prodotti di scarto.

Il materiale, preliminarmente processato all' ILVO (Institute for Agricultural and Fishery Research), è stato usato per l'estrazione di composti organici pigmentati, con l'utilizzo di diversi solventi estraenti. Questi estratti grezzi hanno mostrato in maniera differenziale attività antielmintica, antimicrobica e antibiofilm.
In aggiunta, i gusci dei granchi sono stati utilizzati come substrato per la fermentazione microbica, utilizzando sei diversi ceppi marini con attività proteolitiche/chitinolitiche, precedentemente selezionati all'ILVO. I ceppi *Pseudoalteromonas arctica* e *Pseudoalteromonas issachenkonii* sono risultati i più promettenti per la degradazione efficiente dei prodotti scartati dai granchi. Gli idrolizzati ottenuti dalla fermentazione con i sopra-citati ceppi hanno mostrato attività antielmintica e antibiofilm. È interessante notare che le due attività sono associate a diverse frazioni. L'attività antielmintica è stata rilevata dell'estratto in etile acetato dall'idrolizzato, pertanto potrebbe essere associata alla presenza di metaboliti secondari organici/idrofobici. L'attività antibiofilm è stata invece rilevata nella frazione acquosa rimasta dopo l'estrazione con etile acetato.
La frazione acquosa derivata dalla fermentazione di *P. issachenkonii*, è stata ulteriormente frazionata attraverso estrazione in fase solida (SPE), raccogliendo 6 frazioni a crescente polarità e la frazione eluita con acqua ha dato la maggiore attività, indicando l'accumulo di composti polari con attività antibiofilm.
Il mezzo utilizzato per la fermentazione è composto da 2% di NaCl e 0.1% di acido acetico, con l'aggiunta dei gusci omogenizzati. Senza una fonte di carbonio, rappresentata dalla chitina dei gusci di granchio, i batteri non possono crescere e, conseguentemente, non possono sintetizzare alcun composto. Pertanto, le attività osservate, possono essere associate alla biosintesi di composti bioattivi dai batteri selezionati quando questi degradano e usano la chitina come fonte di carbonio.
La caratterizzazione chimica degli estratti bioattivi tramite GC-MS e LC-MS/MS è in corso, in collaborazione con i partner del consorzio BlueShell, poiché il progetto è ancora in corso.
I risultati mostrati indicano il potenziale uso dei prodotti di scarto per il bioprospecting. In particolare, la bioconversione microbica di questi prodotti in prodotti validi dovrebbe ricevere profonda attenzione perché rappresenta un mezzo sostenibile per sfruttare i prodotti di scarto, fungendo da soluzione alternativa a basso impatto ambientale allo smaltimento dei rifiuti e i problemi associati a esso.

General Introduction

1. Today's societal challenges

1.1 Antibiotic resistance

"*It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them […] and by exposing these 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 drug-resistance. Nowadays, that "prediction" is a fact. Commonly used antibiotics gradually became ineffective to cure infections in community and hospital setting, increasing mortality and morbidity, causing longer hospitalization period and rising social health coast.

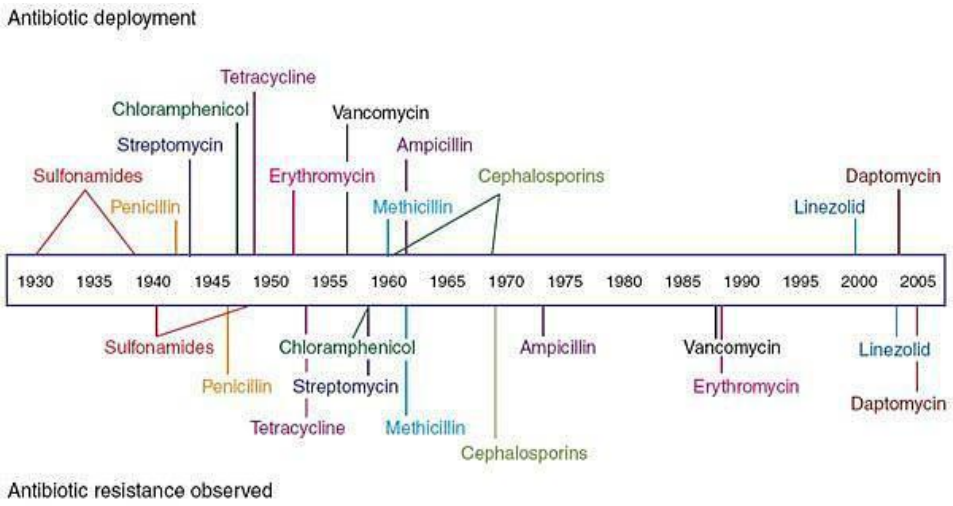


Fig. 1. Antibiotic development in the XX century [1]. The upper panel reports the introduction of the antibiotics in the market, while in the lower pane is reported the year when antibiotic resistance was first observed.

CDC's (Centers for Disease Control and Prevention) Antibiotic Resistance Threats in the United States, 2019 (2019 AR Threats Report) includes the latest national death and infection estimates that underscore the continued threat of antibiotic resistance in the U.S. According to the report, more than 2.8 million antibiotic-resistant infections occur in the U.S. each year, and more than 35,000 people die as a result.

A high number of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), and often, by the ESKAPE pathogens group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baummanii*, *Pseudomonas aeruginosa*, and enterobacteriaceae), so-called to emphasize their capability to "escape" from common antibacterial treatments [2].

Bacteria belonging to Burkholderia cepacia complex (Bcc) also occupy a critical position among Gram-negative multi-host Multidrug-resistant (MDR) pathogens. The Bcc include, at least, 20 closely related species inhabiting different ecological niches, including plants and animals [3, 4, 5]. Bcc opportunistic human pathogens cause problematic lung infections in immune-compromised individuals, including cystic fibrosis patients [6, 7].

Another emerging MDR global opportunistic pathogen is *Stenotrophomonas maltophilia*. It is an environmental bacterium found in aqueous habitats, including plant rhizospheres, animals, foods, and water sources, but it becomes a critical threat in immune-compromised individuals [8]. In particular, *S. maltophilia* is a significant pathogen in cancer patients [9, 10].

The treatment of patients with *Achromobacter xylosoxidans* bacteremia is challenging because this microorganism carries both intrinsic and acquired mechanisms of resistance, often conferring a phenotype of multidrug resistance [11]. In addition, current data on this uncommon entity is mostly limited to very small series or single case reports.

The challenging of antibiotic resistance concerns not only human nosocomial and opportunistic pathogens but also bacteria responsible of food spoilage. The CDC's report included *Salmonella* species among the category "serious threats". *Salmonell*a is a major foodborne pathogen, which causes widespread contamination and infection worldwide. In the last decade, the emergence of several *Salmonella* serotypes resistant to multiple antibiotics in food animals became a significant food safety hazard [12, 13, 14].

The explanation of this dramatic phenomenon relies on several factors. The evolution of resistant strains is a natural process, due to the incredible metabolic capabilities of pathogens to adopt new strategies to survive and to spread these strategies through genetic material exchange [15]. The uncontrolled use of antimicrobial agents, the overuse of antibiotics, and often, the incorrect prescription of them has significantly accelerated the phenomenon

of antibiotic resistance, acting as selective pressure for the emergence of MDR pathogens [16, 17].
To quote Dr. Charles Penn, chairman of WHO's (World Health Organization) Guidelines Review Committee: "*Antibiotics are very often prescribed for no useful purpose. Too many antibiotics are prescribed for viral infections such as colds, flu and diarrhea. Unfortunately, these public misconceptions are often perpetuated by media, for example, through the use of generic terms such as 'germs' and 'bugs*" [18].
Anyway, one primary reason for MDR's rise is the huge decline in antibiotic discovery. After the so-called "golden age" of antibiotic discovery (1940-1960), an extremely productive period which led to the discovery and marketing of almost all antibiotic classes used nowadays, no new classes of antimicrobials were developed. All antimicrobials that entered the market between the introduction of nalidixic acid (1962) and linezolid (2000) were chemical modifications of the existing molecules. This period is referred to as "innovation gap", to describe the lack of novel structural classes introduced to antibacterial armamentarium since 1962 [19]. After 1962, we assisted to a disengagement of the big pharma industries from pursuing antibiotics research for motivation mainly non-scientific and not health-related, but purely economic. The development of new antimicrobial agent had become a more complex, costly and slowly process. On the average, research and development of anti-infective drugs takes around 15-20 years and can cost more than $1 billion dollars [20]. 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 with non-inferior properties.
This dramatic scenario, with the absence of effective antibiotics and the emerging of increasingly stronger bacteria, the humanity may fall in the pre-antibiotic era. One of the most promising strategies to counteract this threat is the identification of novel effective drugs exploiting natural products, in particular focusing the attention on unexplored areas

1.2 Bacterial biofilm

Microorganisms exist as free-floating cells or, more often form a hydrated matrix, called biofilm, attached to a substrate. By definition, a biofilm is a community of cells attached to a surface enclosed in a

complex of extracellular polymeric substances (EPS), like polysaccharides, proteins, DNA and other molecules [21]. Bacterial biofilms have a structurally complex and dynamic architecture and form either on biotic (plants, animals and humans) and abiotic (plastic, glass, metal and minerals) surfaces [22].
Biofilm formation is a dynamic and complex process influenced by several bacterial and/or environmental factors. It occurs in four steps schematically represented in figure 2: first contact, attachment, maturation and dispersion. Factors, that are known to play a role in biofilms formation, are components of bacterial mobility, like flagella, characteristics of the surface, hydrophobicity, rugosity, and characteristics of environment, like temperature, pH, ionic force [23].

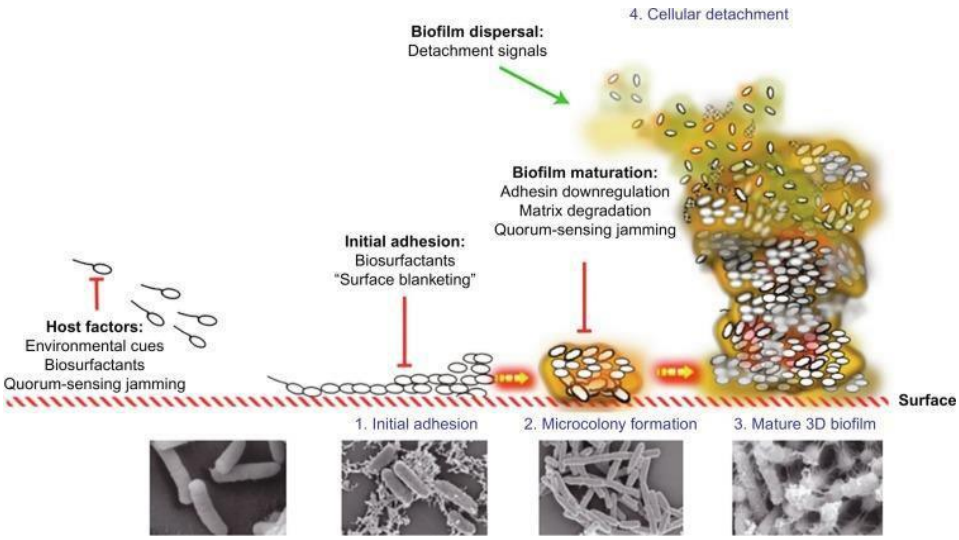


Fig. 2. Stages in the biofilm formation process, including scanning electron microscopy imaging of each stage [24].

Biofilms allow microorganisms to trap nutrients and face up to hostile environmental conditions, allowing them the survival [25]. The ability to form a biofilm, adds greater resistance to anti-infective agents such as antibiotics [26], sanitizers and disinfectants [27], as well as to clearance by host defense [28], thus allowing it to overcome food preservation and other control processes. Bacteria growing in biofilms are up to 1000-fold more resistant compared to their planktonic counterparts [29]. The higher antibiotic resistance in biofilms is due to the exchange of resistance plasmid between strongly aggregated microbial cells [30].

Biofilm-related bacteria are, therefore, a major issue in infections in both animals and humans. In humans, infectious due to biofilms are often related to contamination of medical devices, such as catheters, contact lenses, hearing aids, braces, prosthesis [31, 32]. Unfortunately, bacterial biofilm constitute a dramatic problem not only in clinical domains, but also in non-medical areas.

An important issue, for example, is related to safety of drinking water (DW). The provision of safe DW is considered a top priority in civilized societies. The consumption of microbiologically and chemically contaminated DW has been linked with several health problems [33]. The major recognized source of contamination in DW distribution systems are bacterial biofilms, which can easily develop in the inner walls of pipes [34, 35]. Here, pathogenic bacteria trapped in the biofilm matrix are protected and survive in stress conditions (chlorine, shear stress, temperature) [36]. Hence, when detachment of portions of the biofilms occurs, they enter bulk water, which permits a possible outbreak of disease. According to WHO, death rate due to water born infections exceeds 5 million people per year, in which more than 50% are intestinal infections [37].

The biofilms in the DW distribution system are therefore a threat to animal and human health, and, in addition cause problems in colour, taste and odour due to release of chemical compounds, affecting the chemical quality of DW [38].

The presence of biofilms in both drinking and industrial water distribution systems also cause not strictly health-related problems, affecting for example the efficiency of transport and heat.

The ability of bacteria to adhere with surfaces is a danger also in food industry, with implications in food safety. Bacteria, including foodborne pathogens can easily grow as biofilms on food, leading to severe hygienic problems and economic loss due to spoilage of food [39].

In marine environments the complexity of bacterial biofilms, often referred to as “micro-fouling” is increased by the inclusion of microalgae (especially diatoms), fungi, protozoa, spores of macroalgae and larvae of various invertebrates, leading to a very complex community, referred to as “macro-fouling” [40]. An important implication of the phenomenon of marine biofouling is related to aquaculture. Aquaculture, also referred to as aqua-farming, where marine organisms are cultivated in seawater, usually in sheltered coastal waters, is a very fast expanding industry worldwide. Biofilm formation in water recycling systems especially in

the fry and juvenile fish-handling sector is problematic because biofilms will incorporate and multiply the microflora present in the water. Biofilms in breeding tanks and filtration units, pipelines, and pumps sgould be checked regularly in order to avoid outbreak of diseases, which may cause high financial losses. Diseases can be caused by pathogenic organisms such as species of *Listeria*, *Salmonella*, *Shigella*, *Vibrio*, *Bacillus* and *Aeromonas*, all of which have been found in biofilms in aquaculture environments.

Biofilm, in conclusion, is a serious threat in public health in developing countries, as well as an issue in different industrial domains in developed countries, because it is an example of defense mechanism in numerous bacteria. Safety of both non-medical and medical areas require accurate biofilm control solutions, like limited water and nutrients supply, controlled temperature and well-designed apparatus. However, good design, often, is not sufficient. Currently, there are no therapies that effectively target microbial biofilms, due to their resistance against many antibiotics [41]. The traditional method of control of surface contamination is an effective cleaning and disinfection (sanitation) programme [42, 43]. Generally, disinfectants do not penetrate the intact biofilm matrix and thus do not destroy all the biofilm living cells [44]. The cleaning process can remove up to 90% or more of sessile microorganisms, but cannot kill them. Importantly, cleaning methods can create aerosols that are known to transport microbial cells and relocate viable cells from some areas to others receiving less disinfection [45, 46]. Chemicals commonly used in disinfection processes are acidic compounds, caustic products, aldehyde-based biocides, hydrogen peroxide, chlorine, iodine, ozone, isothiazolinones, phenolics, peracetic acid, surfactants and biguanidines [47]. These chemicals are often toxic to environment.

Therefore, there is the need to find non-toxic alternative solutions for the treatment of bacterial biofilms. Marine organisms have the potential as promising sources of non-toxic compounds with sustainable anti-biofouling/anti-biofilm properties as they can produce molecules that can prevent the biofilm development, by inhibiting the attachment, the microorganism’s growth, and/or the cell-cell communication. Different studies have proven that a wide range of marine organisms and microorganism are a potential source of anti-fouling/anti-biofilm metabolites [48 - 52].

1.3 Helminthic infections

Resistance to common drugs is not limited to bacteria but also to fungi, viruses and especially parasites. Among parasite helminths, a prominent role is played by nematodes. The nematodes, or roundworms, comprise a large number of human and domestic animals pathogens.

According to the latest report of the WHO on soil-transmitted helminth infections, ~ 1.5 billion people were infected with intestinal worms in 2018, predominantly in disadvantaged communities [53]. Helminthic infections are particularly dangerous in children, exacerbate malnourishment and can lead to severe anemia, retarded growth and mental incapacity [54]. The disease burden caused by these parasitic worms is similar to liver cancer and higher than prostate cancer [55] Helminth infections are one of the most significant “neglected tropical diseases”, a diverse group of infections, which are common in underprivileged developing countries [56]. Despite the high impact on human health, these diseases are “forgotten” and do not receive high attention and funding for research on them.

Parasitic worms also have a huge negative impact on agriculture and livestock industry.

Parasitic nematodes cause substantial mortality and morbidity in domesticated and wild animals worldwide, with consequent losses to the global food production annually [57]. For instance, economic losses due to livestock (sheep, goats, cattle and pigs) diseases caused by nematodes of the order Strongylida, have been estimated at tens of billions of dollars per annum globally [58]. Animal parasitic infections are a huge problem for both developing and developed countries, as the livestock industry plays a major role in the economies of both. The livestock field provides not only food but also animal products (e.g. hides, wood and leather), employment, income, a source of organic fertilizer and biogas. In the developing countries, the impact of parasitic infections and disease closely affects the productivity losses. In the developed countries, on the other hand, the main impact is related to the cost of parasite control [59, 60] associated with animal management and pasture utilization, the use of anthelmintics and strategic or integrated anthelmintic treatment programs [61].

Nematode infections of agriculturally important plants also result in huge economic losses worldwide. Phytoparasitic nematodes are

associated with nearly every important agricultural crop and represent a significant constraint on global food security, as well as costly burdens of crop production. Over 4100 species of plant-parasitic nematodes have been identified [62] .Collectively, they cause an estimated $80–$118 billion dollars per year in damage to crops [63, 64].

The most economically important species directly target plant roots of major production crops and prevent water and nutrient uptake resulting in reduced agronomic performance, overall quality and yields. The major genera of phytoparasitic nematodes reported to cause crop losses were *Heterodera*, *Hoplolaimus, Meloidogyne*, *Pratylenchus*, *Rotylenchulus*, and *Xiphinema* [65].

Anthelmintic chemotherapy is limited to three major chemical classes: the benzimidazoles, the imidazothiazoles (e.g levamisole) and the macrocyclic lactones (e.g ivermectin) and most of anthelmintic drugs currently available were introduced decades ago. Inevitably, drug resistance has emerged in human and livestock pathogenic helminths against each class, with some natural isolates showing multidrug resistance [66, 67, 68]. Resistance in nematodes are widespread and have been reported to occur on all continents, becoming a global issue.

Given the impact on nematode infections on animal and human populations and the alarming spread of multi-drug resistant nematodes, there is an urgent need to discover and develop new drugs for the sustainable and effective control of nematodes [69].

Despite this emergency, anthelmintic drug discovery is the poor relation of the pharmaceutical industry [70]. The main reason is to attribute to low funding and interest in research on worm parasitic infections.

1.3.1 *Caenorhabditis elegans* as model system

Traditional high-throughput screening that rely on parasitic worms are costly and labor-intensive and target-based approaches have failed to yield novel anthelmintics. The difficulty in identifying lead compounds at high throughput is related to the complex life cycle of parasitic nematodes. Parasitic nematodes are very hazardous and complex to manipulate in laboratory conditions (especially because they cannot live without their host).

The free-living nematode *Caenorhabditis elegans* may offer a valid alternative as “lab-friendly” model system to search for new

compounds that specifically kill nematodes, despite the many differences with parasites, especially from a genomic point of view. Anyway, it is no more dissimilar to parasitic nematodes than each individual species of parasite is to another [71].
C. elegans is a widespread non-hazardous, non-infectious, non-pathogenic, non-parasitic multicellular organism. Adults can reach the maximum length of 1 mm, and in optimal condition, they can produce about 300 genetically identical progeny in 3 days, allowing the cultivation of nematodes and the rapid establishment of homogenous populations. At 20°C, the development proceeds from embryo through four distinct larval stages (L1-L4) to gravid adults in approximately 72 hours (Fig. XX). In laboratory conditions *C. elegans* can be easily propagated on agar plates or liquid media using the *Escherichia coli* strains OP50 as food source. Moreover, because of their small size, *C. elegans* nematodes can be placed in 96- or 384-well plates for high-throughput screenings [72, 73, 74]. *C. elegans*, therefore, offer a high-throughput screening system for anthelmintic lead compounds that is not possible with parasitic species. The majority of marketed anthelmintics are active against *C. elegans*, and the use of this model system has been instrumental in improving the understanding of the mechanism of action of several anthelmintic compounds, including levamisole, benzimidazole and the amino-acetonitrile derivatives [75, 76, 77].
Moreover, the availability of a fully sequenced genome and the well-developed tools for genetic manipulation allow the understanding of mechanisms of action [78] A study published in Nature seems to have finally proved the usefulness of *C. elegans* as effective and cost-efficient model system for the discovery of nematocidal molecules. In this paper, more than 67,000 compounds were subjected to a primary high-throughput automatized screening against *C. elegans* to find molecules with anthelmintic activity. The 267 positive hits were then tested against two parasites: *Cooperia onchophora* and *Haemonchus contortus*. This second test performed on a small scale, revealed that 103 compounds out of the 267 (38%) were able to kill the two parasites, demonstrating the value of *C. elegans* as a model system for the anthelmintic discovery [79].

1.4 Cancer

Cancer remain one of the most important today's disease worldwide. A global cancer statistics study reported in 2018, approximately 18 million new cases of cancer reported globally, resulting in approximately 10 million deaths [80]. Lung and breast cancer are the most frequently diagnosed cancers worldwide and are the leading causes of cancer-related death in men and women, respectively. Nonmelanoma skin cancer (NMSC) is the most frequently diagnosed cancer in North America, and in Australia, New Zealand, the countries with the highest incidence rates worldwide in men and women, respectively.

The predisposition factor of cancer could be both external and/or associated with lifestyle (tobacco, chemicals, radiation, infectious organisms), and internal (genetic predisposition, immune conditions). Anyway, the highest incidence rates were associated with age and unhealthy lifestyle, which represent the increased risk [81].

The incidence of cancer continues to increase due to environmental changes and life style modernization [82].

The discovery of a cure for cancer has become a feasible goal thanks to progress in biological research and immunotherapy and the important improvements in drug design and manufacturing [83] Cure and prolonged survival have already been achieved for a number of human malignancies, such as lymphomas, testicular cancer, and childhood lymphoblastic leukemia [84, 85, 86].

Despite the significant advances of current therapies, multiple side effects have been reported with chemotherapy, motivating the search for other effective cure with fewer side effects. The research of effective new cure can be done among natural products. Approximately 80% of the approved chemotherapeutic drugs, and more than half of all drugs are based on bioactive natural products [87]. Natural bioactive molecules exhibit cytotoxic effects by attacking macromolecules expressed by cancer cells, such as those in oncogenic signal transduction pathways [88].

Among natural products, marine-derived metabolites became a hot-spot for the research on new anticancer drugs or scaffolds. Over the past 50 years, emerging evidence has shown that many natural products derived from plants and microbes of marine origin, exhibit beneficial effects in the prevention and treatment of cancer, i.e., cytarabine, eribulin mesylate, brentuximab vedotin, and trabectidine

are marine-based drugs used against leukemia, metastatic breast cancer, soft tissue sarcoma and ovarian cancer [89, 90].

Polyphenols, polysaccharides, and alkaloids are among the highly active, biologically potent and predominant anticancer compounds isolated from marine organisms. Polyphenols are categorized into phenolic acids, flavonoids, tannins, catechin, anthocyanidins, epigallocatechin, lignin, epicatechin, epigallate, and gallic acid. Polyphenolic compounds are known for their potential to reduce the mitotic index and decrease the levels of cellular proteins needed for cancer cell proliferation and colony formation. In addition to anticancer effects, the phenols exhibited anti-inflammatory activity, antiviral effects, and inhibited the human platelet aggregation [91, 92, 93].

The other potent group of compounds that is abundantly present in several marine organisms are polysaccharides, primarily alginates, agar, and carrageenans [94]. The main mechanism of action of polysaccharides cytotoxic effect is the activation of the innate immune system [95, 96], leading to attraction of macrophages and natural killer cells to the target site and production of tumoricidal cytokines [97, 98, 99].

Alkaloids derived from marine organisms are divided into four groups, namely indoles, halogenated indoles, phenylethylamines, and other alkaloids. The growth inhibitory activity of these compounds has been successfully demonstrated using various cancer cell lines.

2. Eco-friendly “ocean solutions” to societal challenges

Since the beginning of humankind nature has been contributing considerably to drug discovery for human beings by providing remedial treatments. The process of discovery of natural products from biological resources is defined “bioprospecting”. The core of bioprospecting is the exploration of biodiversity for new resources of social and commercial value. It is carried out by a wide range of industries, the best known being the pharmaceutical, cosmeceutical, nutraceutical and food industry, but also by different branches of agriculture, manufacturing, engineering, construction and many others [100].

One of the most promising nature’s treasures is the marine biota and the marine bioprospecting is increasingly becoming the excellence tool to face up the today’s societal challenges.

The marine environment harbors a huge number of macro and microorganisms that have developed unique metabolic abilities to ensure their survival in diverse and hostile habitats, resulting in the biosynthesis of an array of metabolites with specific activities.
Several of these metabolites are high-value commercial products, which find application in a wide range of industries. Nowadays, marine natural products are unanimously acknowledged as the “blue gold” in the urgent quest for new pharmaceuticals.
Marine bioprospecting is therefore an important field of innovation, but it is important to understand that it relies, like all bioprospecting processes, on biodiversity and it is crucial, for a sustainable growth, to maintain this biodiversity. There is a growing recognition that following an unsustainable ‘business as usual’ approach entails great risk, not only to business, but also to our planet, eroding the ocean’s natural capital on which the sustainable growth depends.
Therefore, there is an urgent need for a sustainable approach to the use of marine organisms as a source. Marine bioprospecting should associate the search for molecules with an analysis of their supply. A bioprospecting program based on the sustainable use of marine resources is important, not only to reach economic benefits, but also to promote the protection and conservation of biodiversity of marine compounds.
The theme of sustainability is one of the pillars of the Europe 2020 strategy for the European growth, which can be summarized in the term “Blue Growth”, in the framework of the development of the Circular bio-economy. The European Commission defines bioeconomy as: “the production of renewable biological resources and the conversion of these resources and waste streams into value added products, such as food, feed, bio-based products and bioenergy.” (European Commission, 2012). The adjective “blue” underlines a focus on aquatic or marine environments.
In other words, the “Blue Growth” encompasses the sustainable use of marine resources for biotechnological applications addressing global challenges of food, energy and health.
Marine resources are microbes (e.g. bacteria, fungi), microalgae, macroalgae (seaweeds), invertabrates (e.g. crustaceans, annelids, molluscs), as well as discards of fish (fish biomass) and other marine organisms. In the framework of the blue bioeconomy activities, these resources can be used for the development of added value products, like high value non-food (cosmetics, pharmaceutics), chemical building blocks, functional food or functional feed.

The blue bioeconomy is an exciting field of innovation because it contributes to the building of an eco-sustainable and highly efficient society.
According to OECD projections, the blue economy is set to grow faster than the general economy and by 2030 could double in size, both in terms of value added and employment [101].
The Directorate General for Maritime Affairs and Fisheries (DG MARE) of the European Commission and the Executive Agency for Small and Medium Sized Enterprises (EASME) have initiated the Blue Bioeconomy Forum (BBF) in 2018 to bring together industry, public authorities, academia, finance and civil society in order to strengthen Europe's competitive position, exploit the potential and ensure the sustainable use of the resources of the emerging blue bioeconomy. In November 2019, the BBF published the Roadmap for the Blue Bioeconomy that should help the blue bioeconomy fulfil its potential and flourish.

2.1 Bioprospecting from marine microorganisms

The marine environment is extremely complex and contains a huge diversity of life forms. It was found that the marine environment, including bottom sediments, represents a giant pool of microbial diversity, numbering up to approximately 3.67×10^{30} microorganism [102].
The major part of marine bacteria is exposed to extreme conditions of pressure, salinity, temperature, oxygen, lack of sunlight. These factors led them to develop the incredible metabolic abilities for the biosynthesis of substances with unusual characteristics, different from their terrestrial counterparts. This group of metabolites are very important to implement the survival strategies of bacteria in adverse conditions, acting as mediators with the external environment and means of intercellular communication [103]. Substances with various biological properties, including antibacterial [104, 105], antifungal [106], antiviral [107], and antiproliferative agents [108] have been found among these complex biomolecules synthesized by marine prokaryotes. Many of these compounds, exhibiting high biological activities, play an important role in life functions of bacteria and are widely used in pharmacology, cosmetics, food industry, and agriculture.
Microorganisms constitute the source of sustainable bioprospecting par excellence. The importance of bacteria as source for

bioprospecting, is related to the feasible and sustainable production of large quantities of secondary metabolites with reasonable cost, by large-scale cultivation and fermentation of the source organisms, in contrast to macroorganisms [109].
Moreover, many of the compounds found in marine invertebrates' organisms may be of microbial origin [110]. In fact, microbial symbionts are often suggested to be the real producers of the secondary metabolites found in marine invertebrates, usually based on the structural similarities to known microbial compounds [111].

2.1.2 Cold adapted bacteria

Cold environments are arguably the most widespread on our planet and in our solar system. Many microorganisms populate Arctic and Antarctic regions. The bacteria that can survive in these environments are known as cold-adapted bacteria [112]. Cold-adapted bacteria can be classified into two groups based on their temperature tolerance: (i) psychrophiles, which can grow at temperatures not exceeding approximately 20°C, and (ii) psychrotrophs (or psychrotolerants), that tolerate a broader range of temperatures – between 0 and 30 °C [113] The diversity, biology and ecology of psychrophilic or psychrotolerant bacteria have been extensively studied in recent years. Both groups of microorganisms share basic molecular and physiological characteristics, which permit their survival in extremely cold environments: (i) increased fluidity of cellular membranes, (ii) the ability to accumulate compatible solutes (e.g. glycine, betaine and trehalose), (iii) the expression of cold-shock, anti-freeze and ice-nucleating proteins, as well as (iv) the production of cold-active enzymes [114].
Recent studies have proven that psychrophilic bacteria may be a promising source of bioactive compounds. These researches have shown that bacteria from Antarctica belonging to genus *Pseudoalteromonas*, produce a variety of bioactive compounds, able to inhibit the growth of different strains belonging to the Burkholderia Cepacia Complex [115, 116]. These strains demonstrated also interesting antibiofilm activity against various pathogens, including *S. aureus* and *P. aeruginosa* [117, 118].
These results emphasize the importance of cold environments as source of new drugs, and certainly, encourage scientific and economic interests in those areas.

2.2 Bioprospecting from marine invertebrates

Bioprospecting from marine invertebrates can face up serious obstacles linked with sustainability.
Currently, the search for new marine natural products typically depends on the harvest of wild specimens. This is a major limitation for the development of new marine drugs and commonly entails two major bottlenecks: sustainability and replicability [119].
Sustainability issues are associated with the large amounts of biomass that are usually required for drug discovery. For example, many pharmacologically interesting secondary metabolite candidates for clinical trials, such as ecteinascidin 743 isolated from the ascidian *Ecteinascidia trubinata*, bryostatins from the bryozoan *Bugula neritina*, or the halichondrins from the sponge *Lissodendoryx*, can be isolated only in minute yields. To use halochondrins for the treatment of cancer, it has been estimated that the annual need of 1-5 kg per year would require that about 3000-16000 metric tons of sponge biomass be collected and processed annually [120]. According to these perspectives, destruction of the environment and/or local extinction of species would or will be inevitable consequences. Another example is given by extensive reef communities which have been disrupted for drug development purposes, affecting negatively also ecological processes of ecosystems of which were part [121].
Replicability is constrained as a result of environmental variability and community level changes to the chemical ecology of the target organisms [122]. Individuals of the same species sampled in different areas, or periods, may not display the same chemical composition and therefore may not guarantee the supply of the target metabolite (a pitfall commonly termed as ‘loss of the source’).
Aquaculture (culture of the organism under artificial conditions) of marine invertebrates may overcome these two bottlenecks as animal biomass can be continuously produced using homogenous environmental conditions, a realization that has prompted much researches on aquaculture for drug production.
Aquaculture trials have been carried out to supply bioactive compounds from marine invertebrates such as the bryozoan *B. neritina*, the ascidian *E. turbinata* and the sponges *Lissodendoryx* sp., and *Acanthella cavernosa*. Most of these have succeeded in producing organisms with economically viable concentrations of bioactive compounds [123, 124, 125].

Aquaculture, therefore, could be a viable and economically feasible option to produce the biomass required to produce compounds for screening and execute the first steps of the NP-based drug discovery pipeline, as well as to obtain commercially quantities of valuable chemicals.

2.3 Seafood by-products as sources for bioprospecting

Seafood products are an important part of the diet worldwide. Moreover, as a source of protein, seafood provide functional components that are essential to human health. However, this industry generates every year huge quantity of waste worldwide. Seafood processing recovers only 20–50% as edible portions and the remaining parts (80–50%) are discarded as “nonedible” by-products /co-products/leftover raw materials, with an average of 20 MT globally [126, 127].

The disposal of marine wastes is becoming an increasingly difficult and costly challenge to the shellfish production and processing industry. The main issue is related to the environmental impact that this waste could have on aquatic ecosystems, since the release of organic material might change significantly the community structure and biodiversity of the benthic assemblage [128]. In addition, fish wastes result in a significant economic loss.

In order to minimize potential adverse impacts on shellfish business and on environment there is a requirement to develop new shellfish waste management options. This need led to amplified interest in the conversion of seafood by-products into commercial products.

By-products containing meat, such as heads, frames and belly flaps, and viscera find already application as feed or fertilizers because of their good nutritional value, containing high quality proteins and lipids with long-chain omega-3 fatty acids. In addition, they are also often rich in micronutrients like vitamins A, D, riboflavin and niacin as well as minerals such as iron, zinc, selenium and iodine [129]. Meat-containing by products are increasingly being used also as co-products for human consumption. Iceland and Norway have long traditions in utilizing by-products from processing of wild Atlantic cod for human consumption. Cod heads are often dried and exported to Africa, while chin medallions and tongues obtained from larger heads are highly regarded domestically by many people [130].

An important category of by-products from marine bioprocessing plants include crustacean shells. Efficient utilization of these marine

by-products also become an environmentally priority due to increased quantity of accumulation as well as slow natural degradation of these materials. Crustacean waste is the major source of the chitin available in the market. Chitin can be identified as a biologically active polysaccharide and thus valuable for many applications. It is a high-molecular weight linear polymer of N-acetyl-D-glucosamine units and can be easily processed into many other bioactive derivatives. Among them, the most common form is chitosan, which result from the removal of considerable amount of acetyl groups from chitin. Much attention has been paid to chitin, chitosan and their oligomers as natural bioactive materials concerning their non-toxicity, biocompatibility and biodegradable nature [131]. These materials have important functional properties that make them to find applications in many fields such as food and nutrition, biomedicine, biotechnology, agriculture and environmental protection. Moreover, chitosan is highly discussed in relation to biomedical and food applications [132]. Hydrolysis of the crustacean waste to obtain chitin and chitosan oligomers is traditionally carried out by chemical treatments. The chemical processing involve the use of acids and generate considerable amount of harmful industrial chemicals. In contrast, enzymatic hydrolysis is more preferable for the preparation of oligomers since this method generate greater yields of oligomers with higher degree of polymerization, and minor environment impact [133].

In addition to chitin and chitosan production form crustacean waste, many researchers proposed the microbial fermentation of these chitin-containing biowaste as an alternative sustainable way to recycling this material and obtain bioactive material. Different enzyme [134, 135, 136] and bioactive material, e.g deodorants, biofertilizers, bio fungicides, carotenoids and antioxidant have been obtained from the microbial fermentation of chitin-containing by products.

Although seafood waste product is currently used in many industries and their commercial applications continue to expand annually, further research is required to exploit their full potential. In particular, through improvement in microbial fermentation and enzymatic hydrolysis, it is possible to convert this material into value added products. With this in mind the sustainable exploitation of seafood by-products become a promising source for bioprospecting, solving the problems related to the management of disposal of marine wastes.

3. Aim of the thesis

This thesis work perfectly fits within the blue bioeconomy field. The aim of the project was the exploitation of three different marine resources in order to understand their biotechnological potential. The project is divided into three chapters, which explore different strategies for the identification of bioactive compounds.

- **Chapter 1**: in this chapter, a “drug-discovery pipeline” was applied and optimized. Classic isolation techniques were used to isolate bacteria from Antarctic sea-ice samples. Then a procedure of bioassay-guided screening was optimized to select the most promising bacteria with multiple bioactivities.

- **Chapter 2**: in this chapter the bioactive potential of extracts derived from the marine abalone gastropod *Haliotis tuberculata*, was evaluated, in view of its importance as an edible item and a commercially important cultivable species. For this purpose, farmed animals were used, underlining the importance of aquaculture as tool for bioprospecting from marine invertebrates.

- **Chapter 3**: in this chapter the bioactivities of fermented/hydrolyzed shellfish by-products were studied. The attention was focused on the crustacean brown crab (*Cancer pagurus*). This work is part of the ERA-MBT BlueShell project.

References

1 Clatwothy, A.E., et al. *Targeting virulence: a new paradigm for antimicrobial therapy*. Nat Chem Bio, 2007; 3(9): 541-8

2 Boucher, H.V., et al. *Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America*. Clin Infect Dis, 2009; 48(1): 1-12

3 Compant ,S.N.J., et al. *Diversity and occurrence of Burkholderia spp. In the natural environment.* FEMS Microbiol Rev, 2008; 32(4): 607-26

4 Peeters, C. et al. *Burkholderia pseudomultivorans sp. Nov., a novel Burkholderia cepacia complex species from human respiratory samples and the rizhosphere*. Syst Appl Microbiol, 2013; 36(7): 483-9

5 Mahenthiralingam, E., et al. *The multifarious, multireplicon Burkholderia cepacia complex*. Nat Rev Microbiol, 2005; 3(2): 144-56

6 Ramsay, K.A., et al. *Factors influencing acquisition of Burkholderia cepacia complex organisms in patients with cystic fibrosi*s. J Clin Microbiol, 2013; 51(12): 3975-80

7 Nash E.F., et al. *"Cepacia syndrome" associated with Burkholderia cepacia (genomovar I) infection in an adolescent with cystic fibrosis*. Pediatr Pulmonol, 2010; 46(5):512-4

8 Brooke, J.S. *Stenotrophomonas maltophilia: an emerging global opportunistic pathogen*. Clin Microbiol Rev, 2012; 25(1): 2-41

9 Safdar, A. and Rolston K.V. *Stenotrophomonas maltophilia: changing spectrum of a serious bacterial pathogen in patients with cancer*. Clin Infect Dis, 2007; 45(12):1602-9

10 Rolston, K.V. *New antimicrobial agents for the treatment of bacterial infections in cancer patients*. Hematol Oncol, 2009; 27(3):107-14

11 Nicolosi, D., et al. *Antibiotic susceptibility profiles of uncommon bacterial species causing severe infections in Italy.* J Chemother, 2009; 21(3):253-60

12 Divek, V.T., et al. *Antibiotic-resistant Salmonella in the food supply and the potential role of antibiotic alternatives for control*. Foods, 2018; 7(10): 167

13 Mohammad, R.Z., et al. *Emergence of resistant salmonella spp.; new challenges, new trends*. Gastroenterol Hepatol Bed Bench, 2011; 4(3): 99–101

14 Wang, X., et al. *Antibiotic resistance in Salmonella typhimurium isolates recovered from the food chain through national antimicrobial resistance monitoring system between 1996 and 2016*. Front Microbiol, 2019; 10: 985

15 Gang, Z., and Jie, F. *The intrinsic resistance of bacteria*. Yi Chuan, 2016; 38(10): 872-80

16 Prevention, C.f.D.C.a. *Antibiotic resistance threats in the United States, 2013*. Centers for Disease Control and Prevention, Office of Infecdtious Disease, 2013

17 Read, A.F. and Woods, R.J. *Antibiotic resistance managment*. Evol Med Public Health, 2015; 2014(1): 147

18 Whiteman, H. *Antibiotic resistance: how has it become a global threat to public health?* Medical News Story, 2014

19 Spelberg, B., et al. *The epidemic of antibiotic resistant infections: a call to action for the medical community from the Infectious Disease Society of America*. Clin Infect Dis, 2008; 46(2): 155-64

20 Birkett, D. et al. *Clinical pharmacology in research, teaching and health care: considerations by IUPHAR, the International Union of Basic and Clinical Pharmacology*. Basic Clin Pharmacol Toxicol, 2010; 107(1): 531-59
21 Branda, S.S., et al. *Biofilms: the matrix reviseted*. Trends Microbiol, 2005; 13(1): 20-6
22 Thenmozhi, R., et al. *Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of Streptococcus pyogenes*. FEMS Immunol Med Microbiol, 2009; 57(3): 284-294
23 Vogeleer, P., et al. *Life on the outside: role of biofilms in environmental persistence of Shiga-toxin producing Escherichia coli.* Front Microbiol, 2014; 5: 317
24 Hadla, M., and Halab, M.A. *Fundamentals of quorum sensing, analytical methods and applications in membrane bioreactors*. Comprehensive Analytical Chemistry, 2018; 81: 95-116
25 Poulsen, L.V. *Microbial biofilm in food processing*. Lebensm-Wiss u-Technol,, 1999; 32: 321-6.
26 Ceri, H., et al. *The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms*. J Clin Microbiol 1999; 37(6): 1771-6
27 Oie, S., et al. *Efficacy of disinfectants against biofilm cells of methicillin-resistant Staphylococcus aureus*. Microbios, 1996; 85(345): 223-30
28 Donlan, R.M. and Costerton, J.W. *Biofilms: survival mechanisms of clinically relevant microorgansims*. Clin Microbiol Rev, 2002; 15(2): 167-193
29 Sauer, K., et al. *Neutral super-oxidised solutions are effective in killing Pseudomonas aeruginosa biofilms*. Biofouling, 2009; 25(1): 45-54
30 Guiton, S.P., et al. *Enterococcal biofilm formation and virulence in an optimized murine model of foreign body-associated urinary tract infections*. Infect Immun. 2010; 78(10):4166-75.
31 Donlan, R.M. *Biofilms and device-associated infections*. Emerg Infect Dis. 2001; 7(2):277-81
32 Rodney, M.D. *Biofilm formation: a clinically relevant microbiological process*. Clin Infect Dis, 2001; 33(8):1387-92.
33 WHO, Guidelines for Drinking Water Quality 4th edn, World Health Organization, 2011
34 Szewzyk, U., et al. *Microbiological safety of drinking water*. Annu Rev Microbiol, 2000; 54: 81-127
35 Sharma, S., et al. *Emerging water-borne pathogens*. Appl Microbiol Biotechnol, 2003; 61(5-6): 424-8
36 Huq, C. A. Whitehouse, C. J. Grim, M. Alam and R. R. Colwell, Curr. Opin. Biotechnol., 2008, 19, 244–247.
37 WHO. *Guidelines for Drinking-water Quality*, Incorporating 1st and 2nd Addenda, Volume 1, Recommendations, 3rd ed, WHO: Geneva, Switzerland 2008.
38 Riley, M.R, et al. *Biological approaches for addressing the grand challenge of providing access to clean drinking water*. J Biol Eng, 2011; 5(1):2.
39 Brooks, J.D and Flint, S.H. *Biofilms in the food industry: problems and potential solutions*. Int J Food Sci Technol, 2008; 43(12):2163-76.

40 Railkin, A.I. *Marine biofouling: colonization processes and defences*. Boca Raton (FL): CRC Press, 2008
41 Gilbert, P., et al. *Biofilms in vitro and in vivo: do singular mechanisms imply cross resistance?* J Appl Microbiol, 2002; 92(31):98S-110S.
42 Møretrø, T, et al. *Control of Salmonella in food related environments by chemical disinfection*. Food Res Int, 2012; 45(2): 532-44
43 Bremer, P.J, et al. *Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms*. Int J Food Microbiol, 2006; 106(3):254-62.
44 Simões, M., et al. *A review of current and emergent biofilm control strategies.* LWT - Food Sci Technol, 2010; 43(4): 573-83
45 Carpentier, B. and Cerf, O. *Biofilms and their consequences, with particular reference to hygiene in food industry*. J Appl Bacteriol, 1993; 75(6):499-511
46 Taormina, P.J and Beuchat, L.R. *Survival of Listeria monocytogenes in commercial food-processing equipment cleaning solutions and subsequent sensitivity to sanitizers and heat*. J Appl Microbiol, 2002; 92(1): 71-80
47 Hermoso, J.A., et al. *Taking aim on bacterial pathogens: from phage therapy to enzybiotics*. Curr Opin Microbiol, 2007; 10(5):1-12.
48 Ganapiriya, V., et al. *Antifouling effect of bioactive compounds from marine sponge Acanthella elongata and different species of bacterial film on larval attachment of Balanus amphitrite (cirripedia, crustacea)*. Brazilian Archives of Biology and Technology, 2012; 55(3): 395-402
49 Zhang, X.Y, et al. *Antifouling potentials of eight deep-sea derived fungi from the South China Sea*. J Ind Microbiol Biotechnol, 2014; 41(4): 741-8
50 Dobretsov, S., et al. *Inhibition of biofouling by marine microorganisms and their metabolites*. Biofouling, 2006; 22(1-2): 43-54
51 El-Gendy, M.M.A., et al. *Novel bioactive metabolites from a marine derived bacterium Nocardia sp. ALAAA 2000*. J Antibiot, 2008; 61(6): 379-86.
52 Selvin, J., et al. *Optimization and production of novel antimicrobial agents from sponge associated marine actinomycetes Nocardiopsis dassonvillei MAD08.* Appl Microbiol Biotechnol, 2009; 83(3): 435-45
53 WHO. *Soil-transmitted Helminth Infections*. 2018
54 Keiser, J., and Utzinger, J., *Efficacy of current drugs against soil-transmitted helminth infections: systematic review and meta-analysis*. JAMA, 2008; 299(16): 1937-48.
55 WHO. *Global health estimates 2016: disease burden by cause, age, sex, by country and by region, 2000-2016*. In: Global Health Estimathes. Genva: World Health Organization, 2018
56 Hotez, P.J., et al. *Rescuing the bottom billion through control of neglected tropical diseases*. Lancet, 2009; 373(9674): 1570–75
57 Anderson, R.C. *Nematode parasites of vertebrates. Their development and transmission*. 2nd ed. Wallingford: CABI Publishing 2000.
58 Roebe, P., et al. *Next-generation molecular-diagnostic tools for gastrointestinal nematodes of livestock, with an emphasis on small ruminants: a turning point?* Adv Parasitol, 2013; 83: 267-333
59 Hawkins, J.A. *Economic benefits of parasite control in cattle*. Vet Parasitol. 1993, 46: 159-73

60 Perry, B.D and Randolph, I.P. *Improving the assesment of the economic impact of parasitic diseases and of their control in production animals*. Vet Parasitol, 1999; 84: 145-168
61 Corwin, R.M. *Economics of gastrointestinal parasitism of cattle*. Vet Parasitol., 1997; 84: 145-168
62 Decraemer, W and Hunt, D. *Structure and classification*. In: Perry R, Moens M, editors. Plant Nematology. Oxfordshire: CAB International 2006; pp.3-32.
63 Nicol, J., et al. *Current nematode threats to world agriculture*. In: Jones J, Gheysen G, Fenoll C, editors. Genomics and Molecular Genetics of Plant-Nematode Interactions. Berlin: Springer Science Business Media 2011; pp. 21-43.
64 Sasser, J. and Freckman D. *A world perspective on nematology: the role of the society*. In: Veech J, Dickson D, editors. Vistas on Nematology; Society of Nematologists, Inc.: Hyattsville, MD .pp.7-14
65 Koenning, S.R et al. *Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994*. J Nematology, 1999; 31(4S): 587-618
66 Awadzi, K., et al. *An investigation of persistent microfilaridermis despite multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana*. Ann Trop Med Parasitol, 2004; 98(3): 231-49
67 Osei-Atweneboana, M.Y., et al. *Prevalence and intensity of Onchocerca volvulus infection and efficacy of ivermectin in endemic communities in Ghana: a two-phase epidemiology study*. Lancet, 2007; 369(9578): 2021-9
68 Sutherland, I. A., and Leathwick, D. M. *Anthelmintic resistance in nematode parasites of cattle: a global issue?* Trends Parasitol, 2011; 27(4):176-81
69 Besier, B. *New anthelmintics for livestock: the time is right*. Trends Parasitol, 2007; 23(1): 530-2
70 Holden-Dye, L. and Walker, R.J. *Anthelmintic drugs*. WormBook, 2007; 1-9.
71 Mitreva, M. et al. Comparative genomics of nematodes. Trends Genet, 2005; 21(10): 573-81
72 Leung, C. K. et al. *An ultra high-throughput, whole-animal screen for small molecule modulators of a specific genetic pathway in Caenorhabditis elegans.* PLoS ONE 2013; 8(4): e62166
73 Kwok, T. C. et al. *A small-molecule screen in C. elegans yields a new calcium channel antagonist*. Nature, 2006; 441(7089): 91-5
74 Petrascheck, M., et al. *An antidepressant that extends lifespan in adult Caenorhabditis elegans*. Nature, 2007; 450(7169):553-6
75 Kaminsky, R. et al. *A new class of anthelmintics effective against drug-resistant nematodes*. Nature, 2008; 452(7184):176-80
76 Holden-Dye, L., and Walker, R. J. *The C. elegans Research Community*, in WormBook 1–13, 2007
77 Jones, A. K., et al. *Chemistry-to-gene screens in Caenorhabditis elegans*. Nat Rev Drug Discov., 2005; 4(4):321-30

78 *C. elegans* Sequencing Consortium. *Genome sequence of the nematode C. elegans: a platform for investigating biology*. Scienc, 1998; 202(5396): 2012-8
79 Burns, A.R., et al. *Caenorhabditis elegans is a useful model for anthelmintic discovery*. Nat Commun, 2015; 6:7485
80 Bray, F., et al. *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries*. CA A Cancer J Clin, 2018; 68(6): 394-424
81 White, M.C., et al. *Age and cancer risk: a potentially modifiable relationship*. Am J Prev Med, 2014; 46(3 Suppl 1):S7-15
82 Gurib-Fakim, A. *Medicinal plants: Traditions of yesterday and drugs of tomorrow.* Mol Asp Med, 2006; 27(1):1-93
83 Ramsay, R.R., et al. *A perspective on multi-target drug discovery and design for complex diseases*. Clin Transl Med, 2018; 7(1):3
84 Garattini, S. and La Vecchia, C. *Perspectives in cancer chemotherapy*. Eur J Cancer, 2001; 37:128-47
85 Ang, K.K.H., et al. *In vivo antimalarial activity of the beta-carboline alkaloid manzamine A*. Antimicrob Agents Chemother, 2000; 44(6):1645–9.
86 Ang, K., et al. *Immune-mediated parasite clearance in mice infected with Plasmodium berghei following treatment with manzamine A*. Parasitol Res, 2001; 87(9): 715-21
87 Newman, D.J. and Cragg, G.M. *Natural products as sources of new drugs from 1981 to 2014*. J Nat Prod, 2016; 79(3): 629-61.
88 Nobili, S., et al. *Natural compounds for cancer treatment and preventio*n. Pharmacol Res, 2009; 59(6): 365-78.
89 Dyshlovoy, S.A, and Honecker, F. *Marine compounds and cancer: 2017 updates*. Mar. Drugs 2018; 16(2). pii: E41
90 Drug Bank. Available online: http://www.drugbank.ca
91 Gawron, A. and Kruk, I. *Cytotoxic effect of xanthotoxol (8-hydroxypsoralen) on TCTC cells in vitro*. Pol J Pharmacol Pharm, 1992; 44(1): 51-7.
92 Mohsen, S.M., and Ammar, A.S.M. *Total phenolic contents and antioxidant activity of corn tassel extracts*. Food Chem, 2009; 112(3): 595-8.
93 Shan, X.U., et al. *Reversal effect of 4'-methylether-scutellarein on multidrug resistance of human choriocarcinoma JAR/VP16 cell line*. Prog Biochem Biophys, 2006; 33: 1061-73
94 Sithranga, B.N., and Kathiresan, K. *Anticancer drugs from marine flora: an overview*. J Oncol, 2010; 2010:214186
95 Gorelik, E. *Augmentation of the antimetastatic effect of anticoagulant drugs by immunostimulation in mice*. Cancer Res, 1987; 47(3): 809-15.
96 Gorelik, E, et al. *Role of NK cells in the antimetastatic effect of anticoagulant drugs*. Int J Cancer, 1984; 33(1): 87–94
97 Dziarski, R. *Synergistic enhancement of T cell responses and interleukin-1 receptor expression by interleukin-1 and heparin or dextran sulfate*. Cell Immunol, 1992; 145(1): 100-10
98 Dziarski, R. *Enhancement of mixed leukocyte reaction and cytotoxic antitumor responses by heparin*. J Immunol,1989; 143(1): 356-65
99 O'Sullivan, G.M., et al. *Langerhans cell migration is modulated by N-sulfated glucosamine moieties in heparin*. Exp Dermatol, 2000; 9(1): 25–33

100 Beattie, A.J., et al. *New products and industries from biodiversity*. In: Hassan, R.; Scholes, R.; Ash, N., editors. Ecosystems and Human Well-Being. Millennium Ecosystem Assessment, Island Press; Washington D.C: 2005. p. 273-95.
101 The Ocean economy in 2030 © OECD 2016
102 Mondol, M., et al. *Diversity of secondary metabolites from marine Baciullus species: chemistry and biological activity*. Mar Drugs, 2013; 11(8): 2846-72
103 Gokulan, K., et al. *Metabolic pathways. Production of secondary metabolites of bacteria*. Encyclopedia of Food Microbiology, 2014; 561-569
104 Schinke, C., et al. *Antibacterial compounds from marine bacteria, 2010-2015*. J Nat Prod, 2017; 80(4):1215-28
105 Tortorella E., et al. *Antibiotics from deep-sea microorganisms: current discoveries and perspectives*. Mar Drugs, 2018; 16(10)
106 Lijian X., et al. *Antibacterial and antifungal compounds from marine fungi.* Mar Drugs, 2015;13(6): 3479-513
107 Tong J., et al. *Antiviral activities and putative identification of compounds in microbial extracts from the Hawaiian coastal waters*. Mar Drugs, 2012; 10(3): 521-38
108 Williams, P.G. *Panning for chemical gold: marine bacteria as a source of new therapeutics.* Trends Biotechnol, 2009; 27(1): 45-52
109 Waites, M.J., et al. *Industrial microbiology: an introduction*. Oxford, UK: Blackwell Science, 2011
110 Jensen, P.R., and Fenical, W. *Marine microorganisms and drug discovery: current status and future potentia*l. In: Fusetani N (Ed). Drugs from the sea. Basel: Karger, 200; 6-29.
111 Trindade-Silva, A.E., et al. *Bryostatins: biological context and biotechnological prospects*. Curr Opin Biotech, 2010; 21(6): 834-842.
112 de Pascale D., et al. *The microbial diversity of Polar environments is a fertile ground for bioprospecting*. Mar Genomics, 2012; 8: 1184-7
113 Helmke, E., and Weyland H. *Psychrophilic versus psychrotolerant bacteria-occurrence and significance in polar and temperate marine habitats.* Cell Moll Biol (Noisy-le-grand), 2004; 4(4): 449-60
114 Russell, N.J. *Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications*. Adv Biochem Eng Biotechnol, 1998; 61: 1-21
115 Papaleo, M.C., et al. *Bioactive volatile organic compounds from Antarctic (sponges) bacteria*. N Biotechnolo, 2013; 30(6): 824-38
116 Maida, I., et al. *Phenotypic and genomic characterization of the Antarctic bacterium Gilisia sp. CAL575, a producer of antimicrobial compounds*. Extremophiles, 2013;18(1): 35-49
117 Papa R., et al. *Anti-biofilm activity of the Antarctic marine bacterium Pseudoalteromonas haloplanktis TAC125*. Res Microbiol, 2013; 164(5): 450-6
118 Papa R. et al. *Anti-biofilm activities from marine cold-adapted bacteria against Staphylococci and Pseudomonas aeruginosa*. Front Microbiol, 2015; 6:1333
119 Council, N.R. *Marine biotechnology in the twenty-first century: problems, promise and products. National Academies Press*, 2002

120 Proksch, P., et al. *Drugs from the sea: opportunities and obstacles*. Mar Drugs, 2003; 1: 5-17.
121 Burke, L. et al. *Reefs at risk revisited*. World Resources Institute, 2011
122 Hay, M. *Marine chemical ecology: what's known and what's next?* J Exp Mar Biol Ecol, 1996; 200(1-2):103-34
123 Hart, J.B., et al. *The halichondrins: chemistry, biology, supply and delivery*. In: Fusetani N (org.). Drugs from the sea. Basel: Karger, 2000; 134-54.
124 Mendola, D. *Aquacultural production of bryostatin 1 and Ecteinascidian 743*. In: Fusetani N (org.). Drugs from the sea. Basel:Karger, 2000; 120-33.
125 Mendola, D. Aquaculture of three phyla of marine invertebrates to yield bioactive metabolites: process developments and economics. Biomol Eng, 2003; 20: 441-458.
126 Suresh, P.V., et a. *Valorization of food processing by-products*. CRC Press, Taylor & Francis, New York, 2013; 685-736
127 Pangestuti R., and Kim S.K. *Bioactive materials derived from seafood and seafood processing by-products*. A. Noomhorm, I. Ahmad, A.K. Anal (Eds.), Functional foods and dietary supplements: Processing effects and health benefits, Wiley-Blackwell, USA, 2014; 139-58
128 Arvanitoyannis, I.S., and Kassaveti, A. *Fish industry waste: treatments, environmental impacts, current and potential uses*. International J Food SciTechnol, 2008; 43: 726-745.
129 Olsen, R.L., et al. *Challenges and realistic opportunities in the use of by-products from processing of fish and shellfish*. Trends Food Sci Technol, 2014; 36: 144-51
130 Rustad, T., et al. *Possibilities for the utilization of marine by-products.* International J Food Sci & Technol, 2011; 46: 2001-14.
131 Kim, S.K, et al. *Subacute toxicity of chitosan oligosaccharide in Sprague-Dawly rats*. Antibiotics. Arzneim-Forsch. Drug Research, 2001; 51: 769-74.
132 Jeon, Y.J., and Kim, S.K. *Antitumor activity of chitosan oligosaccharides produced in an ultra filtration membrane reactor system*. J Microbiol Biotechnol, 200; 12: 503-7
133 Jeon, Y.J, and Kim, S.K. *Continuous production of chitooligo-saccharides using a dual reactor system*. Process Biochem, 2000; 35: 623-32
134 Wang, S.L, and Yeh, P.Y. *Purification and characterization of a chitosanase from a nattokinase producing strain Bacillus subtilis TKU007 using shrimp shell powder as a medium*. Process Biochem, 2008; 43: 1545-52.
135 Wang, S.L., and Yeh, P.Y. *Production of a surfactant-and solvent-stable alkaliphilic protease by conversion of shrimp shell wastes fermented by Bacillus subtilis TKU007*. Process Biochem, 2006; 41: 1545-52
136 Wang, S.L, et al. *A solvent sable metalloprotease produces by Bacillus sp. TKU004 and its application in the deproteinization of squid pen for β-chitin preparation*. Enzyme and Microbial Technology, 2006; 39:724-31

Materials and methods

In this chapter the material and method common in all three chapters are reported.

1. Growth media compositions

M9 Salts Solution

Na_2HPO_4	6.0 g/L
KH_2PO_4	3.0 g/L
NH_4Cl	1.0 g/L
NaCl	0.5 g/L

Marine Broth (MB)

$SrCl_2$	34.0 g/L
NaCl	19.45 g/L
$MgCl_2$	5.9 g/L
Peptone	5.0 g/L
$MgSO_4$	3.24 g/L
$CaCl_2$	1.8 g/L
Yeast extract	1.0 g/L
KCl	0.55 g/L
$NaHCO_3$	0.16 g/L
Ferric citrate	0.1 g/L
KBr	0.08 g/L
Na_2PO_4	0.008 g/L
Na_2SiO_3	0.004 g/L
NaF	0.0024 g/L
H_3BO_3	0.0022 g/L
NH_4NO_3	0.0016 g/L

Modified Marine Broth (mMB)

MB supplemented with peptone 10.0 g and K_2HPO_4 0.3 g

Tryptic Soy broth (TSB):

Tryptone	17.0 g/L
NaCl	5.0 g/L
Soytone	3.0 g/L
K_2HPO_4	2.5 g/L
Dextrose	2.5 g/L

Luria-Bertani Broth (LB)

Tryptone	10.0 g/L
NaCl	10.0 g/L
Yeast extract	5.0 g/L

Brain Heart Infusion broth (BHI)

Infusion from beef heart	250.0 g/L
Infusion from calf brain	200.0 g/L
Proteose peptone	10.0 g/L
NaCl	5.0 g/L
Na_2HPO_4	2.5 g/L
Dextrose	2.0 g/L

TYP

Tryptone	16.0 g/L
Yest extract	16.0 g/L
NaCl	10.0 g/L

Nematode Growth Medium (NGM)

Peptone	2.5 gr/L
NaCl	2.9 gr/L
$CaCl_2$	1 mM
Cholesterol	5 µg/mL
KH_2PO_4	25 mM
$MgSO_4$	1 mM

All media were prepared by dissolving the compounds in distilled water and were sterilised by autoclaving at 121°C for 15 min.
To obtain the solid media 17 gr/L of agar were added.

2. Human and food pathogen used as target

The following human pathogens were used as target to evaluate the antimicrobial activity of the three marine sources reported in the three chapters.

Strain	Species	Gram classification
ILVO 4360	*Achromobacter xylosoxidans*	Negative
AB13	*Acinetobacter baumannii*	Negative
LMG 24068	*Burkholderia metallica*	Negative
ATCC 10536	*Escherichia coli*	Negative
DF12SA	*Klebsiella pneumoniae*	Negative
6538P	*Staphylococcus aures*	Positive
RP62A	*Staphylococcus epidermidis*	Positive
D71	*Stenotrophomonas maltophilia*	Negative
PaO1	*Pseudomonas aeruginosa*	Negative

In addition, the following bacteria responsible for food spoilage were also used as target.

Strain	Species	Gram classification
ILVO MB677 CIS 4b	*Listeria monocytogenes*	Positive
LMG 10395	*Salmonella enteritidis*	Negative
MB4487	*Salmonella thyphimurium*	Negative
ILVO MB4972	*Pseudomonas fragi*	Negative

In addition, the strain *Paenibacillus alvei* and *Pasteurella piscicida,* pathogen bacteria of bees and fishes, respectively, were used as target to evaluate the antimicrobial activity of shellfish by-products derived extracts (Chapter 3)

All the strains, except *L.monocytogenes* were routinely recovered from frozen stocks, grown in Luria-Bertani (LB) broth at 37°C and shaking at 200 rpm for 24h and weekly maintained on LB solid medium at 4°C. *L. monocytogenes* was grown in Brain Heart Infusion (BHI) broth at 37 °C.
The strains *S. aureus* 6538P and *S. epidermidis* RP62A were also used as target to evaluate the antibiofilm activity

Human cancer cells used as target

The following cellular lines of human cancers were used as target to evaluate the anticancer potential: MCF-7 (breast cancer cells), MDA-MB-231, A375 (skin melanoma cells) and HeLa (cervical cancer cells). All the strains were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine, at 37°C an atmosphere of 5% CO_2.

Nematode strain used as target

The *Caenorhabditis elegans* strain N2 Bristol (wild type) was obtained from the *Caenorhabditis* Genetic Center (CGC), University of Michigan, USA. This strain was used as target to evaluate the anthelmintic activity. The strains was recovered from frozen stock and routinely propagated on Nematode Growth Medium (NGM) agar plates, supplemented with *Escherichia. coli* OP50 as nutrient source, and incubated at 20°C
E. coli OP50 cells were routinely grown in LB liquid medium at 37°C and shaking at 200 rpm for 24h and weekly maintained on LB solid medium at 4°C.

CHAPTER 1:

Bacteria from the extreme: *Shewanella* sp. S3A, a promising candidate to counteract helminthic infections and microbial biofilms

Bacteria from the extreme: *Shewanella* sp. S3A, a promising candidate to counteract helminthic infections and microbial biofilms

Abstract

The Antarctic sea-ice is the frozen seawater of the Southern Ocean and it represents an extreme environment suitable for bioprospecting. Antarctic microorganisms are well known as producers of valuable bioactive compounds. Herein, we applied a traditional biodiscovery pipeline for the identification of multi-bioactive producer strains. Antarctic sea-ice aqueous samples were collected from the Ross Sea and used to isolate 40 microorganisms. They were used to create a collection of cell-free fermentation supernatants (FS), containing the compounds produced by each strain in different growth conditions, and released in the external environment. This collection of cell-free FS was used in a preliminary screening aimed at investigate the ability of isolated microorganisms to produce bioactive compounds. In particular, the antimicrobial, antibiofilm, anthelmintic and anticancer potentiality were investigated. The most promising strain selected from the preliminary screening, showing both anthelmintic and antibiofilm activity was identified, via sequencing and analysis of 16s rRNA genes, as *Shewanella antarctica.* The isolate was used for the production of lipopeptide-rich extract, which confirmed both the activities when grown at 4 °C. The minimum toxic concentration toward the helminth *Caenorhabditis elegans* resulted to be 125 µg/mL, with a killing rate of 80%, and the minimum concentration able to inhibit the biofilm formation of *Staphylococcus epidermidis* RP62A resulted to be 250 µg/mL. The bioactive extract was characterized in terms of fatty acids compositions. The full chemical characterization of the extract is still on going. This work shows the validity of the multi-bioactivity screening method based on the creation of a cell-free FS as a fast and effective screening method for bioprospecting of microorganisms.

1.1 Introduction

The marine environment is considered the richest source of biodiversity. The marine microbial biodiversity was estimated to reach approximately 3.67×10^{30} different microorganisms [1].

The unique environments of seawater and oceans represent a promising source for the isolation of new bioactive compounds-producer microorganisms. Marine microorganisms have developed unique metabolic abilities to ensure their survival in the varied and hostile marine habitat, resulting in the biosynthesis of an array of secondary metabolites with unique biological activities and structures not found in terrestrial organisms. Such compounds, known as marine natural products (NP), are generally used by organisms to control ecological relationships that involve defence against predation, competition for space and food, interspecies communication, among other functions [2, 3]. These compounds, therefore, are required to implement the survival strategies of bacteria in adverse conditions, but many of them turn out to be useful for modern society due to their biological activities. A lot of marine secondary metabolites, in fact, have already found a wide range of applications as anti-tumor, anti-proliferative, photoprotective, antibiotic, anti-infective, antifungal, antiviral, antiparasitic, antihelmintic [4 - 8].

The advantage to isolate potential bioactive compounds-producer strains from marine sources, in contrast with macroorganisms, is related to the feasible and sustainable production of large quantities of secondary metabolites with reasonable cost, by large-scale cultivation and fermentation of source organisms [9].

Among marine ecosystems, the Antarctic Sea Ice represents an extreme environment suitable for the research of promising bacteria. When it reaches its maximum, it becomes one of the major ecosystems on the planet, covering 13% of the Earth's surface [10]. Frozen seawater, unlike freshwater ice, is characterized by a network of channels and pores, which vary in size from few micrometres to millilitres. In these channels, brine formed from expelled salts when the ice crystals freeze together is accumulated [11].

Sea ice is highly variable in terms of temperature, salinity, space, and light because of swift transitions between water and ice, with the subsequent rapid physical and chemical changes.The labyrinth of brine hosts many planktonic organisms, including viruses, bacteria, algae, protists, flatworms, and small crustaceans, with a great abundance of bacteria. Biological assemblages are trapped within the brine channels as the ice grows and consolidates, being almost immediately confined to a new habitat quite different from the

one from which they were recruited [12].Therefore, organisms living this habitat are required to possess a great metabolic adaptability, which make these organism potential producers of novel compounds.
Changes in salinity and temperature are the main factors that may have an influence on the microorganisms within the labyrinth of sea ice. The high brine salinity is a strong stress factor for ice-trapped organisms, which have to face percentages of salinity three times that of normal seawater. Conversely, when the ice melts, the released organisms are exposed to hyposaline conditions close to freshwater. The osmotic stress due to the high concentration of dissolved organic matter, requires the adjustment of balance between the external medium and the intracellular medium, by the accumulation or synthesis of organic solutes, such as proline, mannitol, and glycine betaine [13].Temperature is also a key factor which drives the ecological structure of sea-ice. The transition to internal temperature typically ranging from -1 to -15 °C favour the abundance of psychrophilic species (minimum growth temperature $\leq$ 0°C, optimal growth temperature < 15 °C, and maximum growth temperature < 20 °C). The capacity to survive at low temperature requires the maintenance of functional lipid membranes. The degree of membrane fluidity and resistance at low temperature is allowed by the ability of cold-adapted microorganisms to modify their membrane fatty acids composition. Hence, an increase in the proportion of unsaturated fatty acids, a decrease in average chain length and an increase in polyunsaturated fatty acids (PUFAs) is fundamental to retaining membrane fluidity at low temperature [14].
In this work, we report a complete biodiscovery pipeline aimed at the identification of powerful bioactive compounds-producing strains starting from the isolation of bacteria from Antarctic sea-ice samples. A simple and fast newly created multi bioactivity screening was set up. A promising psychrotolerant bacterium, showing anthelmintic and antibiofilm activity, belonging to genus *Shewanella* was selected.

1.2 Materials and methods

Isolation of bacterial strain

In this work, sea-ice samples, collected from four different sampling sites near Terranova Bay, Ross Sea, Antarctica, were used for the

isolation of microorganisms. The seawater samples were filtered through 0.22 µm filters. The filters were mixed with 10 mL of M9 salts solution in 50 mL sterile tubes and incubated at 15°C with agitation at 200 rpm for 24h. Extinction cultures were obtained as 10^{-5} dilutions of the original enrichments and 100 µL aliquots from each dilution were plated on Marine Broth (MB) solid medium plates Plates were incubated at 15°C. After the incubation period, from 15 to 30 days, colonies were picked and grown as pure cultures in MB at 20°C for 2 days. The isolated bacteria were then suspended in glycerol (20% final volume) and stored at -80°C.

Preliminary screening for antimicrobial activity: agar-diffusion test

In order to detect the antimicrobial activity of isolated Antarctic strains, a preliminary screening was performed by a modified agar-diffusion test against the selected target strains listed in the chapter "Material and methods" Isolated Antarctic bacteria were inoculated into a sterile flat bottom 96-well plate in 200 µl of different media. Growth media used for the experiment are listed below and described in the chapter "Materials and methods":

- Marine Broth (MB)
- Modified Marine Broth (mMB)
- Tryptic Soy Broth (TSB)
- Tryptone Yeast Broth

The 96-well plates were incubated at 20°C and shaking at 120 rpm for 48h and used to inoculate sterile 96-Deepwell plates, filled with the 4 different media. The 96-Deepwell plates were incubated at 20°C and shaking at 120 rpm for 5 days to allow the production of secondary metabolites. A replica of the 96-Deepwell plate by transferred, by using a sterilised 96-Pin replicator, on LB plates inoculated with the target bacteria.

The target bacteria, previously grown for 24h in LB or BHI at 37°C and shaking at 200 rpm were inoculated in 50 mL of LB supplemented with agar, before solidification, at a concentration of 0.05 OD/m L and poured in Petri dishes for the agar-diffusion test.

Plates with the target bacteria and the replica of the tester strains were incubated at 20°C for 48h to allow the growth of the tester Antarctic bacteria and then moved at 37°C for 24h to allow the growth of target pathogenic bacteria. For statistical purposes, three independent experiments were carried out.

Antimicrobial activity is identified by the formation of inhibition halos around antimicrobials-producing colonies.

Preliminary screening for other activities: creation of a cell-free fermentation supernatants collection

Preliminary screening for antibiofilm, anthelmintic and anticancer activity was performed through the creation of a cell free-fermentation supernatants (FS) collection. The isolated Antarctic bacteria were grown in 10 ml of the four different media mentioned above in sterile 50 mL tubes at 20°C and shaking at 200 rpm for 5 days. The exhausted culture broths were centrifuged at 7000 rpm for 30 minutes and the supernatants were further filtered through 0.22 µm filters, in order to obtain cell-free FS, containing all the compounds produced and released by bacteria in the extracellular medium.
In order to perform different bioactivity screenings, 200 µL aliquots of each cell-free FS were transferred into a sterile flat bottom 96-Well plate, by creating a master plate to use for the following assays.

Preliminary screening for anticancer activity: cell-viability assay

For the cell viability assay, MCF-7, A375, MDA-MB-231 and HeLa cells were harvested by trypsinization, seeded in sterile round bottom 96-well plates at 4 x 10^4 cells/well in 100 µL of complete DMEM and allowed to adhere overnight at 37°C and 5% CO_2. After 24 h of incubation, the cells were treated with the cell-free FS (10% w/v) for 24 h. The cell-viability was evaluated using the Cell Counting Kit-8 (Sigma). After the 24h-treatment, the exhausted medium was replaced with fresh medium and 10 µL of tetrazolium salt (WST-8) were added to each well. The cells were again incubated at 37°C for 4 h, after which absorbance was read by using the ELx800 microplate reader (BioTek Instruments) at 490 and 630 nm. For this assay cells not treated represented the negative control to determine the normal cell growth. Wells containing only the medium represented the negative control and were used as blank. After subtracting the not-specific OD_{630nm} value from the OD_{490nm} value, the percentage of viable cells was calculated by the formula:

Percentage of cell viability =
[(Test OD - Blank OD) / (Cells OD - Blank OD)] x100

For statistical purposes, two independent experiments with three replicates per trial were carried out.

Preliminary screening for antibiofilm activity: crystal violet assay

S. aureus 6538P and *S. epidermidis* MB5263 were used as target strains to evaluate the effect of cell-free FS on biofilm formation. Samples (10% v/v) were transferred from the master plate into sterile flat bottom 96-well plates, filled with LB broth supplemented with glucose (1% w/v). The target bacteria, previously grown in LB at 37°C and shaking at 200 rpm for 24 h, were diluted using OD/CFU correlation growth curves, and about 2 x 10^5 CFU were dispensed into each well of the prepared plate. Plates were incubated at 37°C for 24 h. After the incubation time, prior to the crystal violet assay, the wells containing the bacterial suspension were analyzed spectrophotometrically at 630 nm to ensure that there was no antibiotic activity. In the crystal violet assay, the planktonic cells and spent media were discarded and weakly adherent cells were removed through washing twice with deionized water, and allowed to air dry before being stained. The biofilms were stained with 100 µL of 0.01 % (w/v) crystal violet solution for 20 min. Subsequently the dye was discarded and the wells were rinsed twice with deionized water. The wells were allowed to dry before solubilisation of the crystal violet with 100 µL of absolute ethanol for 30 min. The optical density was determined at 490nm, using the ELx800 microplate reader (BioTek Instruments). In this assay, wells containing no samples represented the negative control to determine the normal biofilm formation. The percentage of biofilm inhibition was calculated by the formula:

Percentage of biofilm inhibition =
([Control OD – Test OD] / Control OD) x 100

For statistical purposes, two independent experiments with three replicates per trial were carried out

Preliminary screening for anthelmintic activity: nematode toxicity assay

The anthelmintic activity of cell-free FS was evaluated through the *in vitro* liquid toxicity assay.

Synchronised nematodes were obtained by bleaching treatment [16]. Adult nematodes and eggs were harvested from NGM plates with sterile water and treated with a sodium hypochlorite solution for 2 min, mixing gently. The bleaching solution was removed and after washings with S-basal and water, the eggs were transferred on NGM plates supplemented with *E.coli* OP50 and incubated at 20°C. After three days of incubation, the plate contained nematodes synchronised in the 4th larval stage (L4). Around 20-30 L4 nematodes were transferred to each well of sterile 96-well plate containing 100 µL of M9 salts solution supplemented with 5µg/mL cholesterol and *E.coli* OP50 at the concentration of 0.5 OD/mL. Cell free-FS (10% v/v) were added into each well of the prepared plate. The plate was incubated at 20°C and after 24h and 48h the wells were scored for living worms. A worm was considered dead when it no longer responded to touch. The percentage of surviving nematodes after the treatment was obtained by the formula:

Percentage of viable worms =
(N° worms t24h / N° worms t0h) x 100

For statistical purposes, two independent experiment with three replicates per trial were carried out with a unique egg preparation

Molecular identification and phylogenetic analysis of isolated strains

Genomic DNA was used as template for the amplification via PCR of 16S rDNA genes. The genomic DNA was extracted from the isolated bacteria by using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) following the manufacturer's instructions. Concentration and purity of extracted genomic DNA were detected by reading absorbance at 260 nm, 230 nm and 280 nm, using a NanoDrop spectrophotometer.

The PCR reaction 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 Eub 27F (Forward, seq: 5′-AGAGTTTGATCMTGGCTCAG-3') and Univ1492R (Reverse, seq:5′ -GGTTACCTTGTTACGACTT-3′)[17]. The reaction conditions used were: one cycle for iniatial denaturation (95 °C for 5 min), 30 cycles for amplification (95 °C for 90 s, 55 °C for 30 s and 72 °C for 2 min) and final extension step (72 °C for 5 min). The presence of PCR products (1.5 kb) was verified on 1% agarose gel

by electrophoresis. PCR products were then purified using the GeneElute PCR Clean-Up kit (Sigma-Aldrich) following the manufacturer's instructions and sequenced. The DNA sequence output was edited with BioEdit software and submitted to BLAST for the phylogenetic analysis.

Extract preparation

A single colony of bacterial isolate used to inoculate 3 mL of liquid medium in sterile 15 mL bacteriological tubes. After 48 h of incubation at 20°C and shaking at 200 rpm, the pre-inoculum was used to inoculate 125 mL of the same medium in 500 mL flasks, at an initial cell concentration of 0.01 OD600/mL. The flasks were incubated at 20°C and 4°C with shaking at 200 rpm up to5 days. The cultures were then centrifuged at 7000 rpm at 4°C for 30 min and the exhausted culture broth were collected and store at -20°C.
The exhausted culture broths were first used for extraction of biosurfactants lipopeptides, using a combination of acid precipitation and solvent extraction. The aqueous cell-free FS were acidified by addition of concentrated HCl to pH 2.0 and incubated overnight at 4°C to allow the formation of a precipitate. They were then centrifuged at 8000 rpm for 15 min to obtain pellet. The supernatant was removed and stored and the pellet was extracted with methanol for 2h while stirring continuously. The methanol was recovered by filtration, to remove remaining material, and evaporated to dryness using rotatory evaporation a Rotavapor® R-100 (Buchi, AG Switzerland) equipped with a V-100 pump (Buchi, AG Switzerland). The obtained crude extract was weighted, dissolved in 100% DMSO at 50 mg/mL and stored at -20°C.
After removing the precipitate, the residual supernatant was subjected to a further liquid-liquid organic extraction using three volumes of ethyl acetate in separatory funnels. The organic phase collected was evaporated using the rotavapor. The obtained ethyl aceate extract was weighted, dissolved in 100 % DMSO at 50 mg/mL and stored at -20°C until used for biological assays.

Antibiofilm activity

To confirm the antibiofilm activity detected in the preliminary screening with the cell-free FS, extracts were placed into each well of a flat bottom 96-well plate at an initial concentration of 1 mg/mL

and serially 2-fold diluted using LB medium supplemented with glucose (1% w/v). The prepared plate was inoculated with the target bacterium (2 x 10^5 CFU/100µL/well) and incubated as described above. The crystal violet assay was carried out as described above. Wells containing no compounds represented the negative control. In this assay DMSO was also used as control to determine the effect of solvent on biofilm formation.

Anthelmintic activity

To confirm the anthelmintic activity detected in the preliminary screening with the cell-free FS, the toxicity test on *C. elegans* was performed as described above using the extracts at 1 mg/mL. Wells containing no compound represented the negative control to determine the normal nematode's viability. DMSO was also used as control to determine the effect of solvent on worm's viability.

Radical-scavenging activity

To determine the antioxidant potential of the selected strain, extracts were placed into each well of a flat bottom 96-microtiter plate at initial concentration of 1 mg/mL and serially 2-fold diluted using methanol in a final volume of 100 µL. Then 100 µL of a methanolic DPPH (0.5 mM) solution were added to each well and the plate was incubated at room temperature overnight. Ascorbic acid was used as positive control. Well containing no compounds represented the negative control used as blank. The abosarbance at 590 nm was read and the percentage of scavenging activity was calculated by the formula:

Percentage scavenging activity =
([Blank OD – Test OD] / Blank OD) x 100

Fatty acids analysis by GC-MS

Fatty acid methyl esters were prepared from dried extracts using a direct transesterification procedure with 2.5% (v/v) sulfuric acid in methanol as described by De Troch *et al*. [18]. The internal standard (19:0, 6 µg, Sigma-Aldrich, Bornem, Belgium) was added prior to the transesterification procedure. Fatty acid methyl esters were extracted twice with hexane.
Composition analysis of fatty acids was carried out using a gas chromatograph (HP 7890B, Agilent Technologies, Diegem,

Belgium) equipped with a flame ionization detector (FID) and connected to an Agilent 5977A Mass Selective Detector (Agilent Technologies, Diegem, Belgium). The GC was further equipped with a PTV injector (CIS-4, Gerstel, Mülheim an der Ruhr, Germany). A 60m×0.25mm×0.20µm film thickness HP88 fused-silica capillary column (Agilent Technologies, Diegem, Belgium) was used for the gas chromatographic analysis, at a constant Helium flow rate (2 mL/min). The injected sample is split equally between the MS and FID detectors at the end of the GC column using an Agilent capillary flow technology splitter. The oven temperature program was as follows: at the time of sample injection the column temperature was 50°C for 2 min, then gradually increased at 10°C/min to 150°C, followed by a second increase at 2°C/min to 230°C. The injection volume was 2 µL. The injector temperature was held at 30°C for 0.1 min and then ramped at 10°C/s to 250°C and held for 10 min. The transfer line for the column was maintained at 250°C. The quadrupole and ion source temperatures were 150 and 230°C, respectively. Mass spectra were recorded at 70 eV ionization voltage over the mass range of 50 - 550 m/z units.
Data analysis was done with Agilent MassHunter Quantitative Analysis software (Agilent Technologies, Diegem, Belgium). The signal obtained with the FID detector was used to generate quantitative data of all the compounds. Peaks were identified based on their retention times, compared with external standards as a reference (Supelco 37 Component FAME Mix, Sigma-Aldrich, Overijse, Belgium) and by the mass spectra obtained with the Mass Selective Detector. Quantification of fatty acid methyl esters was based on the area of the internal standard (19:0) and on the conversion of peak areas to the weight of the fatty acid by a theoretical response factor for each fatty acid [19, 20].

1.3 Results

Isolation of Antarctic bacteria

Antarctic sea-ice samples were used for the isolation of psychrotolerant microorganisms. The water samples were filtered through 0.22 µM filters in order to concentrate the amount of bacteria dissolved in the liquid samples. For the isolation the MB medium was used, which is a specific medium for heterotrophic marine bacteria's growth, with a salt's composition very similar to the sea's

salts composition. Extinction culture up to 10^{-5} dilutions were plated to increase the probability to observe a major biodiversity, which is not possible in a crowded microenvironment. The incubation time was extended to 30 days to allow the development of slow-growing strains. After 30 days, a morphological analysis was performed in order to collect the colonies, on the basis of differences in colours, sizes and shapes.
In total, 40 different colonies were isolated, were picked and used to obtain pure isolates. The isolated bacteria were used to create a clone collection, stored in glycerol (20% v/v) at -80°C.

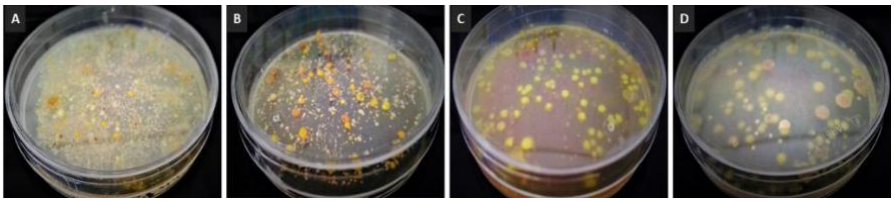


Fig. 1 Extinction cultures of the sea-ice concentrated samples on MB agar

Preliminary screening: selection of bioactive-compounds producing strains

A fast and simple multi-bioactivity screening method was set up to screen the clone collection, in order to select potential bioactive compounds-producing strains. Among the isolated strains, 20 were chosen to be included in the preliminary screening, basing on the most interesting morphological characteristics (colour/shape).
The isolated bacteria were grown in different media in order to create different microenvironments and stimulate different metabolic pathways, leading to the production of different compounds.
To check the antimicrobial activity of the Antarctic bacteria, the halo inhibition assay, based on the diffusion of antimicrobial compounds in a plate inoculated with the target bacteria, was performed. A panel of different target bacteria, including MDR and opportunistic human pathogens and bacteria responsible for food spoilage, was selected.
To evaluate other biological activities a cell-free FS collection was created. The cell-free fermentation supernatant contain compounds produced by the bacteria and released in the extracellular environment. The collection of cell-free FS was screened for the presence of antibifilm, anticancer and anthelmintic activity.
To check the strong biofilm former strains *S. aureus* 6538P and *S. epidermidis* RP62A were used as target.

The anticancer potential was evaluated by performing a cell-viability assay was using the following cell lines as target: MCF-7, A375 and HeLa.
The anthelmintic activity was checked using *C. elegans* as nematode target.
The strain coded S3.A was selected as positive to anthelmintic and antibiofilm screening.
None of the isolated strains showed positivity in the halo inhibition assay (antimicrobial activity) and the screening to evaluate the anticancer potential.
As shown in the following table, the strain S3.A, grown in TYP medium at 20°C , was the most valuable antibiofilm producer-strain, with a biofilm inhibition percentage of 64%. The antibiofilm activity was selective towards *S. epidermidis* RP62A.

	S2.L	S4.B	S3.N	S2.B	S3.A	S3.P	S4.G	S2.D	S3.R	S2.A
TSB	6	0%	7	32%	28	4	11	10	3	23
MB	6	9%	12	26%	24	16	24	6	11	2
TYP	7	28	7	27%	**64%**	26	26	31	9	7
mMB	12	23	6	25%	5	12	15	41	21	25

	S2.C	S1.H	S1.G	S3.O	S4.F	S4.D	S1.B	S1.F	S3.C	S4.H
TSB	3	56	7%	4	5	12	6	17	6	2
MB	2	6	6%	10	9	15	20	3	16	4
TYP	9	9	7%	4	8	15	4	6	27	3
mMB	1	43	3%	6	1	3	5	8	10	7

Tab. 2. Effect of 10% (v/v) cell free-FS on *S. epidermidis* RP62A biofilm formation after the 24h treatment. Antibiofilm activity is expressed as percentage of biofilm inhibition.

As reported in the following table, the strain S3.A, grown in TYP was also able to produce anthelmintic compounds, showing the highest activity, with 80 % of killing rate.

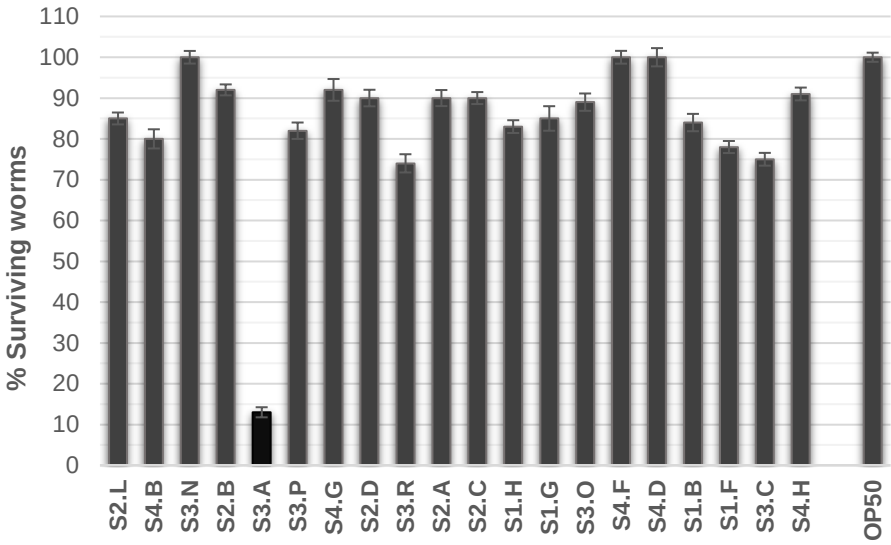


Fig.3. Effect of 10% (v/v) cell-free FS on *C. elegans* viability after the 24h treatment. OP50 represent the normal worms viability. Anthelmintic activity is expressed as percentage of surviving worms. Error bars represent SEM (Standard Error of the Mean).

On the basis of anthelmintic and antibiofilm potential, the strain S3A was selected for further study.

Phylogenetic affiliation of S3A

The 16S rDNA genes of the isolate S3A was amplified by PCR and the obtained sequence was submitted to BLAST for the strain identification. The anthelmintics and antibiofilm producer strain S3A showed the highest score of sequence similarity with *Shewanella Antarctica*.

Antibiofilm activity of S3A-derived extracts

In order to confirm the antibiofilm activity of the cell-free FS derived from S3A detected in the preliminary screening, the strain was grown in TYP at 4° and 20° and the cell-free FS were used for the extraction of extracellular compounds. The antibiofilm assay was carried out using different concentrations of the extracts obtained as described in the section “material and methods” of this chapter. Data obtained revealed that the methanol crude was more active compared with the residual ethyl acetate extract. Moreover, the

bacterium produced more active compounds when it grew at 4°C. The methanol crude extract derived from the growth at 4°C was able to inhibit the biofilm formation of *S. epidermidis* RP62A with a biofilm inhibition percentage of 57 % at 0.25 mg/mL.

Conc. [mg/mL]	1	0,5	0,25	0,125
MeOH extract 4 °C	68	62	57	28
EtOAc extract 4 °C	52	37	35	33
MeOH extract 20 °C	16	9	2	2
EtOAc extract 20 °C	41	22	19	17

Tab. 4. Effect of different concentrations of *Shewanella* S3A-derived extracts on *S. epidermidis* RP62A biofilm formation after 24-h treatment. Antibiofilm activity is expressed as percentage of biofilm inhibition.

Anthelmintic activity of S3A derived extracts

In order to confirm the anthelmintic activity of the cell-free FS derived from S3A detected in the preliminary screening, the strain was grown in TYP at 4°C and 20°C and the cell-free FS were used for the extraction of extracellular compounds. The toxicity test towards *C. elegans* was carried out using 1 mg/mL of the extracts obtained as described in the section “materials and methods” of this chapter The methanol crude extract produced by the strain grown at 4°C showed the highest activity, with a killing rate around 90 %.

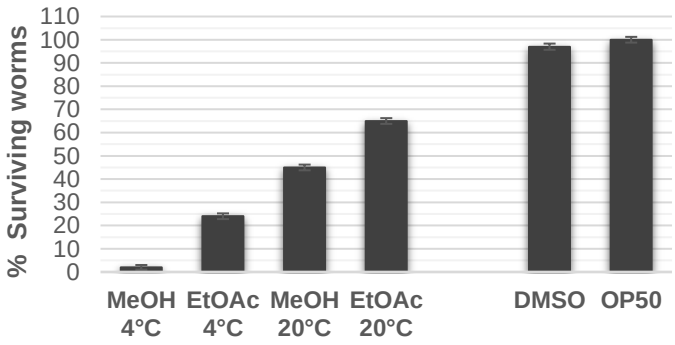


Fig.5. Effect of 1 mg/mL of *Shewanella* S3A-derived extracts on *C. elegans* viability after 24h treatment. Anthelmintic activity is expressed as percentage of surviving worms. DMSO represents the negative control. OP50 represents the normal worms viability. Error bars represent SEM (Standard Error of the Mean).

In order to determine the Minimum Toxic Concentration (MTC) the toxicity test was repeated using 2-fold serial dilutions of the methanol extract at 4°C. The MTC resulted to be 0.125 mg/mL, with killing rate of 80%, as reported in the following graph.

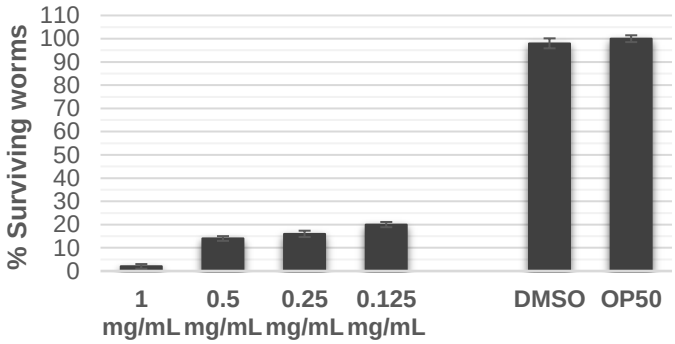


Fig. 6. Effect of different concentrations of *Shewanella* S3A-derived extracts on *C. elegans* viability after the 24h treatment. Anthelmintic activity is expressed as percentage of surviving worms. DMSO represents the negative control. OP50 represents the normal worms viability. Error bars represent SEM (Standard Error of the Mean).

Fatty acid analysis of the methanol extract

Lipopeptide and high molecular weight biosurfactants vary greatly in the lipid portion, therefore the analysis of this component provides detailed structural information for identification of fatty acid structures. To facilitate the analysis by GC-MS, a process which involves cleavage of the link between the carbohydrate or peptide/protein part of the biosurfactants and the lipid portions was carried out. This process allowed the derivatization of the resulting fatty acid chains to fatty acid methyl esters (FAME), easily detectable by GC-MS. The methanol extract obtained from the growth of S3A at 4°C was subjected to this process and the fatty acid analysis was performed. The fatty acid composition, described in the following table, resulted to be: 56% of saturated fatty acids, 39% of monounsaturated fatty acids (MUFa) and 5% of poluunsaturated fatty acids (PUFa).

FAs	FAs %
12:0	3,6
13:0	0,4
iso-14:0	0,3

14:0	7,6
iso-15:0	11,0
14:1	1,1
anteiso-15:0	0,8
15:0	1,5
iso-16:0	1,2
16:0	20,2
16:1+iso-17:0	1,5
cis-9-16:1	29,9
17:0	0,5
16:2n-7	0,1
iso-18:0	0,6
17:1+16:2n-4	1,0
18:0	5,3
cis-9-18:1	7,2
cis-11-18:1	6,3
16:4n-1	1,5
19:0	15,6
18:2n-6	1,0
20:0	0,2
18:3n-3	0,1
20:1 a	0,2
20:1 b	0,8
20:1	0,2
18:4n-3	0,1
CLA	0,7
20:4n-6	0,9
22:2	0,4
20:5n-3	0,7
22:5n-3	0,1

Tab. 7. FAME analysis. Percentages of fatty acids abundance.

Discussion

The marine environment is a good starting point for the isolation of bioactive compounds-producing strains, useful for biotechnological applications. In particular, extreme marine habitats, like the sea-ice, are interesting hot spots, due to their peculiar and diversified microenvironments, that allowed hosted microorganisms the

development of adaptive strategies, including unique secondary metabolites biosynthesis pathways.
In this work, Antarctic sea-ice samples were used to isolate Antarctic bacteria. A simple and fast multi-bioactivity screening platform, based on the creation of a cell-free supernatants allowed the selection of a biotechnological powerful bacterium identified as *Shewanella antarctica*. *Shewanella* S3A isolated in our lab showed both anthelmintic and antibiofilm activity. In particular, the growth of S3A at 4°C allowed the production of a biosurfactants/lipopeptides rich extract with both antibiofilm activity towards *S. epidermidis* RP62A and anthelmintic activity against *C. elegans*. These findings confirm the ability of bacteria to produce bioactive compounds at hostile conditions, like the low temperatures. In fact, the same strain, S3A, grown at 20°C, in the same nutritional conditions, produced a lipopeptide rich extract with a lower activity.
Considering the problem of biofilm producing bacteria, it is becoming really necessary to find new antibiofilm agents. Bacterial biofilms constitute a critical issue in a wide range of clinical domains including medicine, surgery and the food industry [21, 22, 23]. Biofilms also contaminate a wide variety of infrastructure elements such as water and air purification systems optical sensors, and marine and industrial equipment [24, 25]. Furthermore, biofilms can release harmful toxins and even obstruct indwelling catheters [26]. These capabilities increase the infective power of the bacteria.
The increasing need of finding new anthelmintic agents is also moving the research on marine natural products. In fact, today many anthelmintic drugs, because of their repeated and improper use are losing their effectiveness. Moreover, serious concern regarding the environmental impact of the nematicides used for crop protection have prompted the legislation to remove them from use, leaving agriculture at increased risk from nematode pests.
Shewanellae are known to have a great potential for use in biofuel cell applications, as well as for remediation of various environmental pollutants, due to their ability to utilize a diverse array of electron acceptors in absence of oxygen [27]. This ability allows them to survive in diverse habitats, including extreme environments like the sea-ice. Due to their peculiar characteristics, Shewanellae are very promising bioactive compounds producers. Despite the big amount of literature on the biotechnological applications of *Shewanellae*, little is known about their ability to produce bioactive compounds. According to our knowledge, this is the first report of the ability of a *Shewanella* sp. to produce antibiofilm and anthelmintic compounds. Of course, further

study is necessary to identify the active molecules and their mechanism of action, in order to give an added value to the already biotechnologically powerful of *Shewanella* genus.
The chemical characterization of the bioactive extracts is in progress, through GC-MS and HRMS analysis, in collaboration with the Department of Pharmacy of University of Naples “Federico II”.

References

1 Mondol, M., et al. *Diversity of secondary metabolites from marine bacillu species: chemestry and biological activity*. Mar Drugs, 2013; 11(8): 2846-72

2 Bhatnagar, I., and Kim S.K. *Immense essence of excellence: marine microbial bioactive compounds*. Mar Drugs, 2010; 8(10):2673-701

3 Imhoff, J.F., et al. *Bio-mining the microbial treasures of the ocean: new natural products*. Biotechnol Adv, 2011: 29(5):468-82

4 Waters, A.L., et al. *The expanding role of marine microbes in pharmaceutical developpment*. Current Opin in Biotechnol, 2010; 21(6): 780–786.

5 Teasdale, M.E., et al. *Secondary metabolites produced by the marine bacterium Halobacillus salinus that inhibit quorum sensing-controlled phenotypes in Gram-negative bacteria*. Appl Environ Microbiol, 2009; 75(3): 567-572.

6 Nishimura, S., et al. *Marine antifungal theonellamides target 3beta-hydroxysterol to activate Rho1 signaling*. Nat Chem Biol 2010; 6:519-526

7 Kayser, O., et al. *Natural products as potential antiparasitic drugs*. Stud Nat Prod Chem, 2002; 26: 779-848

8 Wright, G.D. *Something old, something new: reviseting natural product in antibiotic drug discovery*. Can J Microbiol, 2014; 60:147-154

9 Waites, M.J., et al. *Industrial microbiology: an introduction*. Oxford, UK: Blackwell Science, 2001

10 Parkinson, C.L., and Gloersen P. *Atlas of satellite observations related to global change*. R.J. Gurney, J. L. Foster and C.L. Parkinson., Eds. (Cambridge Univ. Press, Cambridge, 1993).Eicken, H. *Polar Biol,* 1992; 12:3

11 Palmisano, A.C., and Garrison D.L. in Antarctic Microbiology, Friedman E.I., Ed. (Wiley-Liss, New York, 1993), pp. 167-218

12 Kirst, G.O., Wiencke C. J Phycal 1995; 31:181

13 Hulbert, A.J. *Life, death and membrane bilayers*. J Exp Biol, 2003; 206: 2303-11

14 Brenner, S. *The genetic of Caenorhabditis elegans*. Genetics, 1974; 77:71-94

15 Ballestriero, F., et al*. Identification of compounds with bioactivity against the nematode Caenorhabditis elegans by a screen based on the functional genomics of the marine bacterium Pseudoalteromonas tunicata D2*. Appl Environ Microbiol, 2010; 76(17): 5710-7

16 Weisburg, W.G., et al. *16S ribosomial DNA amplification for phylogenetic study*. J Bacteriol, 1991; 173 (2): p. 697-703

17 de Troch M., et al. *Bioconversion of fatty acids at the basis of marine food webs: Insights from a compound-specific stable isotope analysis*. Marine Ecology Progress Series, 2012; 465: 53–67

18 Ackman, R.G, and Sipos, J.C. *Application of specific response factors in the gas chromatographic analysis of methyl esters of fatty acids with flame ionization detectors*. J Am Oil Chem Soc, 1964; 41:377–8

19 Wolff, R.L., et al. *Evaluation of sequential methods for the determination of butterfat fatty acid composition with emphasis on tran-18:1 acids.*

Application to the study of seasonal variations in French butters. J Am Oil Chem Soc,1995; 72:1471–83

20 Srey, S., et al. Biofilm formation in food industries: a food safety concern. Food Control, 2013; 31(2): 572-85

21 Percival, S.L. Importance of biofilm formation in surgical infection. British J Surg, 2017; 104:e85-e94

22 Khatoon, Z., et al. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. Heliyon, 2018;4(12):e01067

23 Zacheus, O.M., et al. Bacterial biofilm formation on polyvinyl chloride, polyethylene and stainless steel exposed to ozonated water. Water Research, 2000; 34(1): 63-60

24 Mittelmann, M.W. Structure and functional characteristics of bacterial biofilms in fluid processing operations. J Dairy Sci, 1998; 81(10): 2760-64

25 Nickel, J.C., et al. Bacterial biofilms and catheters: A key to understanding bacterial strategies in catheter-associated urinary tract infection. Can J Infect Dis, 1992; 3(5): 261–7.

26 Hau, H.H., and Gralnick, J.A. Ecology and biotechnology of the genus Shewanella. Annu Rev Microbiol, 2007; 61: 237-58

CHAPTER 2:

The lipids-rich extract from the marine edible gastropod *Haliotis tuberculata*: a potential source of bioactive compounds

The lipids-rich extract of the marine edible gastropod *Haliotis tuberculata*: a potential source of bioactive compounds

Abstract

The phylum Mollusca represents one of the largest groups of marine invertabrates. Nowadays molluscan shellfish belonging to the classes Bivalvia and Gastropoda have a commercial interest in fishery and aquaculture. Over the past decades, bioactive properties of bivalve molluscs have been widely investigated and several dietary supplements have been brought to the market but the bioactive potentiality of marine gastropods is poorly documented.
The present study investigated the bioactive properties of the tissue extract derived from *Haliotis tuberculata*, or “green abalone”, an edible abalone species distributed in the Mediterranean Sea and the northeast Atlantic Ocean. A bioactive lipid-rich extract was obtained using ethyl acetate as extracting solvent. It showed antimicrobial activity towards the methicillin-resistant *Staphylococcus epidermidis* strain RP62A, the emerging multi-drug resistant *Stenotrophomonas maltophilia* D71 and *Staphylococcus aures* ATCC 6538P, being the most sensitive strain. It also showed anthelmintic activity, evaluated through the toxicity against the target model helminth *Caenorhabditis elegans.* In addition, the ethyl acetate extract demonstrated a selective cytotoxic activity on the cancer cell lines A375, MBA-MD 231, HeLa and MCF7, at the concentration of 250 µg/mL.
The fatty acid composition present in the the bioactive extract was obtained through the FAME analysis.
The presence of some biologically important secondary metabolites in the extract was also analysed, resulting positive for the presence of alkaloids, terpenes and flavonoids.

2.1 Introduction

The marine life constitute almost 80% of the world biota with a conspicuous part being invertebrate organisms [1]. The phylum Mollusca represents one of the largest and most diverse groups of marine superior organisms, counting approximately 85,000 species [2]. Among the molluscs, the classes Gastropoda and Bivalvia have been widely described and studied.The molluscs are highly consumed seafood because of their nutritive value next to finfishes

and crustaceans. In addition, they are considered good source of bioactive metabolites; many classes of molluscs have shown to produce bioactive compounds with antitumor [3, 4], antileukemic [5], antibacterial [6 - 8] and antiviral [9] properties. Indeed, large populations living in coastal areas have consumed these marine animals for a substantial portion of their diet with beneficial effects on their health [10]. The sauce from a decoction of *Mytilus edulis*, for instance, is traditionally used in China for its immune strenghthening properties and to treat liver and kidney sysfunctions, as well as impotence and menoxemia [11].

Over the past decade, bioactive properties of bivalve molluscs components have been widely investigated. Several dietary supplements, containing mussel extracts, have been brought to the market. The most famous, Lyprinol®, a dietary supplement product containing the lipid extract of the green-lipped mussel, *Perna Canaliculis,* is sold almost worldwide as anti-inflammatory and anti-arthritic remedy.

Hence, the importance of marine molluscs as wealthy resource of biologically active metabolites and the interest for the bioactivity of molluscan extracts is increasing rapidly.

Molluscs are, therefore, good candidates as new functional food with high nutraceutical value.

Nutraceutical companies, as well as consumers are increasingly interested in marine functional foods, with the aim of improving human health as well as reducing medicinal.

A commercially important class of marine gastropods is constituted by abalone, a safe marine organism used as a food source with a rich nutritional value. Abalone is a common name for any of a group of small to very large edible sea snails that belong to the family Haliotidae. They are distinct from other species of marine molluscs in having a single flattened shell, which places them in the genus ‘haliotis’, which means ‘sea ear’ owing to its peculiar shape. Abalone are distributed worldwide in temperate and tropical marine waters. Naturally abalones are found along reefs and rocky shores from sea level to a depth of 30m. Abalone farming started in the late 1950s in Japan and China. At present China, is the largest producer of cultured abalone, but almost full production is consumed domestically. The remaining abalone production is shared between Taiwan, Chile, South Africa, Australia, New Zeland, South Korea, France, Ireland and the United States [12, 13]. As other marine-derived foods, abalones also contribute to health-promoting effects, mainly through nutrients and bioactive components.

Considering the importance of the group and paucity of information the present study aims at evaluate the bioactive potential of extracts derived from the marine abalone gastropod *Haliotis tuberculata* in view of its importance ad an edible item and a commercially important cultivable species.
Also known as “green abalone” *H. tuberculata* is currently the only commercial abalone species in Europe. Ireland and the Channel Islands [14], Scotland [15] and France are currently the main established producing countries. Fishery of *H. tuberculata* is regulated by the Ministry of Agriculture (General Directorate of Fisheries) and supported by the relative legislation (Presidential Degree 86/98 as it has been recently reformed 227/03).

2.2 Materials and methods

Collection of samples

The molluscan specimens provided for this study were obtained through aquaculture production in the GIA/IUECOAQUA facilities in the Canary Islands. They were fed two species of macroalgae *Ulva rigida* and *Hydropuntia cornea*, both were produced in the Land-based Integrated Multi-trophic Aquaculture system (IMTA) of the same aquaculture facilities.

Extraction of hydrophilic/polar components

The shells were removed and the tissue samples were washed with distilled water. Hydrophilic compounds were extracted by homogenising the animal tissue in distilled water in the ratio 100 mL of water for 10 gr of tissue with the help of a minipimer. The resulting suspension was vaccum-filtered with a Büchner funnel and the aqueous supernatant was used for extraction of hydrophilic compounds. The adsorbent resins Amberlite™ XAD-16N and DIAION™ HP-20 were used for the extraction. The resins were activated with methanol, washed with distilled water and added to the aqueous supernatant in a ratio 1/10 (w/v), separately. The mixtures were incubated overnight at 4°C on magnetic stirrers. The resins binding the hydrophilic compounds were then recovered by vacuum-filtering and added to methanol in a ratio 1/3 (w/v) for the recover of compounds. The suspensions were incubated for 3h at 4°C on magnetic stirrers. The resins were removed by vacuum-

filtration and the methanol was recovered and evaporated to dryness using a Rotavapor® R-100 (Buchi, AG Switzerland) equipped with a V-100 pump (Buchi, AG Switzerland). The obtained extracts were weighted and dissolved in 50% DMSO at 50 mg/mL and stored at -20 °C until used for biological assays.

Extraction of lipids and hydrophobic/non-polar components

The shells were removed and the tissue samples were washed with distilled water. The animal tissue was homogenized with the help of a minipimer in different extracting organic solvents. The following solvents were used for extraction: hexane, acetone, chloroform, dichloromethane, ethanol, ethyl acetate, methanol and the mixture methanol/dichlorometane (1:2), more suitable for lipids extraction. The solvents were added in the ratio 100 mL for 10 gr of tissue. The mixtures were incubated in glass flasks overnight at 4°C and shaking at 200 rpm. Subsequently the suspension were vaccumm-filtered with a Büchner funnel and the supernatant solvents were collected and evaporated to dryness by rotatory evaporation at 40°C under reduced pressure. The resultant viscous greenish – brownish exacts were weighted and dissolved in 100% DMSO at 50 mg/mL and stored at -20 °C until used for biological assays.

Preliminary screening for antimicrobial activity: agar-diffusion test

Preliminary screening for antimicrobial activity of the polar and non polar extracts was performed by the agar-diffusion test against the selected target strains listed in the chapter “Material and methods”. The target bacteria, previously grown for 24h in LB or BHI at 37°C and shaking at 200 rpm were inoculated in 50 mL of LB supplemented with agar, before solidification, at a concentration of 0.05 OD/m L and poured in Petri dishes for the agar-diffusion test. When the media were well solidified, holes were punched aseptically with a sterile tip and a volume (10 µL) of each extract corresponding to 500 µg was introduced into the wells. Then agar plated were incubated at 37°C for 24 h to allow the growth of the target bacteria. The extracts diffuse in the agar medium inhibiting the growth of the target microbial strain resulting in an inhibition halo if there is the presence of antimicrobial compounds.

For statistical purposes, three independent experiment were carried out.

Antibacterial activity

To confirm the antibacterial activity detected by the agar-diffusion test, a broth micro dilution method was carried out, allowing the determination of the lowest concentration (MIC, Minimum Inhibitory Concentration) of extract that completely inhibits the growth of the target bacterial strain. The extracts were placed into each well of a sterile 96-well microtiter plate at an initial concentration of 500 µg/mL and were serially 2-fold diluted using LB broth.
The growth of the target bacterium, previously grown for 5-8 h in liquid medium at 37°C and shaking at 200 rpm, was measured by reading the absorbance at 600 nm and about 40,000 CFU were dispensed into each well of the prepared plate, containing the dilutions of the samples.
Wells containing only the medium and no compounds represented the negative control. DMSO (2% (v/v)) was used as control to determine the effect of the solvent on bacterial cell growth. Wells containing only the target bacterium and no compounds represented the reference control of the normal bacterial growth. Plates were incubated at 37 °C for 24 h and after the incubation time growth of target bacteria was measured by reading the absorbance at 600 nm using the ELx800 microplate reader (BioTek Instuments).
For statistical purposes, two independent experiments with three replicates per trial were carried out

Minimum bactericidal concentration

The minimum bactericidal concentration (MBC) was determined from broth dilution MIC tests.
After MIC determination of the extracts, an aliquot of 10 µl from all wells in which no visible bacterial growth was observed were plated in fresh LB plates without the target pathogen. The plates were then incubated overnight at 37°C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the MIC plates.

Anthelmintic activity

The anthelmintic activity of extracts was evaluated by performing an *in vitro* liquid toxicity assay, using *C. elegans* as target nematode. Synchronised nematodes were obtained by bleaching treatment. Adult nematodes and eggs were harvested from NGM plates with sterile water and treated with a sodium hypochlorite solution for 2 min, mixing gently. The bleaching solution was removed and after washings with S-basal and water, the eggs were transferred on NGM plates supplemented with *E.coli* OP50 and incubated at 20°C. After three days of incubation, the plate contained nematodes synchronised in the 4th larval stage (L4). Around 20-30 L4 nematodes were transferred to each well of sterile 96-well plate containing 100 µL of M9 salts solution supplemented with 5µg/mL cholesterol and *E.coli* OP50 at the concentration of 0.5 OD/mL. Extracts were added at the concentration of 1 mg/mL into each well of the prepared plate. The plate was incubated at 20°C and after 24h and 48h the wells were scored for living worms. A worm was considered dead when it no longer responded to touch. The percentage of surviving nematodes after the treatment was obtained by the formula:

Percentage of viable worms =
(N° worms t24h / N° worms t0h) x 100

For statistical purposes, two independent experiment with 3 replicates per trial (with a unique egg preparation) were carried out with a unique egg preparation.

Anticancer activity

For the cell viability assay, MCF-7, MDA-MB-231, A375 and HeLa cells were harvested by trypsinization, seeded in sterile round bottom 96-well plates at 4 x 10^4 cells/well in 100 µL of complete DMEM and allowed to adhere overnight at 37°C and 5% CO_2. After 24 h of incubation, the cells were treated with the cell-free FS (10% w/v) for 24 h. The cell-viability was evaluated using the Cell Counting Kit-8 (Sigma). After the 24h-treatment, the exhausted medium was replaced with fresh medium and 10 µL of tetrazolium salt (WST-8) were added to each well. The cells were again incubated at 37°C for 4h, after which absorbance was read by using the ELx800 microplate reader (BioTek Instuments) at 490 and 630 nm. For this assay cells not treated represented the negative control to determine the normal cell growth. Wells containing only the medium

represented the negative control and were used as blank. After subtracting the aspecific OD_{630nm} value from the OD_{490nm} value, the percentage of viable cells was calculated by the formula:

Percentage of cell viability =
[(Test OD – Blank OD) / (Cells OD – Blank OD)] x100

For statistical purposes, two independent experiments with three replicates per trial were carried out.

Characterization of extract's stability

In order to characterize the extract's stability, the bioactive extract was subjected to different treatments, prior to check the activity. To determine the thermo-stability, it was incubated in a water bath for 1h at 70 °C and 100 °C.
To determine the pH stability, it was dissolved in DMSO acidified with HCl 1M up to pH 2 and pH 4.

Preliminary qualitative chemical evaluation of the extract

The identification of the chemical constituents present in the bioactive extract was carried out using various general detection reagents as described Roopalatha. Briefly, stock solutions at 10 mg/mL of the bioactive extract were prepared. These extract along with negative control were tested for the presence of alkaloids (Dragendorff's test; Mayer's test; Hager's test), terpenoids, (Salkowski test), flavonoids (Shinoda test; alkaline reagent test), and saponins (foam test; olive oil test).

Fatty acids analysis by GC-MS

Fatty acid methyl esters were prepared from dried extracts using a direct transesterification procedure with 2.5% (v/v) sulfuric acid in methanol as described by De Troch *et al*.[17]. The internal standard (19:0, 6 µg, Sigma-Aldrich, Bornem, Belgium) was added prior to the transesterification procedure. Fatty acid methyl esters were extracted twice with hexane.
Composition analysis of fatty acids was carried out using a gas chromatograph (HP 7890B, Agilent Technologies, Diegem, Belgium) equipped with a flame ionization detector (FID) and connected to an Agilent 5977A Mass Selective Detector (Agilent Technologies, Diegem, Belgium). The GC was further equipped with

a PTV injector (CIS-4, Gerstel, Mülheim an der Ruhr, Germany). A 60m×0.25mm×0.20µm film thickness HP88 fused-silica capillary column (Agilent Technologies, Diegem, Belgium) was used for the gas chromatographic analysis, at a constant Helium flow rate (2 mL/min). The injected sample is split equally between the MS and FID detectors at the end of the GC column using an Agilent capillary flow technology splitter. The oven temperature program was as follows: at the time of sample injection the column temperature was 50°C for 2 min, then gradually increased at 10°C/min to 150°C, followed by a second increase at 2°C/min to 230°C. The injection volume was 2 µL. The injector temperature was held at 30°C for 0.1 min and then ramped at 10°C/s to 250°C and held for 10 min. The transfer line for the column was maintained at 250°C. The quadrupole and ion source temperatures were 150 and 230°C, respectively. Mass spectra were recorded at 70 eV ionization voltage over the mass range of 50 - 550 m/z units.
Data analysis was done with Agilent MassHunter Quantitative Analysis software (Agilent Technologies, Diegem, Belgium). The signal obtained with the FID detector was used to generate quantitative data of all the compounds. Peaks were identified based on their retention times, compared with external standards as a reference (Supelco 37 Component FAME Mix, Sigma-Aldrich, Overijse, Belgium) and by the mass spectra obtained with the Mass Selective Detector. Quantification of fatty acid methyl esters was based on the area of the internal standard (19:0) and on the conversion of peak areas to the weight of the fatty acid by a theoretical response factor for each fatty acid [18, 19].

2.3 Results

Preliminary antimicrobial screening

The hydrophilic and hydrophobic obtained extracts were evaluated for their antimicrobial activity through the agar-diffusion test. The ethyl acetate extract was selected as the most active showing inhibition halos against *S. aureus*, *S. epidermidis* and *S. maltophilia*.

Antibacterial activity: MIC and MBC

In order to confirm the antibacterial activity detected in the agar-diffusion assay the ethyl acetate extract was used to perform a broth-dilution assay against the selected target bacteria.

The extract confirmed the antimicrobial activity against the three pathogens. The MIC resulted to be 125 µg/mL against *S. maltophilia* and *S. epidermidis* and 62.5 µg/mL against *S. aureus,* as showed in the following table reporting the absorbance at 600 nm after the overnight incubation at 37 °C.

	Conc. [µg/mL]					
Target pathogen	**500**	**250**	**125**	**62.5**	**31,25**	**Target control**
S. maltophilia	0,000	0,000	0,000	0,239	0,295	0,364
S. aureus	0,000	0,000	0,000	0,081	0,278	0,502
S. epidermidis	0,000	0,040	0,058	0,106	0,203	0,216

Tab. 1. Effect of different concentrations of the *H. tuberculata*-derived ethyl acetate extract on the growth of target pathogen bacteria after the O/N incubation at 37°C. Antimicrobial activity is expressed by the low OD_{600nm} value in presence of the extract compared with the control OD_{600nm} value associated to the normal growth of the pathogen in absence of any treatment.

In order to determine if the detected inhibitory effect was associated to bacteriostatic or bactericidal activity, the minimum bactericidal concentration was detected, by subculturing the MIC test in LB plates. The results are reported in the following table.

	Conc. [µg/mL]			
Target pathogen	**500**	**250**	**125**	**62.5**
S. maltophilia	-	-	+	+
S. aureus	-	-	+	+
S. epidermidis	+	+	+	+

Tab. 2. Bacteriostatic/bactericidal concentration of *H. tuberculata*-derived ethyl acetate extract. The symbol + indicated growth of the subculture (bacteriostatic effect) and the symbol – indicates not growth of the subculture (bactericidal effect).

The ethyl acetate extract derived from *H. tuberculata* showed to have a bacteriostatic effect on *S. epidermidis*, with the growth of the subculture also at the highest tested concentration. It showed bactericidal effect on *S. maltophilia* and *S. aureus*, with the minimum bactericidal concentration corresponding to 250 µg/mL.
The antimicrobial activity was evaluated also with the ethyl acetate extract at different treatments temperature/pH.

	Conc. [µg/mL]					
Extract treatment	**500**	**250**	**125**	**62.5**	**31.25**	**Target control**
pH 2	0,000	0,000	0,000	0,040	0,264	0,410
pH 4	0,000	0,000	0,000	0,018	0,233	
70 °C	0,000	0,000	0,000	0,079	0,306	
100 °C	0,000	0,000	0,000	0,070	0,365	
Control	0,000	0,000	0,000	0,015	0,200	

Tab. 3. Effect of different concentrations of *H. tuberculata*-derived ethyl acetate extract subjected to different treatments on the growth of *S. aureus* after the O/N incubation at 37°C. Control is the not treated extract. Antimicrobial activity is expressed by the low OD_{600nm} value in presence of the extract compared with the control OD_{600nm} value associated to the normal growth of the pathogen in absence of any treatment.

	Conc.[µg/mL]					
Extract treatment	**500**	**250**	**125**	**62.5**	**31.25**	**Target Control**
pH 2	0,000	0,070	0,117	0,173	0,220	0,222
pH 4	0,000	0,090	0,138	0,171	0,220	
70 °C	0,043	0,098	0,113	0,149	0,191	
100 °C	0,030	0,072	0,102	0,134	0,167	
Control	0,000	0,022	0,138	0,178	0,213	

Tab. 4. Effect of different concentrations of *H. tuberculata*-derived ethyl acetate extract subjected to different treatments on the growth of *S. epidermidis* after the O/N incubation at 37°C. Control is the not treated extract. Antimicrobial activity is expressed by the low OD_{600nm} value in presence of the extract compared with the control OD_{600nm} value associated to the normal growth of the pathogen in absence of any treatment.

	Conc. [µg/mL]					
Extract treatment	**500**	**250**	**125**	**62,5**	**31,25**	**Target control**
pH 2	0,000	0,000	0,161	0,233	0,368	0,362
pH 4	0,000	0,000	0,000	0,172	0,333	
70 °C	0,000	0,000	0,062	0,174	0,242	
100 °C	0,000	0,000	0,051	0,132	0,201	
Control	0,000	0,000	0,000	0,189	0,250	

Tab. 5. Effect of different concentrations of *H. tuberculata*-derived ethyl acetate extract subjected to different treatments on the growth of *S. maltophilia* after the

O/N incubation at 37°C. Control is the not treated extract. Antimicrobial activity is expressed by the low OD_{600nm} value in presence of the extract compared with the control OD_{600nm} value associated to the normal growth of the pathogen in absence of any treatment.

Based on shown results, the extract resulted to be very stable, maintaining the antimicrobial activity after the treatment at higher temperature and acid pH. The MIC value is the same against *S. aureus* in presence of both the control extract (not treated) and all the treated extracts (62.5 µg/mL). The extract at pH 2 showed a lower activity against *S. maltophilia,* with a MIC value of 250 µg/mL instead of 125 µg/mL as for the control and the other treatments. As regard to *S. epidermidis*, the extract is less active after all the treatments, with a MIC value of 250 µg/mL instead of 125 µg/mL as for the control.

Anthelmintic activity

The hydrophilic and hydrophobic extracts obtained from *H. tuberculata* were evaluated for their anthelmintic activity by performing a toxicity test on *C. elegans*, as target helminth.
The ethyl acetate extract also in this case showed the highest activity.
In order to determine the Minimum Toxic Concentration (MTC) the toxicity test was performed with different concentrations of the ethyl acetate extract. MTC resulted to be 400 µg/mL, with killing rate of approximately 100%, as reported in the following graph.

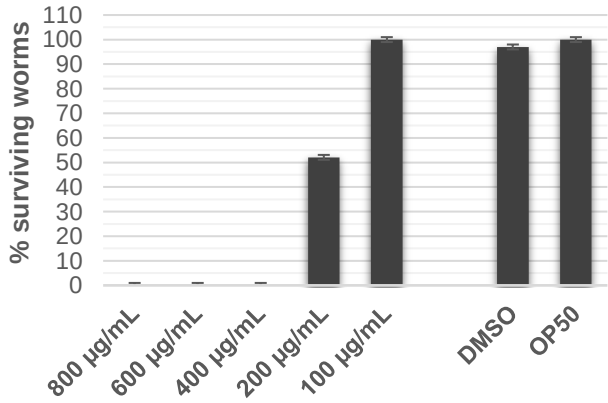


Fig. 6. Effect of different concentrations of *H. tuberculata*-derived ethyl acetate extract on *C. elegans* viability after the 24h treatment. Anthelmintic activity is

expressed as percentage of surviving worms. DMSO represents the negative control. OP50 represents the normal worms' viability. Concentrations are in µg/mL. Error bars represent SEM (Standard Error of the Mean).

The anthelmintic activity was evaluated also after the pH/temperature treatment. As shown in the following table, the extract is resistant to treatments, maintaining the same activity as the control, at 1 mg/mL, with killing rate higher that 90%.

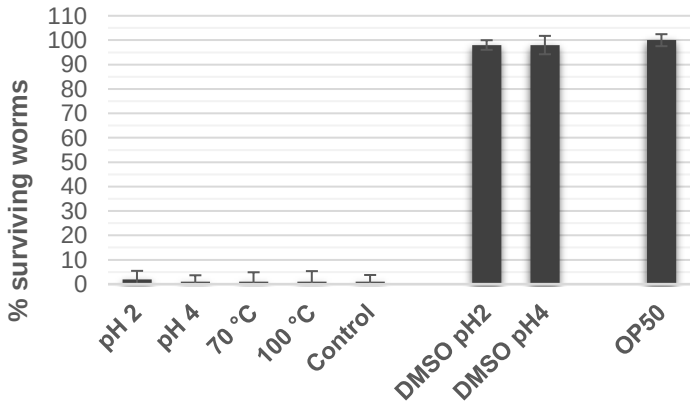


Fig 7. Effect of 1 mg/mL of *H. tuberculata*-derived ethyl acetate extract T/pH treated on *C. elegans* viability after the 24h treatment. Anthelmintic activity is expressed as percentage of surviving worms. DMSO represents the negative control. OP50 represents the normal worms' viability. Error bars represent SEM (Standard Error of the Mean).

Anticancer activity

The hydrophilic and hydrophobic extracts obtained from *H. tuberculata* were evaluated for their ability to kill cancer cells, through a cell-viability assay based on the conversion of a tetrazolium salt, the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), into a soluble orange product, the formazan, by cellular dehydrogenases. The reaction cannot take places in dead cells and thus, the amount of formazan produced is directly proportional to the number of living cells. The cell death is therefore detectable by the decrease in absorbance at 570 nm, compared to the absorbance of cells not treated.
The ethyl acetate extract also in this case showed the highest activity. It showed the highest activity on MCF-7 cells, with a killing

rate of approximately 85% at 125 µg/mL after the 24h treatment, followed by the activity on A375 and MDA-MB-231 cells, with a killing rate of approximately 50% at 125 µg/mL after 48h of treatment; the lowest activity was detected on HeLa cells, with a killing rate of approximately 90% at 250 µg/mL after 48h of treatment.

		Conc. [µg/mL]			
Target cells		**500**	**250**	**125**	**62**
A375	**24h**	1	40	63	100
	48h	1	3	44	100
MDA-MB 231	**24h**	3	8	52	100
	48h	1	4	50	100
HeLa	**24h**	0	20	100	100
	48h	1	10	100	100
MCF7	**24h**	16	15	16	100
	48h	10	12	14	80

Tab. 8. Effect of different concentrations of *H. tuberculata*-derived ethyl acetate extract on viability of four different target cancer cells. Anticancer activity is expressed as percentage of viable cells after the treatment for 24 and 48h.

In order to determine the selective toxicity on cancer cells, at the tested concentration, the cell viability assay was performed using normal cell lined as target.
The ethyl acetate extract showed to be toxic on Vero cells at the highest concentration (500 µg/mL), with a killing percentage of 76%, while it is not toxic at lower concentration. This is compatible with the toxic concentration of 250 µg/mL. The extract is less toxic on HFFF2 cells, with a killing percentage of 50% at 500 mg/mL, a low toxicity that is compatible with the “therapeutic” (toxic on cancer cells) concentration of the extract (250 µg/mL).

	Conc. [µg/mL]			
Target cells	**500**	**250**	**125**	**62.5**
VERO	24	98	100	100
HFFF2	50	95	100	100

Tab. 9. Effect of different concentrations of *H. tuberculata*-derived ethyl acetate extract on viability of four different normal cell lines. Anticancer activity is expressed as percentage of viable cells after the treatment for 24 and 48h.

Preliminary chemical characterization

The bioactive ethyl acetate extract was evaluated for the presence of biologically important secondary metabolites, co-extracted with lipids. A general detection method based on the use of general reagent was used. The screening showed the presence of alkaloids, terpenes and flavonoids. Alkaloids were detected from the formation in the extract of a creamy precipitate when reacting with Mayer's and Hager's reagents. Terpenes were detected from the formation of a reddish/brown colour at interface after the Salkowski reaction. Flavonoids were detected from the formation of a greenish/blue colour after the Shinoda reaction.

Fatty acids analysis

In order to characterize the lipid-rich ethyl acetate extract, the identification of fatty acids was carried out. To facilitate the analysis by GC-MS, a process which involves cleavage of the link between the carbohydrate or peptide/protein part of the biosurfactants and the lipid portions was carried out. This process allowed the derivatization of the resulting fatty acid chains to fatty acid methyl esters (FAME), easily detectable by GC-MS.
The fatty acid composition, described in the following table, resulted to be 45% of saturated fatty acids, 22% of monounsaturated fatty acids (MUFa) and 33% of poluunsaturated fatty acids (PUFa), with palmitic acid (C:16), being the major component (25.7%).

FAs	FAs %
12:0	0,1
13:0	0,1
14:0	7,2
iso-15:0	0,2
anteiso-15:0 F	0,1
15:0	1,3
iso-16:0	0,2
16:0	25,7
16:1+iso-17:0	0,6
cis-9-16:1	1,6
17:0	1,4
16:2n-7	0,4

iso-18:0	0,3
17:1+16:2n-4	0,3
17:1	0,2
18:0	6,2
cis-9-18:1	7,6
cis-11-18:1	8,6
16:4n-1	0,1
19:0	2,4
18:2n-6	9,3
18:3n-6	0,1
20:0	0,1
18:3n-3	2,0
20:1 a	1,9
20:1 b	1,4
20:1	0,2
18:4n-3	0,9
CLA	0,7
20:2n-6	0,6
20:4n-6	11,1
22:2	3,1
20:5n-3	2,4
22:4	1,3
22:5n-3	1,8
22:6n-3	0,7

Tab. 10. FAME analysis. Percentage of fatty acids abundance

2.4 Discussion

A wide range of bioactive substances are being isolated and characterized from the marine-derived food. The molluscs are very good source for human consumption as well as bioactive compounds.
The aim of the present study was to assess the bioactive properties of the tissue extract of edible gastropod *H. tuberculata*, in order to evaluate its potential for biotechnological applications.
On the basis of a literature survey, the most bioactive marine molluscs compounds can be classified as typical primary metabolites, such as proteins/peptides and lipids.

Therefore, in order to obtain two classes of metabolites, the animal tissue was subjected, separately, to aqueous and organic extractions, in order to obtain, hydrophilic and hydrophobic compounds, respectively. The aqueous extract was used for the extraction of polar compounds and proteins/peptides/amminoacids through the use of adsorbent resins (Amberlite®XAD16N and Diaion®HP-20). The organic extraction were carried out using different solvents and allowed the extraction of lipids.

The ethyl acetate extract was selected as the most promising, showing antibacterial, anthelmintic and anti-cancer activity. It showed antibacterial activity against *S. aureus*, *S. maltophilia* and *S. epidermidis*, with minimum inhibitory concentrations of 125 µg/mL, 62.5 µg/mL and 62.5 µg/mL, respectively. The minimum bactericidal concentration resulted to be 250 µg/mL against *S. aureus* and *S. maltophilia*, while only bacteriostatic activity was detected against *S. epidermidis*. In addition, the extract showed to be temperature and pH resistant, maintaining the antibacterial activity after the treatments at higher temperature (70 °C and 100 °C), and when dissolved in acidified DMSO (pH 2 and pH 4).

The anthelmintic activity resulted in the minimum toxic concentration of 400 µg/mL.

The ethyl acetate extract resulted to specifically possess anticancer activity at the “therapeutic” concentration of 250 µg/mL against the target cancer cells: MCF-7, MBA-MD 231, HeLa and A375, with cytotoxic percentages ranging from 59% and 92% after 24h of treatment and from 88% and 97% after 48h of treatment. At the same concentration, the extract did not show toxicity on the normal cell lines Vero and HFFF2.

The fatty acid composition present in the ethyl acetate extract was obtained by the FAME analysis, resulting in 45 % saturated fatty acids, 22 % monounsaturated fatty acid (MUFa) and 33 % polyunsaturated fatty acids (PUFa), with the palmitic acid (C:16) being the most represented fatty acid (25.7%).

Further studies need to be carried out to fully characterize the lipid composition of this bioactive marine oil derived from *H. tuberculta*. Among the major groups of molluscs primary metabolites, lipids have so far shown the highest potential for the commercial development of health beneficial functional foods or dietary supplements, the most famous being Lypirinol®, the marine oil extracted from the New Zeland green lipped mussel *Perna canaliculus,* with anti-inflammatory properties [20, 21].

A preliminary chemical evaluation of the ethyl acetate extract, using general detection reagents, was also carried out to identify the most frequent secondary metabolites isolated from molluscs, eventually co-extracted together with lipids. Alkaloids, terpenes and flavonoids were identified in the ethyl acetate extract derived from *H. tuberculata*. All of these compounds are well known for their biological activities. Alkaloids have been isolated in reasonably large numbers from both classes (gastropods and bivalves) of molluscs [22]. Alkaloids have been associated with medicinal uses for century and one of their common biologicasl properties is their cytotoxicity with possible interaction with cell wall and DNA [23]. Therefore, from the above results it can be assumed that the presence of these components may have contributed to these activities of the extract. In marine gastropods, terpenes constitute the major class of secondary metabolites. A broad range of the biological properties of terpenoids is described, including cancer chemopreventive effects, antimicrobial, antifungal, antiviral, antihyperglicemic, anti-inflammatory and antiparasitic activities [24]. The extract was also positive for flavonoids. Phenolic compounds also have been reported from molluscs because these natural molecules are relatively easy to assimilate [22]. Phenols have been found to possess antimicrobial activity and as natural antioxidant [25, 26].

Further studies need to be carried out to determine the full characterization of the lipid part and to identify the secondary metabolites among the detected structural classes. The lipid study it is of major challenging, because the increasing instability during purification processes limits the investigation of single lipid components, hence analysis mostly focus on the characterizations of lipid extracts or fractions rather than pure compounds [27].

However, the results obtained in the present study show the potential of the marine oil derived from *H. tuberculata*, suggesting the importance of this species as a promising candidate for further investigation aimed at the development of functional foods, food ingredients, as well as pharmaceuticals.

This is particularly interesting if we consider that, contrarily to other marine invertebrates, the bioprospecting from farmed molluscs and the supply of derived bioactive compounds, can be carried out in a sustainable way [28]. Molluscan shellfish aquaculture provides high quality and high value seafood for human consumption. In addition, filter-feeding molluscan shellfish have the potential to positively affect aquaculture environments, providing beneficial ecosystem

services, such as nutrient extraction and water filtration, to the environment in which they are grown. Shellfish aquaculture is considered the most environmentally sustainable culture of marine invertebrate, and will continue to expand with the development of new technologies and improved production practices [29].

This is the first report, based on our literature survey, on the bioactive potential of tissue extract derived from the marine gastropod *H. tuberculata,* giving a value added to this economic important species.

References

1 McCarthy, P.J, and Pomponi, S.A .*A search for new pharmaceutical drugs from marine organisms*. Marine Biomed Res, 2004; 1-2

2 Rosenberg, G. *A New Critical Estimate of Named Species-Level Diversity of the Recent Mollusca.* American Malacological Bulletin, 2014; 32 (2): 308-22

3 Ciavatta, M.L., et al. *Marine Mollusk-derived agents with antiproliferative activity as promising anticancer agents to overcome chemotherapy resistance*. Med Res Rev, 2017; 37(4): 702-801

4 Faicloth, G., et al. *Kahalide F and ES285: potent anticancer agents from marine molluscs*. Molluscs, 2006; 363-79

5 Miosky, D.L., et al. *Leukemia cell specific protein of the bivalve mollusc Mya arenaria*. J Invertebr Pathol, 1989; 53(1): 32-40

6 Dolashka, P., et al. *Antimicrobial activity of molluscan hemocyanins from Helix and Rapana snails*. Curr Pharm Biotechnol, 2016; 17(3): 263-70

7 Periyasamy, N. et al. *Antimicrobial activities of the tissue extracts of Babylonia spirata Linnaeus, 1758 (Mollusca:Gastropoda) from Thaxhanguda, southeast coast of India* . Asian Pac J Trop Biomed, 2012; 2(1): 36-40

8 Annamalai, N., et al. *Antibacterial activities of green mussel (Perna viridis) and edible oyster (Crassostrea madrasensis)*. Res J Microbiol, 2007; 2(12): 978-982

9 Dang, V.T. et al. *In vitro antiviral activity against herpes simplex virus in the abalone Haliotis laevigata*. J Gen Virol, 2011; 92(3): 627-37

10 Brusca, R.C, and Brusca, G.J. *Invertebrates*. Massachussetts USA: Sinauer Associates, Inc., Sunderland.1990

11 Li, P.,and Ding, X. *The manufacture and nutritional analysis of the functional natural sauce from the decoction of Mytilus edulis*. Zhongguo Tiaoweipin, 2006; 2:17-9.

12 Troell, M. et al. *Abalone farming in South Africa: an overview with perspective on kelp resources, abalone feed, potential for on-farm seaweed production and socioeconomic importance.* Aquaculture, 2006; 257: 266-81

13 Freeman, K.A. *Aquaculture and related biological attributes of abalone species in Australia – a review.* Fisheries Research Report. Department of Fisheries, Perth, Western Australia No.128, 2001

14 Huchette, S.M.H., and Clavier, J. *Status of the ormer (Haliotis tuberculata L.) industry in Europe*. J Shellfish Res, 2004; 23: 951-55

15 Kelly, M.S., and Owen, P.V. *Growth of the abalone Haliotis tuberculata L. at Scottish sea temperatures.* Aquacult Res, 2002; 33: 729-33

16 Brenner, S. *The genetic of Caenorhabditis elegans*. Genetics, 1974; 77: 71-94

17 De Troch, M., et al. *Bioconversion of fatty acids at the basis of marine food webs: Insights from a compound-specific stable isotope analysis*. Marine Ecology Progress Series 2012; 465: 53-67

18 Ackman, R.G., and Sipos, J.C. *Application of specific response factors in the gas chromatographic analysis of methyl esters of fatty acids with flame ionization detectors*. J Am Oil Chem Soc, 1964; 41:377-8

19 Wolff, R.L., et al. *Evaluation of sequential methods for the determination of butterfat fatty acid composition with emphasis on tran-18:1 acids*. *Application to the study of seasonal variations in French butters*. J Am Oil Chem Soc, 1995; 72:1471–83
20 Halpern, M.G. *Anti-inflammatory effects of a stabilized lipid extract of Perna canaliculus (Lyprinol®).* Allergie et immunologie, 2000; 32(7): 272-8
21 Gibson, S.L.M., and Gibson, R.G. *The treatment of arthritis with a lipid extract of Perna canaliculus: a randomized trial*. Complementary Therapies in Medicine, 1998; 6(3): 122-6
22 Benkendorff, K. *Molluscan biological and chemical diversity: secondary metabolites and medicinal resources produced by marine molluscs*. Biol Rev, 2010; 85(4): 757-75
23 Wink, M. *Modes of action of allochemical alkaloids: interaction with neuroreceptors, DNA, and other molecular targets*. J Chem Ecol, 1998; 24(11): 1881-1937
24 Bergman, M., et al. *Medically useful plant terpenoids: biosynthesis, occurrence and mechanism of action.* Molecules, 2019; 24(21): 3961.
25 Alves, M.J. et al. *Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies*. J Appl Microbiol, 2013; 115(2): 346-57
26 Ganan, M. et al. *Antimicrobial activity of phenolic compounds of wine against Campylobacter jejuni*. Food Control, 2009; 20(8): 739-42
27 Grienke, U., et al. *Bioactive compounds from marine mussels and thei effects on human health*. Food Chemestry, 2014; 142: 48-60
28 O'Beirn, F.X., et al. Shellfish aquaculture, Methods of sustainable. In: Christou P., Savin R., Cosat-Pierce B.A., Mistzal I., Whitelaw C.B.A. (eds) Sustainable Food Production. Springer, New York, NY. 2013; 9174-96
29 Shumway, S.E. et al. *Shellfish aquaculture — In praise of sustainable economies and environments*. World Aquaculture, 2003, 34(4)

CHAPTER 3:

BlueShell:
Exploring shellfish by-products as source of blue bioactivities

BlueShell:
Exploring shellfish by-product as sources of blue bioactivities

Seafood industry every year generates about 50-70% of nonedible by-products, the most abundant coming from crustaceans and mollusks. Apart from use for the extraction of chitin/chitosan, this marine biomass is used to either make fertilizer / low value products or is sent to landfill, incinerated or dumped at sea. BlueShell addressed this problem by exploring three typical shellfish by-products; shrimp (*Pandulus borealis*) shells, crab (*Cancer pagurus*) shells and defect mussels (*Mytilus edulis*), for potential bioactive compounds targeted at the sustainable supply of safe, healthy foods. As part of the BlueShell project, this work aimed at analyzing the biological activities of these by-products. The shellfish by-products were used for extraction of pigmented organic compounds and the obtained raw extracts showed antimicrobial, antibiofilm and anthelmintic activities. In addition, crab shells were also used as substrate for bacterial fermentation using six different marine strains. The strains *P. arctica* and *P. issachenkonii* resulted the most promising for the efficient bioconversion of crab by products into bioactive compounds with anthelmintic and antibiofilm activity.

3.1 Introduction

Food industry generates large quantities of by-products most of which are still underutilized. These food wastes may often contain several usable substances of high value, including compounds possessing important health benefits. Generation of waste during the processing of food is unavoidable and disposal can be one very challenging. If this waste is not properly disposed, it can have a negative impact on the environment, generating risks to human health, and a loss of income to the waste generator.

One of the food industry which most contribute to generation of food waste worldwide is the seafood industry. Seafood processing recovers only 20–50% as edible portions and the remaining parts are discarded as “nonedible” by-products /co-products/leftover raw materials (heads, tails, skins, scales, viscera, backbones, and shells), with an average of 20 MT globally [1 ,2]. Among seafood, crustacean and shellfish most contribute to generate huge quantities of by-products. According to FAO reports, every year from 6 to 8 million tons of waste crab, shrimp, prawn, lobster and krill shells are

produced globally [3]. With approximately 75% of the total weight of crustaceans ending up as by-products and the current lack of acceptable waste management options there is a potentially large environmental hazard concern [4].
Especially in developing countries, which cannot afford the disposal of this waste material because it can be very costly, seafood wastes are thrown away at the sea, burned, landfilled, or simply left out to spoil [5].
In developed countries, the shells-containing seafood waste is mainly utilized for the extraction of chitin, which is the second most available polysaccharide after cellulose. Chitin is a fairly ubiquitous compound produced by many organisms: fungi and algae cell walls, insects' exoskeletons, mollusks (endoskeleton of cephalopods) and crustaceans' shells. Annually, it has been estimated that living organisms generate about 100 billion tonnes of chitin [6]. However, commercially chitin is mainly recovered from marine sources, such as the crustaceans processing industries. In fact more than 10,000 tons could be available every year from shellfish waste which would provide sufficient raw material if the appropriate commercial procedures for value-added processes were developed.
The amounts of chitin from shell wastes vary with species and seasons, but, in general, the exoskeletons contain about 30-40% proteins, 30-50% minerals (mainly calcium carbonate), and 20-30% chitin along with others compounds such as pigments (e.g., astaxanthin) and lipids [7].
Chitin is obtained from crustaceans' exoskeletons after demineralization and deproteinisation treatments. However, one of the limitations in the use of this biopolymer on a large-scale is its water insolubility. Therefore, it is often converted into water-soluble derivatives, the most important and famous being chitosan. Chitosan is obtained after the deacetylation of chitin and it is the only natural cationic polysaccharide known [8] Chitin and its derivatives are renewable, biocompatible, biodegradable, and non-toxic compounds that have many biological properties such as: anti-cancer [9], antioxidant [10], antimicrobial [11] and anti-coagulantc [12] properties. In addition, they are used as biomaterials in a wide range of applications: for biomedical purposes such as for artificial skin, bones, and cartilage regeneration [13, 14], for food preservation such as for edible films [15] and for pharmaceutical purposes such as for drug delivery [16, 17]. Chitin polymers are largely used in cosmetic industry. They represent important

components of skin care product (creams, lotions, nail lacquers, and makeup), hair care (shampoos, hair colorant, and hair spray) and oral care (toothpaste, mouthwashes, and chewing gum) products [18].
All these properties attributed to chitin and its derivatives are associated to the presence of nitrogen, which make it different from cellulose. Cellulose and chitin are polysaccharides with structural similarity, but chitin has an acetamide group ($NH\text{-}CO\text{-}CH_3$) at C-2 (general formula: $C_8H_{13}O_5N$) in place of the hydroxyl group in cellulose [19]. Nitrogen-containing chemical, widely used in the pharmaceutical industry, carbon dioxide fixation, textiles and beyond, are crucial for modern life.
Beyond the settled utilization of shellfish waste for extraction of chitin, the potential of shellfish by-products is still largely unexplored. This work, which is part of the ERA-NET project BlueShell, aims at investigating different aspects of bioactivity in fermented/hydrolysed shellfish by-products which can serve as a basis for new high-value products aiding a sustainable supply of safe, health promoting foods. The selected shellfish by-products are peels from the shrimp *Pandalus borealis*, shells from brown crab *Cancer pagurus* and defect mussels of the species *Mytilus edulis.*

3.2 Materials and methods

Processing of shellfish by-products

Shells from the brown crab *Cancer pagurus,* were provided by Irish Fish Canners (Donegal, Ireland). Shrimp shells from industrial shrimp peeling were provided by Räkor och Laxgrossisten AB (Gothenburg, Sweden) and whole defect mussels were collected from local Belgian sources. All the by-products were processed at the Institute for Agricultural and Fishery Research (ILVO), in Belgium. They were kept at -20°C before homogenization. After thawing, the material was homogenized in a Seydelmann® vacuum cooking cutter (K64AC8, Germany) at 0-4°C for approximate 5 min.

Extraction of organic pigmented compounds from raw by-products

The homogenized raw material was used for extraction of organic pigmented compounds. Aliquots of the raw material were left to agitate in different organic solvents separately, overnight at 4°c and

200 rpm. The organic solvents used were methanol, ethyl acetate, hexane, chloroform, dichloromethane and acetone. The resulting suspensions were vacuum-filtered with a Büchner funnel and the supernatant solvents were collected and evaporated to dryness by rotatory evaporation at 40°C under reduced pressure, using a Rotavapor® R-100 (Buchi, AG Switzerland) equipped with a V-100 pump (Buchi, AG Switzerland). The resultant viscous yellowish-greenish-reddish exacts were weighted and dissolved in 100% DMSO at 50 mg/mL and stored at -20 °C until used for biological assays.

Proteolitic bacteria used for fermentation

The following marine bacteria were previously selected at ILVO, based on their proteolytic activity, and used for the bioconversion of the crab waste material: *Pseudoalteromonas arctica* DSM 18437, *Pseudoalteromonas issachenkonii* LMG 19697, *Pseudoalteromonas carrageenovora* DSM 6820, *Pseudoalteromonas nigrifaciens* LMG 2227, *Pseudoalteromonas rubra* DSM 6842, *Pseudoalteromonas tunicata* DSM 14096. Mother culture of all marine bacteria was routinely prepared by culturing in DifcoTM marine broth (BD, Sparks, USA) followed by an incubation step for 72 h with shaking (110 rpm) at 21°C, with the exception of *Pseudoalteromonas rubra* DSM 6842T and *Pseudoalteromonas tunicata* DSM 14096T at 25°C.

Microbial fermentation of crab by-products

Starter cultures of the selected proteolytic marine bacteria were prepared by transferring 100 µL of each mother culture into 10 mL of sterile marine broth and incubated at 21 °C or 25 °C for 72 h until they reached a cell concentration of 10^7 cfu/ml. The fermentation procedure of crab processing side streams by marine bacteria is described as follow: 10 ± 0.02 g of the homogenized crab processing side streams was weighted in a 250 ml sterile Erlenmeyer flask, then mixed with 40 ml sterile distilled water containing 2% (w/v) NaCl and 0.1% (v/v) acetic acid. 1 ml of starter culture was added to start the fermentation. The fermentation was carried out at 21 or 25 °C, 110 rpm. After 72 h, the fermentation was terminated by heating the flasks in a water bath at 98°C for 10 min in order to inactive the enzymes. The medium was then filtered with a piece of gauze,

pellets was pressed and collected as press cakes. The filtrates were centrifuged at 4000 rpm for 20 min. Supernatant was collect as hydrolysates; residues were collected as sludge (wet pellets).

Extract preparation from hydrolysates

The hydrolysates obtained by the microbial fermentation of crab by-products were used for the extraction of organic compounds. They were subjected to liquid-liquid organic extraction using three volumes of ethyl acetate in separatory funnels. The organic phase was collected and evaporated using the Rotavapor. The obtained ethyl aceate extract was weighted, dissolved in 100 % DMSO at 50 mg/mL and stored at -20°C until used for biological assays.
Aliquots of the residual aqueous phases were evaporated to dryness under nitrogen flux. The obtained raw aqueous fractions wer weighted, dissolved in sterile water at 50 mg/mL and stored at -20°C until used for biological assays. The bioactive aqueous phases were stored and further processed.

Solid Phase Extraction of aqueous microbial hydrolysate

After the ethyl acetate extraction to remove organic compounds from the microbial hydrolysates, the bioactive residual aqueous phases were fractionated via Solid Phase Extraction (SPE), using Chromabond SiOH column cartridges (Macherey-Nagel, Duren, Germany). After the activation of the column with chloroform and the desalting with water, the sample was loaded onto the column, collecting the aspecific not bound fraction and different solvent systems of increasing polarity were applied to elute different fractions of the samples. Five fractions were obtained corresponding to the solvent systems: methanol/chloroform (1:9), methanol/chloroform (2:8), methanol/chloroform (1:1), methanol and water. The fractions were collected, evaporated with the Rotavapor and dissolved in 100 % DMSO at 50 mg/mL and stored at -20 °C until used for biological assays.

Antibacterial activity

To evaluate the antimicrobial activity of the samples derived from the organic extractions and the microbial fermentation of crab waste, a broth micro dilution method was carried out, allowing the

determination of the lowest concentration (MIC, Minimum Inhibitory Concentration) of extract that completely inhibits the growth of the target bacterial strain. The samples were placed into each well of a sterile 96-well microtiter plate at an initial concentration of 1 mg/mL and were serially 2-fold diluted using LB broth.
The growth of the target bacterium, previously grown for 5-8 h in liquid medium at 37°C and shaking at 200 rpm, was measured by reading the absorbance at 600 nm and about 40,000 CFU were dispensed into each well of the prepared plate, containing the dilutions of the samples.
Wells containing only the medium and no compounds represented the negative control. DMSO (2% (v/v)) was used as control to determine the effect of the solvent on bacterial cell growth. Wells containing only the target bacterium and no compounds represented the reference control of the normal bacterial growth. Plates were incubated at 37 °C for 24 h and after the incubation time growth of target bacteria was measured by reading the absorbance at 600 nm using the ELx800 microplate reader (BioTek Instuments).
For statistical purposes two independent experiments, with three replicates per trial were carried out.

Antibiofilm activity

S. aureus 6538P and *S. epidermidis* MB5263 were used as target strains to evaluate the effect of the obtained samples on biofilm formation.
The samples were placed into each well of a sterile 96-well microtiter plate at an initial concentration of 1 mg/mL and were serially 2-fold diluted using LB broth supplemented with 1% glucose.
The target bacteria, previously grown in LB supplemented with 1% glucose at 37°C and shaking at 200 rpm for 24 h, were diluted using OD/CFU correlation growth curves, and about 2 x 10^5 CFU were dispensed into each well of the prepared plate. Plates were incubated at 37°C for 24 h. After the incubation time, prior to the crystal violet assay, the wells containing the bacterial suspension were analyzed spectrophotometrically at 630 nm to ensure that there was no antibiotic activity. In the crystal violet assay, the planktonic cells and spent media were discarded and weakly adherent cells were removed through washing twice with deionized water, and allowed to air dry before being stained. The biofilms were stained with 100 µ L of 0.01 % (w/v) crystal violet solution for 20 min.

Subsequently the dye was discarded and the wells were rinsed twice with deionized water. The wells were allowed to dry before solubilisation of the crystal violet with 100 µL of absolute ethanol for 30 min. The optical density was determined at 490nm, using the ELx800 microplate reader (BioTek Instuments). In this assay, wells containing no samples represented the negative control to determine the normal biofilm formation. For statistical purposes, two independent experiments with 3 replicates per trial were carried out. The percentage of biofilm inhibition was calculated by the formula:

Percentage of biofilm inhibition =
([Control OD – Test OD] / Control OD) x 100

Anthelmintic activity

The anthelmintic activity of extracts was evaluated by performing an *in vitro* liquid toxicity assay, using *C. elegans* as nematode target.
Synchronised nematodes were obtained by bleaching treatment. Adult nematodes and eggs were harvested from NGM plates with sterile water and treated with a sodium hypochlorite solution for 2 min, mixing gently. The bleaching solution was removed and after washings with S-basal and water, the eggs were transferred on NGM plates supplemented with *E. coli* OP50 and incubated at 20°C. After three days of incubation, the plate contained nematodes synchronised in the 4th larval stage (L4). Around 20-30 L4 nematodes were transferred to each well of sterile 96-well plate containing 100 µL of M9 salts solution supplemented with 5µg/mL cholesterol and *E. coli* OP50 at the concentration of 0.5 OD/mL. Extracts were added at the concentration of 1 mg/mL into each well of the prepared plate. DMSO was used as control to evaluate thye effect of the storage solvent of the test samples on worms viability. The plate was incubated at 20°C and after 24h and 48h the wells were scored for living worms. A worm was considered dead when it no longer responded to touch.
The percentage of surviving nematodes after the treatment was obtained by the formula:

Percentage of viable worms =
(N° worms t24h / N° worms t0h) x 100

For statistical purposes, two independent experiment with three replicates per trial were carried out with a unique egg preparation.

3.3 Results

Antimicrobial activity of organic raw extracts

The organic pigmented extracts obtained from the three raw shellfish by-products were evaluated for their antimicrobial activity against the selected target bacteria listerd in the chapter “Materials and methods”. The raw extracts derived from mussels showed antimicrobial activity, while no activity was detected from the crab- and shrimp-derived samples. In particular the extracts obtained with ethyl acetate, dichloromethane, and in a less extent, hexane.
The results are reported in the following table as percentage of bacterial growth inhibition. The ethyl acetate extract, at 1 mg/mL was able to inhibit the growth of almost all target bacteria, except *L. monocytogenes* and *S. epidermidis.* The dichloromethane extract, at 1 mg/mL was also very active against almost all target bacteria, except *L. monocytogenes* and *A. xylosoxidans*. The hexane extract showed a lower activity, inhibiting the growth of 5 target bacteria out of 11 used as target.

Target bacteria	EtOAc	Hexane	DCM
P. aeruginosa	74	ND	93
A. baumanii	70	90	89
A. xylosoxidans	100	100	0
S. maltophilia	100	100	100
S. aureus	100	0	100
S. epidermidis	0	0	100
S. thyphimurium	86	0	69
L. monocytogenes	0	0	0
S. enteriditis	100	0	100
P. alvei	100	86	100
P. piscicida	100	100	100

Table 1. Effect of mussels-derived organic pigmented raw extracts on bacterial growth. The extracts obtained with ethyl acetate (EtOAc), n-hexane and dichloromethane (DCM), resulted to be active and are reported in this table. Antimicrobial activity is expressed as percentage of target bacteria growth inhibition, after the overnight incubation at 37°C in presence of 1 mg/mL of extracts.

Anthelmintic activity of organic raw extracts

The organic raw extracts obtained from the three shellfish by-products and the extracts were evaluated for their anthelmintic using the helminth *C. elegans* as target.
Some of the organic raw extracts obtained from both crab and shrimp by-products showed anthelmintic activity, while no activity was detected from the mussel-derived extracts.
The methanol and acetone extracts obtained from the crab shells showed the highest anthelmintic activity, with a killing rate of more than 90%, as shown in the following graph, reporting the percentage of surviving worms after the 24h-treatment with 1 mg/mL of extracts.

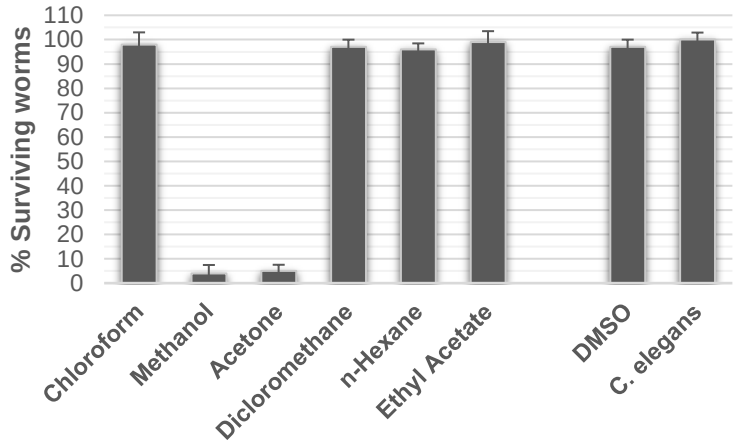


Fig. 2 Effect of crab-shells derived organic raw extracts on *C. elegans* viability. Anthelmintic activity is expressed as percentage of surviving worms after the 24h-treatment with 1 mg/mL of extracts. DMSO represents the effect of the storage solvent on worms' viability

The raw organic extracts obtained from shrimp peels showed to be more toxic against *C. elegans*, with the hexane extract being the most active, able to kill more than 90% of worms, at 1 mg/mL, as reported in the following graph, showing the percentage of surviving worms after 24 of treatment with the extracts.

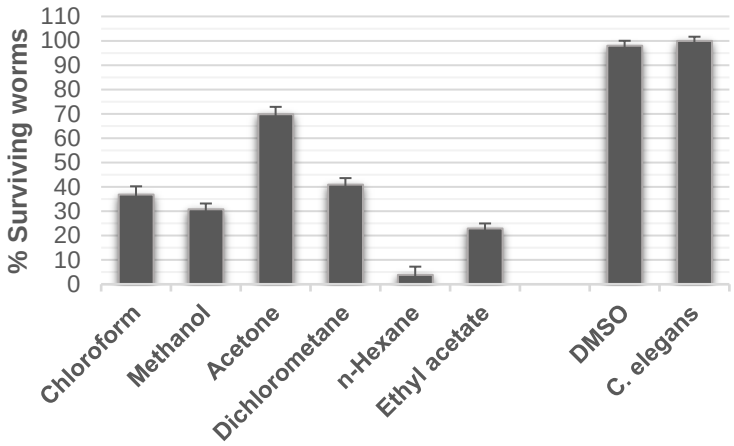


Fig. 2 Effect of shrimp-shells derived organic raw extracts on *C. elegans* viability. Anthelmintic activity is expressed as percentage of surviving worms after the 24h-treatment with 1 mg/mL of extracts. DMSO represents the effect of the storage solvent on worms' viability.

Antibiofilm activity of organic raw extracts

The organic raw extracts obtained from the three shellfish by-products and the extracts were evaluated for their ability to inhibit bacterial biofilm formation, using S. aureus and S. epidermidis as target bacteria. The shrimp-derived extract showed antibiofilm potential against *S. epidermidis*, while no activity was observed from the other samples.

All shrimp shells-derived raw extracts, except hexane, showed an antibiofilm potential. The ethyl acetate and acetone extract resulted to be the most active, with average percentage of biofilm inhibition around 70% at the minimum tested concentration, followed by the chloroform and methanol extract, with a biofilm inhibition percentage of 57 and 52%, respectively, at 125 µg/mL, and the dichloromethane extract with a biofilm inhibition of 21% at 125 µg/mL.

Conc. [µg/mL]	500	250	125
MeOHl	58	62	52
Hexane	0	0	0
DCM	65	37	21
EtOAc	71	70	72
CHL	72	66	57
Acetone	68	68	68

Table 3. Effect of shrimp shells-derived organic raw extract on *S. epidermidis* biofilm formation. Antibiofilm activity is expressed as percentage of target bacteria biofilm formation, after the overnight incubation at 37°C in presence of different concentration of extracts.

Biological activities of crab-fermented hydrolysates

In addition to organic raw extracts, crab by-products were also used as carbon source for bacterial fermentation of six different marine strains, all belonging to *Pseudoalteromonas* genus, with chitinolytic/proteolytic activities previously isolated and selected at ILVO. The obtained bacterial hydrolysates were used for the extraction of secondary metabolites (with ethyl acetate) produced by bacteria fermenting the crab shells and the most polar fraction, not extracted with ethyl acetate, was subjected to fractionation by Solid Phase Extraction, obtaining five fractions of different polarity. The bacteria *Pseudoalteromonas issachenkonii* LMG 19697 and *Pseudoalteromonas arctica* DSM 18437showed the ability to synthetize bioactive compounds when fermented on crab-shells. Interestingly, two different activities were detected from the different samples. The ethyl acetate extract of both hydrolisates showed anthelmintic activity, indicating the presence of hydrophobic anthelmintic secondary metabolites, while the residual aqueous raw fractions showed antibiofilm activity against *S. epidermidis*, indicating the presence of polar metabolites/peptides able to prevent the microbial biofilm formation.
In the following graph the anthelmintic activity of ethyl acetate extracts, expressed as percentage of surviving worms is reported. The ethyl acetate extracts obtained from the fermentative hydrolysates of *P. arctica*, *P. issachenkonii* and *P. rubra* showed very toxic activity against *C. elegans*, with a killing rate of 99%. The ethyl acetate extracts obtained from the other three fermentative hydrolysates were not toxic. The residual aqueous raw fractions of all fermentative hydrolysates did not show anthelmintic activity.

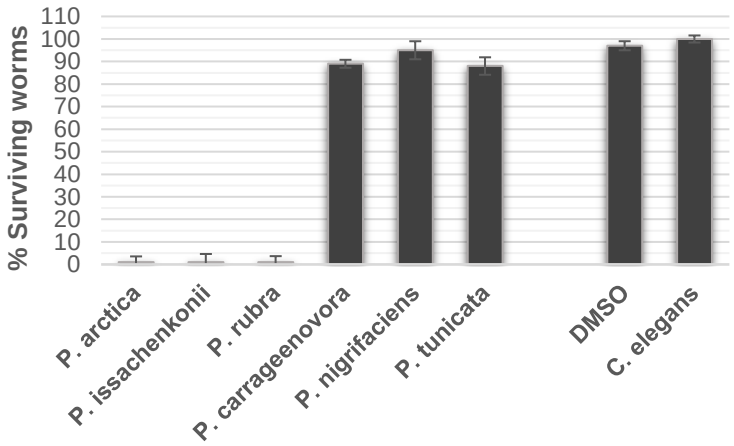


Fig. 4 Effect of fermentative hydrolysates-derived ethyl acetate extracts on *C. elegans* viability. Anthelmintic activity is expressed as percentage of surviving worms after the 24h-treatment with 1 mg/mL of extracts. DMSO represents the effect of the storage solvent on worms' viability.

In the following table, the antibiofilm activity of the aqueous raw fractions obtained after the ethyl acetate extraction from the fermentative hydrolysates of *P. arctica* and *P. issachenkonii*, is reported. Both the samples showed the activity up to the minimum tested concentratation (125 µg/mL), with percentage of biofilm inhibition of 45% and 42% for *P. arctica* and *P. issachekonii*, respectively. No antibiofilm activity was detected from the ethyl acetate extracts and no activity was detected from the other 4 bacteria.

Conc. [µg/mL]	500	250	125
P. arctica	67	50	45
P. issachenkonii	69	57	42

Tab. 5. Effect of crab-derived fermentative hydrolysates (aqueous fraction) on *S. epidermidis* biofilm formation. Antibiofilm activity is expressed as percentage of target bacteria biofilm formation, after the overnight incubation at 37°C in presence of different concentrations of fraction.

The residual aqueous raw fraction obtained from fermentation of crab shells by *P. issachenkonii* was further fractionated via Solid Phase Extraction, obtaining five fraction of different polarities. The fraction eluted with water showed the highest activity, indicating the ability of bacteria to synthesize highly polar compounds able to

inhibit the bacterial biofilm formation, when using crab shells as carbon source. The fraction eluted with water showed a higher activity compared to the source aqueous raw fraction, with a percentage of biofilm inhibition of 77% at 125 µg/mL, indicating the concentration of polar bioactive compounds.

	Conc. [µg/mL]	500	250	125
	Aqueous raw fraction	70	57	43
SPE Fractions	**Not bound**	1	1	1
	MeOH/CHL (1:9)	0	0	0
	MeOH/CHL (2:8)	0	0	0
	MeOH/CHL (1:1)	0	0	0
	MeOH	22	11	9
	MeOH/H_2O (1:1)	53	16	5
	H_2O	78	78	77

Tab. 6. Effect of aqueous raw fraction and derived SPE fractions obtained from the fermentative hydrolisate of *P. issachenkonii* on *S. epidermidis* biofilm formation. Antibiofilm activity is expressed as percentage of target bacteria biofilm formation, after the overnight incubation at 37°C in presence of different concentrations of fractions.

The observed activities are associated to production of bioactive compounds by bacteria when they degrade and use the crab shells as carbon source. The used media are composed of 2% (w/v) NaCl and 0.1% (v/v) and in such condition, bacteria cannot grow without the addition of a carbon source, represented by crab-by products and, consequently, the production of secondary metabolites cannot take place.

3.4 Discussion

About 50-70% of annual shellfish production (1.5 Mt) ends up as by-products. Apart from use for chitin/chitosan production, this marine biomass is either used to make fertilizer / low value products or is sent to landfill, incinerated or dumped at sea. BlueShell addressed this problem by exploring three typical shellfish by-products; shrimp (*Pandulus borealis*) shells, crab (*Cancer pagurus*) shells and defect mussels (*Mytilus edulis*), for potential bioactive compounds targeted at the sustainable supply of safe, healthy foods. This work, as part of the Blueshell project, aimed at evaluate the antimicrobial, anthelmintic and antibiofilm activities of the selected shellfish by-products.

The shellfish by-products, processed at ILVO, were used for extraction of pigmented organic compounds with different organic solvents. Mussel-derived raw extract showed antimicrobial activity, with the ethyl acetate and dichloromethane extracts being the most active and inhibiting the growth of 9 target bacteria out of 11 selected. Both crab and shrimp shells-derived raw extracts exhibited anthelmintic activity. In particular, the methanol and acetone extracts obtained from crab shells and the hexane extract from shrimp peels, with killing rate higher than 90%. Shrimp-derived extracts also exhibited the ability to inhibit the biofilm formation of *S. epidermidis,* with the ethyl acetate and acetone extracts being the most active.

In addition, crab shells were also used as substrate for bacterial fermentation using six different marine strains with chitinolitic/proteolytic activity, previously selected at ILVO. The strains *P. arctica* and *P. issachenkonii* resulted the most promising for the efficient degradation of crab by-products. The hydrolysates obtained from the fermentation with the above mentioned strains showed both anthelmintic and antibiofilm activity. Interestingly, the two activities were associated to different fractions. The anthelmintic activity was detected from the ethyl acetate extract, thus it may be linked with the synthesis of organic/hydrophobic secondary metabolites. The antibiofilm activity was, instead, found in the residual aqueous fraction remaining after the ethyl acetate extraction. The aqueous raw fraction derived from fermentation of *P. issachenkonii,* was further fractionated by Solid Phase Extraction, collecting six fraction of increasing polarity and the fraction eluted with water showed the highest activity, indication the accumulation of polar compounds with antibiofilm activity.

The media used for fermentation are composed of 2% (w/v) NaCl and 0.1% (v/v), with the addition of homogenized crab shells. Without a carbon source, represented by chitin of crab shells, bacteria cannot grow and, consequently, they cannot synthetize any compounds. Thus, the observed activities, can be associated to the biosynthesis of bioactive compounds by the selected bacteria when they degrade and use chitin as carbon source.

The obtained bioactive samples need to be further analyzed. The chemical characterization by GC-MS and LC-MS/MS is ongoing, in collaboration with the partner of the BlueShell Consortium, since the project is still on going. However, the showed result indicate the potential use of shellfish by-products for bioprospecting. In

particular, the bioconversion of shellfish by-products into valuable product should receive deep attention because it represents a sustainable tool to exploit discard products, being an alternative environmentally friendly solution to waste disposal and problems associated to it.

References

1 Suresh P.V., and Prabhu, G.N. Seafood. M. Chandrasekaran (Ed.), *Valorization of food processing by-products*, CRC Press, Taylor & Francis, New York, 2013: 685-736

2 Pangestuti, R and Kim, S.K. *Bioactive materials derived from seafood and seafood processing by-products.* A. Noomhorm, I. Ahmad, A.K. Anal (Eds.), Functional foods and dietary supplements: Processing effects and health benefits, Wiley-Blackwell, USA, 2014: 139-58

3 *Food and Agriculture Organization of the United Nations. The State of World Fisheries and Aquaculture* (FAO, 2014).

4 Kuddus, S.M, and Ahmad, R.I.Z. (2013). *Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinas*e. J Genet Eng Biotechnol, 2012; 11: 39-46.

5 Xu,Y., et al. *Transformation of the matrix structure of shrimp shells during bacterial deproteination and demineralization*. Microb Cell Fact, 2013; 5: 12-90

6 Kim, S. K. Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological Activities and Applications (CRD Press, 2011).

7 Vani, R., and Stanley, S.A. *Studies on the extraction of chitin and chitosan from different aquatic organisms*. Adv Biotechnol,2013; 12: 12-5

8 Du, J., et al. *Comparative evaluation of chitosan, cellulose acetate, and polyethersulfone nanofiber scaffolds for neural differentiation.* Carbohydr Polym, 2014; 99: 483-90

9 Salah, R., et al. *Anticancer activity of chemically prepared shrimp low molecular weight chitin evaluation with the human monocyte leukaemia cell line, THP-1*. Int J Biol Macromol, 2013; 52: 333-9.

10 Sarbon, N.M., et al. *Chitosan extracted from mud crab (Scylla olivicea) shells: physicochemical and antioxidant properties*. J Food Sci Technol, 2015; 52(7):4266-75

11 Sahariah, P., and Másson, M. *Antimicrobial Chitosan and Chitosan Derivatives: A Review of the Structure-Activity Relationship*. Biomacromolecules, 2017; 18(11): 3846-68

12 Hu, Z., et al. *Chitosan-based composite materials for prospective hemostatic applications*. Mar Drugs, 2018; 16(8).

13 Dash, M., et al. *Chitosan - a versatile semi-synthetic polymer in biomedical applications.* Progress in Polymeric Science, 2011; 36(8): 981-1014

14 Parvez, S, et al. *Preparation and characterization of artificial skin using chitosan and gelatin composites for potential biomedical applicatio*n. Polymer Bulletin, 2012; 69(6): 715-31

15 Muzzarelli, R.A.A., and Muzzarelli, C. *Chitosan chemistry: relevance to the biomedical sciences*. Advanc Pol Sci, 2005; 186: 151-209.

16 Kato, Y., et al. *Application of chitin and chitosan derivatives in the pharmaceutical field*. Curr Pharm Biotechnol, 2003; 4(5): 303-9.

17 Riva, R., et al. *Chitosan and chitosan derivatives in drug delivery and tissue engineering*. Advanc Pol Sci, 2011; 244: 19-44

18 Gautier, S. Chitin-glucan, a natural cell scaffold for skin moisturization and rejuvenation. Int J Cosmet Sci, 2008; 30(6):459-69

19 Thirunavukkarasu, N. *Production of chitin from two marine stomatopods Oratosquilla spp. (Crustacea).* J Chem Pharm Res, 2011; 3(1):353-359

General discussion

This thesis addresses marine bioprospecting as weapon to face up to modern societal challenges, such as the emergence of MDR bacteria, the need of finding effective and low-cost anthelmintic drugs, effective and not toxic anticancer drugs and the need of finding new compounds to apply in different industries in order to counteract microbial biofilms.
Marine bioprospecting is an important field of innovation, but it important to understand that this process should not affect negatively the marine biodiversity, which is the natural capital on which the sustainable growth depends. Marine bioprospecting should associate the search for molecules with an analysis of their supply. A bioprospecting program based on the sustainable use of marine resources is important, not only to reach economic benefits, but also to promote the protection and conservation of biodiversity of marine compounds.
In this thesis, three different marine resources were exploited, taking into consideration also the aspects related to the sustainable supply.

In the first chapter, a fast and effective screening method, based on the creation of a cell-free supernatant collection, was optimized and allowed the selection of a promising strain, identified as *Shewanella antarctica*, able to produce anthelmintic and antibiofilm activity. In particular, the observed activity was higher when the strain was grown at low temperature, demonstrating the powerful metabolic abilities of marine bacteria and the adaptation to extreme environments.
Marine bacteria, in particular from extreme environments are very promising starting points for bioprospecting. In addition, microorganisms constitute the source of sustainable bioprospecting par excellence. The importance of bacteria as source for bioprospecting, is related to the feasible and sustainable production of large quantities of secondary metabolites with reasonable cost, by large-scale cultivation and fermentation of the source organisms, in contrast to macroorganisms.
One of the limit of bioprospecting from marine microorganisms is the high difficulty to isolate rare or novel strains since they are not cultivable in laboratory. Molecular-based approaches demonstrated that approximately less than 1 % of microorganisms present in an environmental sample is cultivable *in vitro* and it drastically limits scientists knowledge of microbial life and their derived products.

Efforts should address innovation in this field, in order to find new strategies to isolate uncultivable bacteria.

In the second chapter the bioactive potential of the edible marine gastropod *Haliotis tuberculata* was explored. The results obtained showed the potential of the marine oil (with antimicrobial, anthelmintic and anticancer activity) derived from this species, suggesting its importance as promising candidate for further investigation aimed at the development of functional foods, food ingredients, as well as pharmaceuticals.
Marine macroorganisms are also considered very promising starting point materials for the discovery of novel compounds, but, in contrast to microorgansims, bioprospecting from marine invertebrates can face up serious obstacles linked with sustainability. Sustainability issues are associated with the large amounts of biomass that are usually required for drug discovery. The massive harvest of wild specimens would inevitably bring to the destruction of the surrounding environment and/or local extinction of species. In addition, another issue is related to the replicability. Individuals of the same species sampled in different areas, or periods, may not display the same chemical composition and therefore may not guarantee the supply of the target metabolite.
Aquaculture of marine invertebrates may overcome the problems related to sustainability and replicability, as animal biomass can be continuously produced using homogenous environmental conditions, and could therefore be a viable and economically feasible option for bioprospecting from macroorgansims.
Bioprospecting of marine invertebrate should be applied only that organisms that can be farmed in commercial quantities without affecting the environment and the biodiversity.
Contrarily to other marine invertebrates, the bioprospecting from farmed molluscs and the supply of derived bioactive compounds, can be carried out in a sustainable way. Moreover, filter-feeding molluscan shellfish have the potential to positively affect aquaculture environments, providing beneficial ecosystem services, such as nutrient extraction and water filtration, to the environment in which they are grown. Shellfish aquaculture is considered the most environmentally sustainable culture of marine invertebrate.

In the third chapter the bioactive potential of shellfish by-products was investigated and, in particular, the microbial bioconversion of

this waste material into valuable products. Two marine bacteria with proteolytic activity, *Pseudoalteromonas issachenkonii* and *Pseudoalteromonas artica* showed the ability to efficiently convert crab shells into anthelmintic and antibiofilm compounds.
The promising results show the importance of seafood by-products as source for bioprospecting. The exploitation of this starting material, not only, is very sustainable, but it would solve the problems related to the management of disposal of the abundant marine wastes.

In conclusions, the results obtained from this thesis project demonstrated the huge potential of marine organisms for bioprospecting to address the societal challenges and want to underline the possibility to exploit marine resources without affecting our planet biodiversity.

Appendix: Curriculum

List of publications

- **Tortorella E**., Tedesco P., Palma Esposito F., January G.G., Fani R., Jaspars M. de Pascale D. Antibiotics from Deep-Sea microorganisms: current discoveries and perspectives. Mar Drugs, 2018; 16(10)

- Corral P., Esposito FP., Tedesco P, Falco A., **Tortorella E.**, Tartaglione L., Festa C., D'Auria MV, Gnavi G. Varese G.C. de Pascale D. Identification of a sorbicillinoid-producing Aspergillus strain with antimicrobial activity against Staphylococcus aureus: a new polyextremophilic marine fungus from Barents Sea. Mar Biotechnol (NY), 2018; 20(4): 502-11

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

- Scala E., Di Caprio R., Cacciapuoti S., Caiazzo G., Fusco A., **Tortorella E.**, Fabbrocini G., Balato A. A new T helper 17 cytokine in hidradentis suppurativa: antimicrobial and proinflammatory role of interleukin-26. Br J Dermatol, 2019; 181(5): 1038-1045

List of oral communications

- **Emiliana Tortorella**-Progressive Report. "BlueShell" 1st Mid-Term meeting. January 2018. Naples, Italy

- **Emiliana Tortorella**-Progressive Report. "BlueShell" 2nd Mid-Term meeting. January 2019. Dublin, Ireland

- **Emiliana Tortorella**-Final Report. Ocean Medicine final GA meeting. December, 2019. Naples, Italy.

List of poster communications

- **Emiliana Tortorella.** "*From the hidden potentiality of Ocean to the Industry*". Summer school "Toward a bio-based economy" 2018, 2nd edition. Milan, Italy.

- **Emiliana Tortorella**. "*The lipid extract of the edible marine gastropod mollusc Haliotis tuberculata: a potential source of bioactive compounds*". ENABLE 2018. Copenhagen, Denmark.

- **Emiliana Tortorella**. "*The lipid extract of the edible marine gastropod mollusc Haliotis tuberculata: a potential source of bioactive compounds*". BIOPROSP_19. Troms, Norway

Experiences in foreign laboratories

- 3 months at Advance Science Ltd, Galway, Ireland (from 2/06/2018 to 2/09/2018)
  Tutor: Dr. Dara Scott

- 2 months at Sea4Us, Lisbon, Portugal (from 5/12/2018 to 5/02/2019)
  Tutor: Prof. Pedro Lima

- 1 month at University of Gent, Belgium (from 15/09/2019 to 15/10/2019)
  Tutor: Prof. Marleen de Troch

During my PhD, I have spent 2 months in the company Sea4Us in Lisbon, headed by Dr. Pedro Lima, conducting experiments to evaluate the cell toxicity of bioactive extracts and fractions and extending the experimental research activity to topics related to new assays for the isolation of marine compounds for the treatment of the chronic pain from marine sources.
I also spent 1 month at the University of Ghent, in the research group of Prof. Marleen De Troch in order to learn how to perform the fatty acids analysis and how to use GC-MS, necessary for my work.
Finally I spent 3 months at Advance Science Ltd, located in Galway, a leader company in the production of seaweeds-based

complementary feeds for bees, headed by Dr. Dara Scott. Here I learnt how a settled company works and this experience allowed her to understand how to translate the results obtained from academia to the industrial fields. During the period at Advance Science, I contributed to the communication management of the company with the collection of data about the Italian beekeeping industry and with an article about the benefits of seaweed for animals and bees in particular, that was published in the website of HiveAlive.

marine drugs

MDPI

Review

Antibiotics from Deep-Sea Microorganisms: Current Discoveries and Perspectives

Emiliana Tortorella [1,†], Pietro Tedesco [1,2,†], Fortunato Palma Esposito [1,3,†], Grant Garren January [1,†], Renato Fani [4], Marcel Jaspars [5] and Donatella de Pascale [1,3,*]

1 Institute of Protein Biochemistry, National Research Council, I-80131 Naples, Italy; e.tortorella@ibp.cnr.it (E.T.); tedesco@insa-toulouse.fr (P.T.); f.palma@ibp.cnr.it (F.P.E.); g.january@ibp.cnr.it (G.G.J.)
2 Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, INSA, 31400 Toulouse, France
3 Stazione Zoologica "Anthon Dorn", Villa Comunale, I-80121 Naples, Italy
4 Department of Biology, University of Florence, Sesto Fiorentino, I-50019 Florence, Italy; renato.fani@unifi.it
5 Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Aberdeen, Scotland AB24 3UE, UK; m.jaspars@abdn.ac.uk
* Correspondence: d.depascale@ibp.cnr.it; Tel.: +39-0816-132-314; Fax: +39-0816-132-277
† These authors equally contributed to the work.

Received: 23 July 2018; Accepted: 27 September 2018; Published: 29 September 2018

Abstract: The increasing emergence of new forms of multidrug resistance among human pathogenic bacteria, coupled with the consequent increase of infectious diseases, urgently requires the discovery and development of novel antimicrobial drugs with new modes of action. Most of the antibiotics currently available on the market were obtained from terrestrial organisms or derived semisynthetically from fermentation products. The isolation of microorganisms from previously unexplored habitats may lead to the discovery of lead structures with antibiotic activity. The deep-sea environment is a unique habitat, and deep-sea microorganisms, because of their adaptation to this extreme environment, have the potential to produce novel secondary metabolites with potent biological activities. This review covers novel antibiotics isolated from deep-sea microorganisms. The chemical classes of the compounds, their bioactivities, and the sources of organisms are outlined. Furthermore, the authors report recent advances in techniques and strategies for the exploitation of deep-sea microorganisms.

Keywords: antibiotics; deep-sea; marine microorganisms; extreme habitat; marine sediments

1. Introduction

The deep-sea is one of the less explored and extreme environments on Earth [1]. The characteristics of the deep-sea that make it an extreme environment include (i) pressure increases by one atmosphere (atm) for every 10-m increase in water depth, so pressure varies from 20 atm at the shelf-slope break to >1000 atm in the deepest parts of the trenches; (ii) temperature generally drops with increasing depth reaching values around 2 °C on the abyssal plain; (iii) the oxygen concentration in the bottom waters can be much less than that of the surrounding region, or even zero, depending on the balance between the rate at which oxygen is supplied (by exchange with the atmosphere and as a byproduct of photosynthesis by marine plants in the euphotic zone) and the rate at which it is consumed; and (iv) light intensity declines exponentially with depth in the water column because incident photons are absorbed or scattered, and total darkness prevails below 250 m deep [2]. The definition of deep-sea environment is still variable. Older designations describe the deep-sea as having a depth above 200 m, but more commonly, it is considered a depth $\leq$1000 m as standard. For many years, the difficulty to

reach the bottom of the ocean has been the main issue for studying deep-sea life. Recently, thanks to the improved acoustic technology and the improved access by Remotely Operated Vehicles (ROV) and submersibles, deep ocean environments became more accessible, unveiling the presence of biological activity [3]. The access to deep-sea organic material combined with the application of culture-dependent and -independent methods demonstrated the presence of an unexpected microbial biodiversity [4–6]. Microorganisms inhabiting these harsh environments developed unique strategies to survive, especially to the high pressure. In fact, most of them are piezotolerant and piezophilic microorganisms [7], but the lack of appropriate instrumentation limits the cultivation of these strains. Their adaptation to biochemical and physiological processes is mirrored in modifications to gene regulation and primary/secondary metabolic pathways that result in the expression of novel natural products (NPs).

In the last 50 years, over 30,000 marine natural products (NPs) have been found and approximately 2% of those have been isolated from deep-sea organisms [8]. Among microbial NPs, antibiotics are one of the most interesting molecules, especially for biotechnological and pharmaceutical applications. The discovery of novel antibiotics is necessary to counteract the spread of multidrug-resistant (MDR) bacteria [9], and the exploitation of unexplored deep-sea microorganisms, such as bacteria and fungi, and their related products, could lead to the isolation of new antibiotics.

This review reports on novel bioactive compounds with antibiotic activity isolated from deep-sea bacteria and fungi. We also provide an update on the current state-of-the-art of deep-sea bioprospecting, discussing bottlenecks and current advances in the field, from sampling techniques and cultivation to metagenomic approaches.

2. Antimicrobial Compounds from Marine Microorganisms

2.1. Antimicrobial Compounds from Bacteria

Marine microorganisms represent a significant source for the discovery and development of new antibiotics due to their rich biodiversity and genetic capacity to produce unique metabolites. It is recognized that many taxonomically novel species are promising sources of new bioactive compounds [10]. In particular, marine bacteria derived from deep-sea sediments have shown to be a rich source of secondary metabolites with novel structures and excellent biological activities, including antimicrobials [11–15]. Most natural antibiotics are biosynthesized by bacteria belonging to the high GC Gram-positive bacteria. In particular, actinomycetes represent the most important source of bioactive natural products with clinical or pharmaceutical applications [16]. Most of the antimicrobial compounds shown below (Compounds **1**–**12**) were isolated from deep-sea derived actinomycetes (Figures 1–8; Table 1).

Table 1. A schematic summary of the new compounds with antimicrobial activity isolated from deep-sea derived microorganisms. Details are reported in the text.

	Organism	Depth	Geographic Location	Compound	Molecular Class	Antimicrobial Activity Against	Ref.
Bacteria	*Marinactinospora thermotolerans* (SCSIO 00652)	3865 m	South China Sea, People's Republic of China	**Marthiapeptide A** (**1**)	Cyclic peptide	*M. luteus; S. aureus; B. subtilis; B. thuringiensis*	Zhou et al., 2012
	Streptomyces scopuliridis (SCSIO ZJ46)	3536 m	South China Sea, People's Republic of China	**Desotamide B** (**2**)	Cyclic peptide	*S. aureus; S. pneumoniae;* MRSE shhs-E1	Song et al., 2014
	Streptomyces drozdowiczii (SCSIO 10141)	1396 m	South China Sea, People's Republic of China	**Marfomycins A, B, E** (**3,4,5**)	Cyclic peptide	*M. luteus*	Zhou et al., 2014
	Streptomyces sp. (SCSIO 01127)	1350 m	South China Sea, People's Republic of China	**Lobophorin F** (**6**)	Spirotetronate poliketides	*S. aureus; E. faecalis*	Niu et al., 2011
	Streptomyces sp. (12A35)	2134 m	South China Sea, People's Republic of China	**Lobophorin H** (**7**)	Spirotetronate poliketides	*B. subtilis*	Pan et al., 2013

Table 1. *Cont.*

	Organism	Depth	Geographic Location	Compound	Molecular Class	Antimicrobial Activity Against	Ref.
Bacteria	*Verrucosispora* sp. (AB 18-032)	289 m	Japanese Sea	**Abyssomicin C (8)**	Spirotetronate poliketides	MRSA; vancomycin-resistant *S. aureus*	Bister et al., 2004
	Streptomyces niveus (SCSIO 3406)	3536 m	South China Sea, People's Republic of China	**Marfuraquinocins A, C, D (9,10,11)**	Sesquiterpene derivative	*S.aureus;* MRSE shhs-E1	Song et al., 2013
	Streptomyces sp. (NTK 937)	3814 m	Saharan debris flow near the Canary Islands	**Caboxamycin (12)**	Alkaloid	*B. subtilis; S. lentus; S. epidermidis*	Hohmann et al., 2009
Fungi	*Emericella* sp. (SCSIO 05240)	3258 m	South China Sea, People's Republic of China	**Emerixanthones A, B, C, D (13,14,15,16)**	Xanthone-derivative	*E. coli; K. pneumoniae; S. aureus; E. faecalis; A. baumannii; A. hydrophila*	Fredimoses et al., 2014
	Engyodontium album (DFFSCS021)	3739 m	South China Sea, People's Republic of China	**Engyodontiumone H (17)**	Xanthone-derivative	*E. coli; B. subtilis*	Yao et al., 2014
	Spiromastix sp.	2869 m	South Atlantic Ocean	**Spiromastixones A, B, C, D, E, F, G, H, K, L, M, N, O (18,19,20,21,22,23,24,25, 26,27,28,29,30,31,32)**	Depsidone analogs	*S. aureus; B. thuringiensis; B. subtilis;* MRSA; MRSE; *E. faecalis; E. faecium*	Niu et al., 2014
	Penicillium sp. (F23-2)	5080 m	Chinese Sea, People's Republic of China	**Penicyclones A, B, C, D, E, F (33,34,35,36,37)**	Ambuic acid analogs	*S.aureus*	Guo et al., 2015

Tian et al. first discovered and classified the type-strain of the novel marine actinomycete, *Marinactinospora thermotolerans* SCSIO 00652, isolated from a sediment collected from site E410 (1°58.742′ N 11°00.228′ E; black soft mud at 3865 m depth) in the northern South China Sea [17]. Thereafter, Zhou et al. purified a new polythiazole cyclic peptide, referred to as marthiapeptide A (**1**), from this organism. Marthiapeptide A (**1**) exhibited strong antibacterial activity against *Micrococcus luteus*, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC 6633, and *B. thuringiensis*, with MIC values of 2, 8, 4, and 2 μg/mL, respectively [18]. Three new cyclic hexapeptides, named desotamides B, C, and D were identified from *Streptomyces scopuliridis* strain SCSIO ZJ46, which was isolated from a South China Sea sample sediment collection at a depth of 3536 m (120°0.250′ E, 20°22.971′ N). Among the new compounds identified, desotamide B (**2**) showed an antimicrobial activity against *S. aureus* ATCC 29213, *Streptoccocus pneumoniae* NCTC 7466, and methicillin-resistant *Staphylococcus epidermidis* (MRSE) shhs-E1, with MIC values of 16, 12.5, and 32 μg/mL, respectively [19]. New cyclic congeners, marfomycins A (**3**), B (**4**), and E (**5**), were isolated from the South China Sea-derived *Streptomyces drozdowiczii* SCSIO 10141. The producer strain of marfomycins A, B, and E was isolated from a sediment collected at a depth of 1396 m (118°58.2475′ E, 22°2.3689′ N) [20]. Unique *N*-terminally formylated side chain and five nonproteinogenic amino acid residues characterize marfomycins A (**3**), B (**4**), and E (**5**). Marfomycins A (**3**), B (**4**), and E (**5**), exhibited a selective anti-infective activity against *M. luteus*, among a panel of Gram-positive and Gram-negative bacteria, with MIC values of 0.25, 4, and 4 μg/mL, respectively. The antimicrobial agents marthiapeptide A (**1**), desotamides B (**2**), and marfomycins A (**3**), B (**4**), and E (**5**) (Figure 1) represent new deep-sea derived cyclic peptides. Cyclic peptides and depsipeptides are secondary metabolites of microorganisms and plants, with a recognized broad spectrum of biological properties [21–23]. This class of NP represents a valuable source for the discovery of new therapeutics, due to their favorable properties, such as resistance to enzymatic degradation [24]; a large surface area, which provides high affinity and selectivity for the targets [25]; and limited conformational flexibility [26,27], which enhances binding properties. Moreover, they often possess better membrane penetration properties [28]. As a result, they have much longer half-lives in vivo than their acyclic counterparts, and are thus of great interest in the field of drug discovery [29]. Cyclic antimicrobial peptides (AMPs) have emerged as good antimicrobial candidates due to their aforementioned characteristics [21–29] and high activity [30,31].

The molecular details of the action of cyclic AMPs, that is, whether these short peptides can open and stabilize pores, are still unclear, as well as the molecular basis for their antibacterial activity.

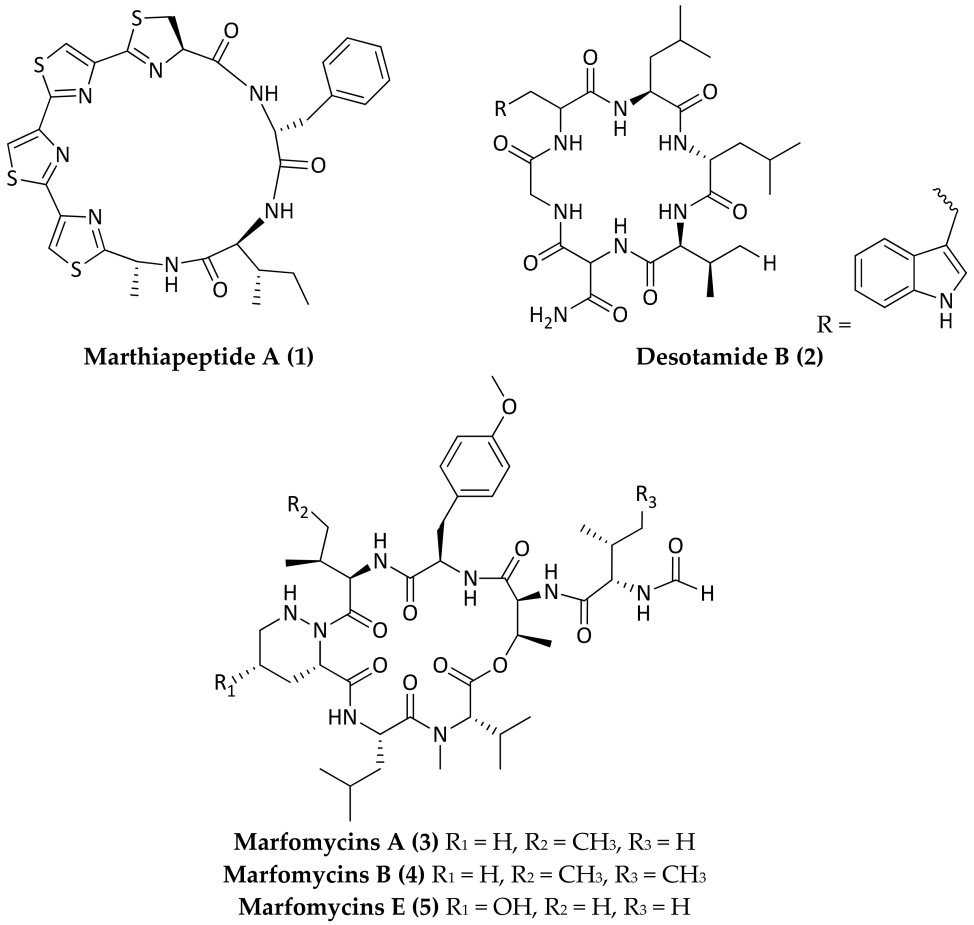


Figure 1. Molecular structure of new cyclic peptides with antimicrobial activity isolated from deep-sea bacteria. Compound (**1**), marthiapeptide A, is a polythiazole cyclic peptide isolated from *M. thermotolerans* SCSIO 00652. Compound (**2**), desotamide B, is a cyclic hexapeptide isolated from *Streptomyces scopuliridis* SCSIO ZJ46. Compounds (**3**–**5**), marfomycins A, B, and E, are cyclic hepta-depsipeptides isolated from *Streptomyces drozdowiczii* SCSIO 10141.

Another family of microbial metabolites with potent antitumor and antibiotic properties is collectively designated as spirotetronate polyketides. Three new spirotetronate polyketides with antibacterial activity isolated from deep-sea Gram-positive bacteria are shown in Figure 2.

Two different research groups identified the already-known lobophorins B and two new spirotetronate antibiotics, designated lobophorins F (**6**) and H (**7**), from actinomycetes isolated from deep-sea sediments of the South China Sea [32]. Both lobophorins F (**6**) and H (**7**) were extracted from *Streptomyces* sp. strains (SCSIO 01127 and 12A35, respectively). The two strains were isolated from a sediment sample collected at the depth of 1350 m (111°54.693′ E, 08°56.003′ N) [33] and 2134 m (17°59.928′ N, 111°36.160′ E), respectively [34]. Lobophorin F (6) showed antibacterial activity against *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 with MIC values of 8 μg/mL for both of the strains. Lobophorin H (**7**) showed antibacterial activity against *B. subtilis* CMCC63501 with a MIC value of 3.13 μg/mL. Based on the inhibitory activity exhibited against Gram-positive bacteria, lobophorins F (**6**) and H (**7**) may potentially find application in antibacterial drug development. Moreover, the discovery of more spirotetronate antibiotics analogs helps to elucidate the structure–activity relationships and potential applications of these compounds. Lobophorin F and H are analogs of lobophorin B, which was previously isolated from an alga-associated actinobacterium; lobophorin B is

structurally related to another spirotetronate antibiotic, named kijanimicin [35]. Recent studies indicate that kijanimicin binds to the TetR family of transcriptional regulators that control the expression of various cytoplasmic proteins in prokaryotes [36].

Bister et al. isolated three new polycyclic polyketide-type antibiotics, named abyssomicins B, C, and D from the rare actinomycete *Verrucosispora* strain AB 18-032, which originated from a sediment sample collected in the Japanese Sea at a depth of 289 m [37]. Abyssomicin C (**8**) showed antibiotic activity against methicillin-resistant *S. aureus* (MRSA) and a vancomycin-resistant *S. aureus* strain with MIC values of 4 µg/mL and 13 µg/mL, respectively. Abyssomicin C is considered a representative member of class I spirotetronate. Abyssomicin C and its atropisomer inhibit para-aminobenzoic acid (pABA) biosynthesis, these compounds are the first known substances derived from a bacterial source that inhibit the biosynthesis of pABA. The biosynthesis of pABA is an attractive target in the field of new antibiotics discovery since it is found in many microorganisms but not in humans, and blocking the pABA pathway damages bacteria since it is a biosynthetic precursor of folic acid, which is essential for DNA synthesis/repair and cell survival [35].

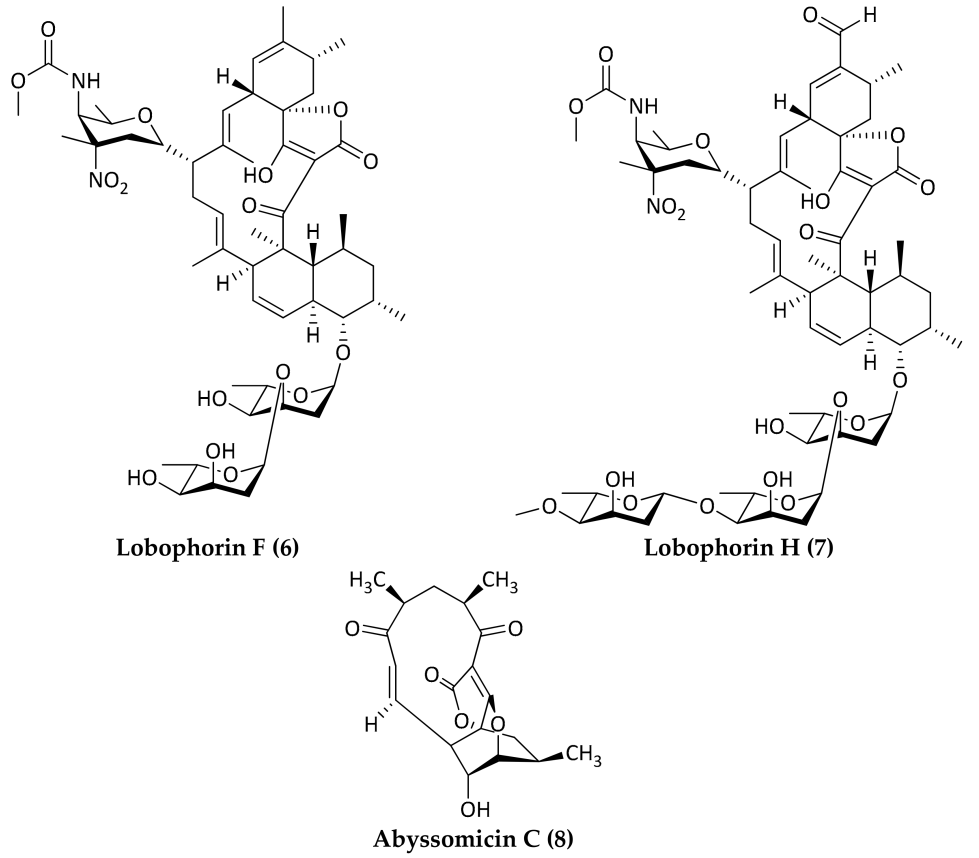
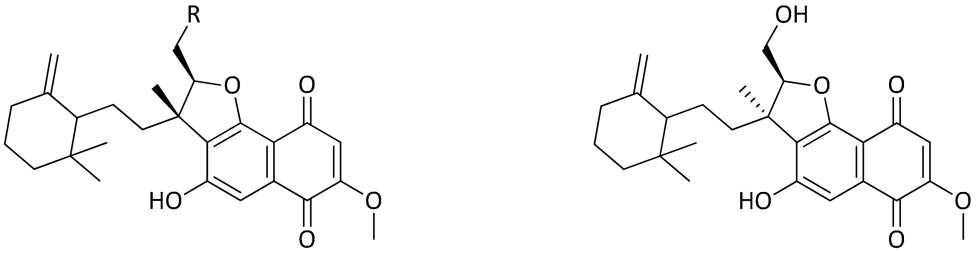


Figure 2. Structures of new spirotetronate polyketides with antimicrobial activities isolated from deep-sea bacteria. Lobophorin F (**6**) was isolated from *Streptomyces* SCSIO 01127. Lobophorin H (**7**) was isolated from *Streptomyces* sp. 12A35*S*; Abyssomicin C (**8**) was isolated from *Verrucosispora* strain AB 18-032.

In 2013, three new sesquiterpene derivatives, named marfuraquinocins A (**9**), C (**10**), and D (**11**), were purified from the fermentation broth of *Streptomyces niveus* SCSIO 3406 isolated from a South China Sea sample sediment (120°0.250′ E, 20°22.971′ N) obtained from a depth of 3536 m [38]. Marfuraquinocins A (**9**), C (**10**), and D (**11**) (Figure 3) exhibited antibacterial activity against *S. aureus* ATCC 29213 with equivalent MIC values of 8 µg/mL; moreover marfuraquinocins C (**10**) and D (**11**) showed antibacterial activity against methicillin-resistant *S. epidermidis* (MRSE) shhs-E1 with MIC values of 8 µg/mL.

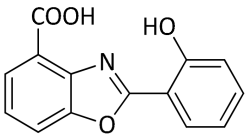


Marfuraquinocin A (9) R = H
Marfuraquinocin C (10) R = OH

Marfuraquinocin D (11)

Figure 3. Structures of Marfuraquinocins A (**9**), C (**10**), and D (**11**) isolated from *Streptomyces niveus* SCSIO 3406.

Hohmann et al. detected caboxamycin (**12**) (Figure 4), a new antibiotic of the benzoxazole family, in extracts of the strain *Streptomyces* sp. NTK 937 originated from an Atlantic Ocean deep-sea sediment (27°02′392 N, 18°29′022 W) at a depth of 3814 m [39]. Caboxamycin showed inhibitory activity against the Gram-positive bacteria *B. subtilis* (IC_{50} = 8 μM) and *Staphylococcus lentus* (IC_{50} = 20 μM) and the opportunistic pathogen *S. epidermidis* (IC_{50} = 43 μm). Notable molecular targets of caboxamycin are phosphodiesterases, which are essential regulators of cyclic nucleotide signaling with diverse physiological functions.



Caboxamycin (12)

Figure 4. Structure of caboxamycin (**12**).

2.2. Antimicrobial Compounds from Fungi

Since the discovery of the antibiotic cephalosporin in 1948, marine fungi have been considered an excellent source of bioactive compounds. Thanks to their unique adaptive capabilities, marine fungi are able to colonize different marine habitats, even the most extreme ones, including deep-sea environments. Although several studies have reported that fungi are abundant and diverse in these habitats [40], it is anticipated that many remain to be discovered. Access to the actual fungal biodiversity present in the deep-sea could lead to the discovery of new bioactive compounds useful for drug discovery [41]. The first antimicrobial compound isolated from an *Aspergillus* sp. strain originating from deep-sea sediments was gliotoxin, which is able to inhibit the growth of the Gram-positive bacteria *S. aureus* and *B. subtilis* [42]. The fungus was isolated from the mud of Seto Inland Sea in Japan. Subsequent to this discovery, other new antibiotics were detected from deep-sea fungi but they still represent a minority compared to molecules produced by marine fungi isolated from surface waters [43]. Zhang et al. [44] isolated 13 novel fungal species (among them some new phylotypes) from deep-sea sediments in the South China Sea and many of them were able to produce antimicrobial compounds against pathogenic bacteria and fungi, like *Micrococcus luteus*, *Pseudoaltermonas piscida*, *Aspergerillus versicolor*, and *A. sydowii* [44].

Prenylxanthones are an important group of naturally occurring secondary metabolites endowed with a wide range of biological and pharmacological activities [45,46]. Four new antifungal and antibacterial prenylxanthones, emerixanthones A–D (**13**–**16**) (Figure 5), were identified from the deep-sea fungus *Emericella* sp. SCSIO 05240, isolated in the South China Sea (3258 m). These molecules are able to inhibit *Escherichia coli* (ATCC 29922), *Klebsiella pneumoniae* (ATCC 13883), *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *Acinetobacter baumannii* (ATCC 19606), and *Aeromonas hydrophila* (ATCC 7966) [47]. Another compound belonging to the xanthone class, engyodontiumone H (**17**)

(Figure 6), was purified from *Engyodontium album* DFFSCS021, a deep-sea fungus collected at 3739 m depth in the South China Sea. This molecule exhibited inhibitory activity against *E. coli* and *B. subtilis* with MIC values of 64 μg/mL and 32 μg/mL, respectively [48]. A study carried out by Huang et al. showed that xanthone derivatives could act as antibiotics by blocking the enzyme I (EI) of the bacterial phosphoenolpyruvate-dependent phosphotransferase system (PTS) [49].

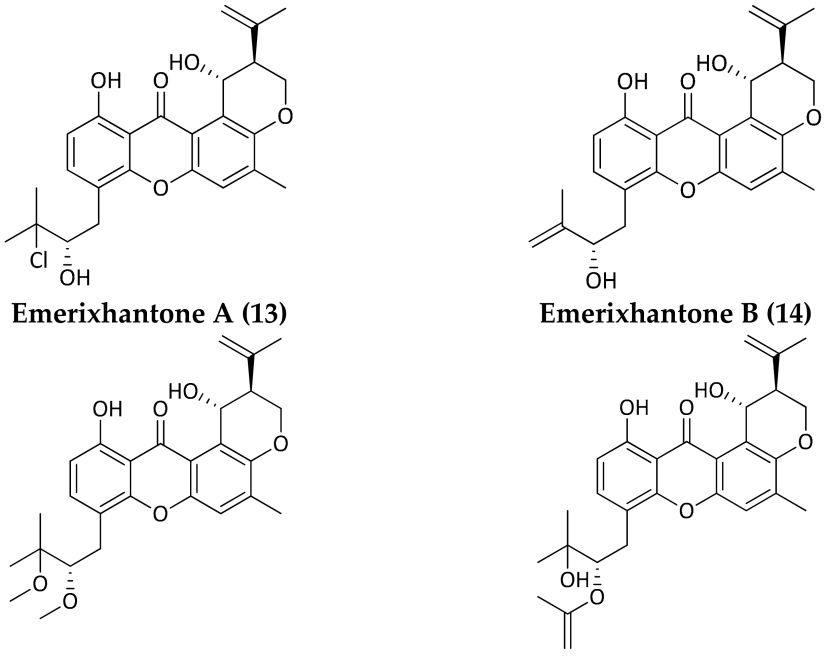


Figure 5. Structures of Emerixanthones A–D (**13**–**16**).

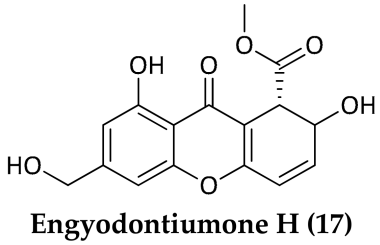
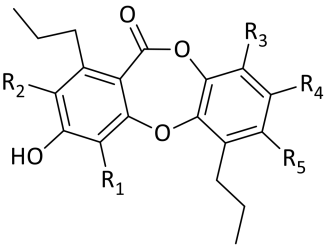


Figure 6. Structure of Engyontiumone H (**17**).

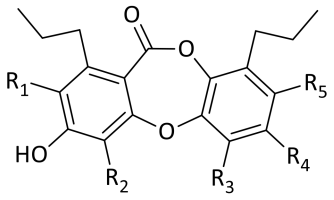
Fifteen new depsidone-based analogs, spiromastixones A–O (**18**–**32**) (Figure 7), have been isolated from an unidentified *Spiromastix* sp. fungus, collected at a depth of 2869 m in the South Atlantic Ocean. These compounds showed an antimicrobial activity towards *S. aureus*, *B. thuringiensis*, and *B. subtilis* with MIC values ranging from 0.125 to 8.0 μg/mL. Moreover, some of them displayed inhibitory effects on methicillin-resistant strains of both *S. aureus* (MRSA) and *S. epidermidis* (MRSE), and also against vancomycin-resistant *E. faecalis* and *E. faecium* (VRE) strains [50].

New chlorinated azaphilone pigments showing antibacterial and cytotoxic activities have been recently identified from a deep-sea fungus *Chaetomium* sp. strain NA-S01-R1, isolated at a depth of 4050 m in the West Pacific Ocean [51].



Spiromastixones A–H (Type A)

(18) A: $R_1 = R_2 = R_3 = R_5 = H$, $R_4 = OH$
(19) B: $R_2 = Cl$, $R_1 = R_3 = R_5 = H$, $R_4 = OH$
(20) C: $R_1 = Cl$, $R_2 = R_3 = R_5 = H$, $R_4 = OH$
(21) D: $R_2 = R_3 = H$, $R_1 = R_5 = Cl$, $R_4 = OH$
(22) E: $R_3 = R_5 = H$, $R_1 = R2 = Cl$, $R_4 = OH$
(23) F: $R_1 = R_2 = R_5 = Cl$, $R_4 = OH$, $R_3 = H$
(24) G: $R_1 = R_2 = R_5 = Cl$, $R_3 = H$, $R_4 = OMe$
(25) H: $R_1 = R_2 = R_3 = Cl$, $R_5 = H$, $R_4 = OH$

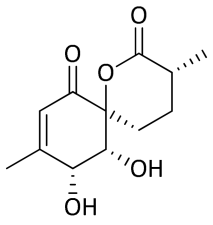


Spiromastixones K–O (Type B)

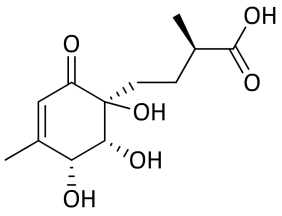
(28) K: $R_1 = R_2 = R_5 = Cl$, $R_3 = H$, $R_4 = OMe$
(29) L: $R_1 = R_2 = R_3 = R_5 = Cl$, $R_4 = OMe$
(30) M: $R_1 = R_2 = Cl$, $R_3 = R_5 = H$, $R_4 = OH$
(31) N: $R_1 = R_2 = R_5 = Cl$, $R_3 = H$, $R_4 = OH$
(32) O: $R_1 = R_2 = R_3 = R_5 = Cl$, $R_4 = OH$

Figure 7. Structures of Spiromastixones A–O (**18**–**32**).

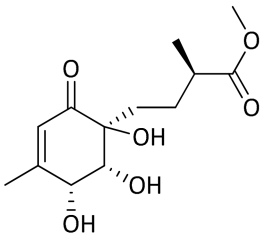
Several analyses of fungal genomes demonstrated that the number of predicted biosynthetic genes exceeds the number of new molecules obtained so far, leaving many compounds yet to be discovered [52]. The application of the OSMAC approach (one strain many compounds) could stimulate the expression of cryptic genes leading to the identification of novel antibiotics as demonstrated by Wenqiang Guo et al. [53]. This study reported that a deep-sea-derived fungus *Penicillium* sp. F23-2, collected from Jiaozhou Bay in China at a depth of 5080 m [54], produced five new ambuic acid analogs, penicyclones A−E (**33**–**37**), which exhibited antimicrobial activity against *S. aureus* with MIC values ranging from 0.3 to 1.0 μg/mL. This fungus showed a different metabolites production when grown on rice-based medium instead of PYG and PD media [53]. Improving isolation methods and exploring different cultivation approaches of deep-sea fungi could become an important weapon against multidrug-resistant bacteria.



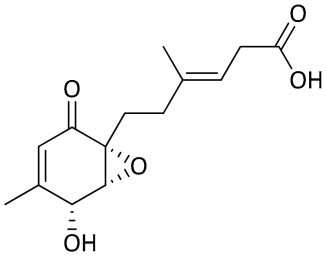
Penicylone A (33)



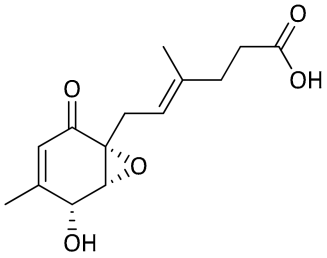
Penicyclone B (34)



Penicyclone C (35)



Penicyclone D (36)



Penicyclone E (37)

Figure 8. Structures of Penicyclones A–E (**33**–**37**).

3. Improving the Biodiscovery Pipeline for Deep-Sea Antibiotics

As shown in Section 2, several antimicrobial compounds have been discovered thus far from deep-sea environments. Nonetheless, we are very likely scratching the surface of a wider reservoir of molecular scaffolds. The discovery of novel antibiotics is per se linked to the discovery of novel biological resources (microorganisms and/or their genetic material) but there are still many bottlenecks limiting the success of biodiscovery campaigns for antimicrobials, and more generally for bioactive compounds, from this extreme ecosystem(s). To improve the current situation, innovations aimed at realizing the exploitation of deep-sea microorganisms are essential. In this section, we deal with the major limitations for deep-sea microbial investigations and report current knowledge and innovations available to researchers.

3.1. Sampling Techniques

Marine sediments experience extremely high pressures, with approximately 58% of the seafloor's surface being at of 4000 m and above, with up to 40 MPa of pressure. One of the major limitations of deep-sea sampling for bacteria isolation is that most of microorganisms inhabiting these sites are obligate piezophiles, unable to grow at atmospheric pressure but especially sensitive to shifts in pressure [55–58]. Hence, pressurized transport and treatment of sub-sea floor sediments to the surface is a crucial step to preserve microbial viability and diversity. In 2002, the German Project OMEGA developed a Multiple Autoclave Corer (MAC) able to collect four 0.6 m cores maintaining in situ conditions (e.g., pressure and temperature) for geological and microbiological purposes. The cores, collected at −776 m in the North Pacific Ocean, were still pressurized after two months [59]. Further efforts developed a Dynamic Autoclave Piston Corer (DAPC) designed for a maximum water depth of 2000 m. The research group of Parkes and colleagues established a complete system, which includes a 'hydrate autoclave coring equipment' (HYACE), a pressurized core subsampling and extrusion system (PRESS), and pressurized chambers for prokaryotic enrichment and isolation (DeepIsoBUG) [60]. After the core is taken from the sea-floor and brought on the vessel without depressurization (up to 25 MPa), it passes to subcoring and slicing system to obtain a sample subcore (20 mm) that is further sliced. The slice is then transferred to a low-pressure vessel (max 25 MPa), which, through shaking, produces a slurry that can be used as an inoculum. The slurry is then transferred to high-pressure culture vessels at different pressures (up to 100 Mpa) containing enrichment media. The isolation chamber has 12 agar plates attached to a motor-driven chain, to select individual plates [60]. Thus, sediments never experience depressurization, helping to maintain the integrity and the value of the samples. The most recent development in deep-sea sampling involves utilization of robots, which is defined as "Soft robotic grippers" [61]. Galloway et al. have built a remotely operated vehicle (ROV) equipped with a robotic-hand able to delicately handle marine microorganisms. In their pilot study, the Deep Reef ROV and the soft robotic grippers were brought to the Gulf of Eilat in the northern Red Sea. The device was employed at depths between 100 and 170 m and proved to be able to grab soft specimen without damage. Despite its current limitations (it can operate at limited depths and only with macroorganisms), this device could significantly help in the study and exploration of the deep-sea. Future developments, as foreseen by Galloway, can include technical modification to add complete robotic-hands and perform experiments underwater, and the possibility to add RNA preservers (e.g., RNAlater) to facilitate transcriptomic experiments. This can aid the research on marine symbionts, known producers of metabolites, and help in the discovery of novel genes involved in the production of antimicrobial compounds.

3.2. Isolation and Cultivation Techniques

Isolation and cultivation of deep-sea microorganisms started in the late 1950s, when Zobell and Morita, true pioneers in this field, succeeded in the development of titanium vessels for bacterial

growth that could reach 100 MPa of pressure. They were not able to isolate obligate piezophilic bacteria but only piezotolerant strains [62].

To obtain the first obligate piezophilic strains, it was necessary to wait until 1979, when Yayanos et al. were able to isolate spirillium CNPT-3 from an amphipod collected in Philippines' trenches using a pressure-retaining trap [63].

In the following years, many piezophiles have been isolated, but the majority of them represented few bacterial taxa and did not reflect the extent of deep-sea biodiversity [64,65].

Classical isolation methods require enrichment steps and nutrient-rich media, which often retrieve dominant fast-growing taxa. Innovative isolation techniques have been used to isolate novel strains such as using a dilution-to-extinction cultivation method employing a natural seawater medium to obtain a novel member of *Roseobacter* clade within alphaproteobacteria [66]. The strain, named PRT1, is an obligate psychropiezophile and resulted in being the slowest-growing (minimal doubling time, 36 h) and lowest cell density-producing piezophile obtained to date, with an optimal growth at 80 MPa and 10 °C [66].

Cultivation of hyperthermophilic and piezophilic bacteria inhabiting deep-sea hydrothermal vents proved to be even more challenging, as predominant strains are often chemolithoautotrophs.

To overcome this obstacle, Takai et al. [67] developed a piezophilic cultivation technique that allows the growth of deep-sea chemolithoautotrophs, including methanogenic bacteria. The system was designed by using a combination of syringe and piston and could reach temperatures from 116 °C at 0.4 MPa to 122 °C at 20 MPa. The technique proved useful for the isolation of a new hyperthermophilic methanogenic strain i.e., *Methanopyrus kandleri* strain 116 [68]. In the follow-up work, the authors applied this system to cultivate H_2- and/or sulfur-oxidizing chemolithoautotrophs bacteria from a thermal vent chimney. Their efforts resulted in the isolation of novel strains, one of which belongs to a novel genus of the previously uncultivated group, defined as *Thioprofundum lithotrophica*. The second bacterium was associated to a new genus of the Rhodobacteraceae family and was named *Piezobacter thermophilus* [67].

The use of specific bioreactors was also effective for the isolation of methanogenic piezophilic strains. Using a bioreactor with polyurethane sponges, the so-called down-flow hanging sponge (DHS) bioreactor, the research group of Aoki and coworkers were able to cultivate microorganisms from deep-sea sediments of 2533 m below sea surface. Methane was used as carbon sources, and the bioreactor was operated in a continuous mode. Using ^{13}C-labeled methane experiments, the group was able to confirm the growth of an anaerobic oxidation of methane (AOM) community, in the bioreactor. At the end of a long incubation period (2013 days), researchers confirmed that the predominant microbial components belonged to archeal anaerobic methanotroph groups [69]. A more recent approach for the cultivation of anaerobic deep hyperthermophilic communities was performed using bacterial immobilization. In this study a mixture of microorganisms, isolated from an active thermal vent of Rainbow field at the Mid Atlantic Ridge (2275 m of depth), were immobilized into beads and used to inoculate a bioreactor in continuous mode at higher temperature (>50 °C). The culture was maintained for 45 days proving to be an effective technique for the cultivation of these microorganisms [70].

3.3. Metagenomics

Despite progress in improving cultivation of deep-sea microorganisms, the majority of these microorganisms remained difficult to cultivate under laboratory conditions. This also means that our capacity to obtain antibiotics from deep-sea microorganisms through traditional approaches is limited. Recent progress in DNA amplification, sequencing, and analysis has provided powerful tools to overcome these limitations. Metagenomics is a cultivation-independent technique with extraordinary potential for the study and exploitation of extreme environments [71]. This approach was successfully applied also to deep-sea environments and in the last decades it has shed some light on the biodiversity and phylogeny of bacteria, fungi, and viruses inhabiting this environment [15,72,73].

The potential of metagenomics for the discovery of natural products discovery had also been explored in the last few years. Using a functional approach, Fujita et al. were able to clone and express a biosynthetic gene cluster from a metagenomics library obtained from East China Sea sediments (1000 meter below sea surface). They generated a library in *E. coli* of about 60,000 clones with an average insert size of about 35 kbp. This library was then screened for the production of metal-binding compounds, using a chrome azurol metal ion indicator assay. In this way, a clone producing the bioactive siderophore bisucarberin was detected. The analysis of the gene clusters suggested that it was likely derived from an uncultured bacterium [74].

Using a similar approach, two metagenomic libraries were obtained using subsurface sediments collected at the depth of 3006 m in the Indian Ocean. Chen et al. [75] screened libraries for analgesic and cytotoxic activities and then selected clones which were further studied. The authors were then able to identify three indole alkaloids with promising bioactivities [75,76]. The main limitation of this metagenomics approach is related with recombinant expression. Systems based on heterologous production in *E. coli* are often inadequate to express deep-sea derived gene clusters. Thus, developing expression platforms based on piezophile microorganisms could be pivotal to increase the success rate of metagenomics. In this context, some progress has already been made. Indeed, a conjugal transfer and knock-out system have been constructed and validated for the strain *Pseudoalteromonas* SM9913, isolated from deep-sea sediment at 1855 m depth near the Okinawa Trough [77]. Yang et al. [78] have recently set up a low-temperature-inducible protein expression vector (pSW2) based on a filamentous phage (SW1) of the deep-sea bacterium *Shewanella piezotolerans* WP3. This vector can be used to successfully transform bacteria belonging to the genus Shewanella [78].

However, the continuous development of sequencing platforms and bioinformatics tools is making the metagenomic sequence-based approach more straightforward and effective for drug discovery purposes [79,80]. The cost of genomes and metagenomic sample sequencing is constantly decreasing, thus allowing investigations at different levels, including at large-scale. Borchet and coworkers who investigated the microbiome of three different deep-sea sponge species, collected from depths of 760 to 2900 m below sea level, using a 454 pyrosequencing for their secondary metabolomic potential, gave an example of the usefulness of this approach. They specifically targeted domains of the subunit of PKS (polyketide synthase) and NRPS (nonribosomal peptide synthase). Their data suggested the presence of many PKS and NRPS of microbial origins, indicating a huge basin of secondary metabolites [80].

Moreover, thanks to the studies of secondary metabolites biosynthetic pathways, there are many bioinformatics tools that can rapidly predict the presence of specific genes, e.g., PRISM [81], antiSMASH [82], and BAGEL [83,84]. Recently, Jackson et al. [85] sequenced the genomes of thirteen Streptomyces strains, isolated from shallow and deep-sea sponges and performed genome-mining using antiSMASH 3.0. They identified 485 clusters coding for PKS, NRPS, terpenes, and bacteriocins; the majority of which, showed little or no homology to previously reported secondary metabolite biosynthetic clusters.

4. Conclusions

Technologies developed over the last ten years have enabled a focus on microbial communities for the search for novel natural bioactive compounds from deep-sea habitats. Recent reports show that deep-sea microorganism-derived natural compounds may provide a new source for the development of drugs against cancer, infectious diseases, and other human ailments.

The first step for the discovery of novel compounds should be the isolation of novel strains, which can be studied by polyphasic taxonomy. For the initial isolation, low nutrient media can be used to mimic the low nutrient content of deep-sea environments. However, it is quite possible that not all the biosynthetic gene clusters (BGC) will be expressed under laboratory conditions. Therefore, one of the most important research areas is the activation of cryptic gene clusters. Dereplication employing advanced techniques such as MS and NMR [86] improved the speed and reliability of

natural product based drug discovery program. Next-generation DNA sequencing (NGS) [79] and advanced bioinformatics tools, including AntiSMASH, BAGEL, SBSPKS, and SMURF, allow the discovery and analysis of BGCs quickly. This approach, combined with new heterologous expression and pathway engineering methods, would pave a new way for the production of novel metabolites and eventually drug leads. Given the ongoing interest in deep-sea-derived natural products, it is also important to study the mechanism of actions of novel compounds discovered. Identification and manipulation of deep-sea gene clusters will make it possible for combinatorial biosynthesis to expand structural diversity.

Increased interests in natural product drug discovery over the last decade can be accelerated via academic and biotechnology industry collaboration. Commercialization of deep-sea-derived natural products and derivatives is in its infancy but some are approaching clinical trials. Therefore, it is imperative to catalogue deep-sea habitat microorganisms for the development of new drug leads for future drugs to treat human diseases.

Author Contributions: E.T., F.P.E., P.T., G.G.J., R.F., and M.J.: Writing-Review & Editing the review article; D.d.P.: Final supervision.

Funding: This research was funded by the MarPipe project: Improving the flow in the pipeline of the next generation of marine biodiscovery scientists, funded through the European Commission H2020-MSCA-ITN-ETN scheme, GA721421.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Corinaldesi, C. New perspectives in benthic deep-sea microbial ecology. *Front. Mar. Sci.* **2015**, *2*, 1–12. [CrossRef]
2. Thistle, D. The deep-sea floor: An overview. In *Ecosystems of the Deep Oceans*; Tyler, A., Ed.; Elsevier Science: Amsterdam, The Netherlands, 2003; Volume 28, pp. 6–8.
3. Danovaro, R.; Carugati, L.; Berzano, M.; Cahill, A.E.; Carvalho, S.; Chenuil, A. Implementing and Innovating Marine Monitoring Approaches for Assessing Marine Environmental Status. *Front. Mar. Sci.* **2016**, *3*. [CrossRef]
4. Schrenk, M.O.; Kelley, D.S.; Delaney, J.R.; Baross, J.A. Incidence and diversity of microorganisms within the walls of an active deep-sea sulfide chimney. *Appl. Environ. Microbiol.* **2003**, *69*, 3580–3592. [CrossRef] [PubMed]
5. Skropeta, D. Deep-sea natural products. *Nat. Prod. Rep.* **2008**, *25*, 1131. [CrossRef] [PubMed]
6. Thaler, A.D.; Van Dover, C.L.; Vilgalys, R. Ascomycete phylotypes recovered from a Gulf of Mexico methane seep are identical to an uncultured deep-sea fungal clade from the Pacific. *Fungal Ecol.* **2012**, *5*, 270–273. [CrossRef]
7. Jebbar, M.; Franzetti, B.; Girard, E.; Oger, P. Microbial diversity and adaptation to high hydrostatic pressure in deep-sea hydrothermal vents prokaryotes. *Extremophiles* **2015**, *19*, 721–740. [CrossRef] [PubMed]
8. Skropeta, D.; Wei, L. Recent advances in deep-sea natural products. *Nat. Prod. Rep.* **2014**, *31*, 999–1025. [CrossRef] [PubMed]
9. Ventola, C.L. The Antibiotic Resistance Crisis. *Pharm. Ther.* **2015**, *40*, 277–283.
10. Goodfellow, M.; Fiedler, H.P. A guide to successful bioprospecting: Informed by actinobacterial systematics. *Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **2010**, *98*, 119–142. [CrossRef] [PubMed]
11. Abdel-Mageed, W.M.; Milne, B.F.; Wagner, M.; Schumacher, M.; Sandor, P.; Pathom-aree, W.; Goodfellow, M.; Bull, A.T.; Horikoshi, K.; Ebel, R.; et al. Dermacozines, a new phenazine family from deep-sea dermacocci isolated from a Mariana Trench sediment. *Org. Biomol. Chem.* **2010**, *8*, 2352–2362. [CrossRef] [PubMed]
12. Huang, H.; Yang, T.; Ren, X.; Liu, J.; Song, Y.; Sun, A.; Ma, J.; Wang, B.; Zhang, Y.; Huang, C.; et al. Cytotoxic angucycline class glycosides from the deep sea actinomycete *Streptomyces lusitanus* SCSIO LR32. *J. Nat. Prod.* **2012**, *75*, 202–208. [CrossRef] [PubMed]
13. Li, S.; Tian, X.; Niu, S.; Zhang, W.; Chen, Y.; Zhang, H.; Yang, X.; Zhang, W.; Li, W.; Ju, J.; et al. Pseudonocardians A-C, new diazaanthraquinone derivatives from a deap-sea actinomycete *Pseudonocardia* sp. SCSIO 01299. *Mar. Drugs.* **2011**, *9*, 1428–1439. [CrossRef] [PubMed]

14. Sato, S.; Iwata, F.; Yamada, S.; Kawahara, H.; Katayama, M. Usabamycins A-C: New anthramycin-type analogues from a marine-derived actinomycete. *Bioorganic Med. Chem. Lett.* **2011**, *21*, 7099–7101. [CrossRef] [PubMed]
15. Zhang, W.; Liu, Z.; Li, S.; Yang, T.; Zhang, Q.; Ma, L.; Tian, X.; Zhang, H.; Huang, C.; Zhang, S.; et al. Spiroindimicins A-D: New bisindole alkaloids from a deep-sea-derived actinomycete. *Org. Lett.* **2012**, *14*, 3364–3367. [CrossRef] [PubMed]
16. Bérdy, J. Bioactive Microbial Metabolites. *J. Antibiot. (Tokyo)* **2005**, *58*, 1–26. [CrossRef] [PubMed]
17. Tian, X.P.; Tang, S.K.; Dong, J.D.; Zhang, Y.Q.; Xu, L.H.; Zhang, S.; Li, W.J. *Marinactinospora thermotolerans* gen. nov., sp. nov., a marine actinomycete isolated from a sediment in the northern South China Sea. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 948–952. [CrossRef] [PubMed]
18. Zhou, X.; Huang, H.; Chen, Y.; Tan, J.; Song, Y.; Zou, J.; Tian, X.; Hua, Y.; Ju, J. Marthiapeptide A, an anti-infective and cytotoxic polythiazole cyclopeptide from a 60 L scale fermentation of the deep sea-derived Marinactinospora thermotolerans SCSIO 00652. *J. Nat. Prod.* **2012**, *75*, 2251–2255. [CrossRef] [PubMed]
19. Song, Y.; Li, Q.; Liu, X.; Chen, Y.; Zhang, Y.; Sun, A.; Zhang, W.; Zhang, J.; Ju, J. Cyclic hexapeptides from the deep South China sea-derived Streptomyces scopuliridis SCSIO ZJ46 active against pathogenic gram-positive bacteria. *J. Nat. Prod.* **2014**, *77*, 1937–1941. [CrossRef] [PubMed]
20. Zhou, X.; Huang, H.; Li, J.; Song, Y.; Jiang, R.; Liu, J.; Zhang, S.; Hua, Y.; Ju, J. New anti-infective cycloheptadepsipeptide congeners and absolute stereochemistry from the deep sea-derived Streptomyces drozdowiczii SCSIO 10141. *Tetrahedron* **2014**, *70*. [CrossRef]
21. Ballard, C.E.; Yu, H.; Wang, B. Recent Developments in Depsipeptide Research. *Curr. Med. Chem.* **2002**, *9*, 471–498. [CrossRef] [PubMed]
22. Jimeno, J.; Faircloth, G.; Sousa-Faro, J.F.; Scheuer, P.; Rinehart, K. New Marine Derived Anticancer Therapeutics—A Journey from the Sea to Clinical Trials. *Mar. Drugs* **2004**, *2*, 14–29. [CrossRef]
23. Lee, Y.; Phat, C.; Hong, S.C. Structural diversity of marine cyclic peptides and their molecular mechanisms for anticancer, antibacterial, antifungal, and other clinical applications. *Peptides* **2017**, *95*, 94–105. [CrossRef] [PubMed]
24. March, D.R.; Abbenante, G.; Bergman, D.A.; Brinkworth, R.I.; Wickramasinghe, W.; Begun, J.; Martin, J.L.; Fairlie, D.P. Substrate-based cyclic peptidomimetics of Phe-Ile-Val that inhibit HIV-1 protease using a novel enzyme-binding mode. *J. Am. Chem. Soc.* **1996**, *118*, 3375–3379. [CrossRef]
25. Ariawan, A.D.; Webb, J.E.; Howe, E.N.; Gale, P.A.; Thordarson, P.; Hunter, L. Cyclic peptide unguisin A is an anion receptor with high affinity for phosphate and pyrophosphate. *Org. Biomol. Chem.* **2017**, *15*, 2962–2967. [CrossRef] [PubMed]
26. Rezai, T.; Yu, B.; Millhauser, G.L.; Jacobson, M.P.; Lokey, R.S. Testing the conformational hypothesis of passive membrane permeability using synthetic cyclic peptide diastereomers. *J. Am. Chem. Soc.* **2006**, *128*, 2510–2511. [CrossRef] [PubMed]
27. Batiste, S.M.; Johnston, J.N. Evidence for Ion-Templation During Macrocyclooligomerization of Depsipeptides. *J. Am. Chem. Soc.* **2018**, *140*, 4560–4568. [CrossRef] [PubMed]
28. Ahlbach, C.L.; Lexa, K.W.; Bockus, A.T.; Chen, V.; Crews, P.; Jacobson, M.P.; Lokey, R.S. Beyond cyclosporine A: Conformation-dependent passive membrane permeabilities of cyclic peptide natural products. *Future Med. Chem.* **2015**, *7*, 2121–2130. [CrossRef] [PubMed]
29. Zorzi, A.; Deyle, K.; Heinis, C. Cyclic peptide therapeutics: Past, present and future. *Curr. Opin. Chem. Biol.* **2017**, *38*, 24–29. [CrossRef] [PubMed]
30. Jelokhani-Niaraki, M.; Hodges, R.S.; Meissner, J.E.; Hassenstein, U.E.; Wheaton, L. Interaction of gramicidin S and its aromatic amino-acid analog with phospholipid membranes. *Biophys. J.* **2008**, *95*, 3306–3321. [CrossRef] [PubMed]
31. Jelokhani-Niaraki, M.; Prenner, E.J.; Kay, C.M.; McElhaney, R.N.; Hodges, R.S. Conformation and interaction of the cyclic cationic antimicrobial peptides in lipid bilayers. *J. Pept. Res.* **2002**, *60*, 23–36. [CrossRef] [PubMed]
32. Jiang, Z.D.; Jensen, P.R.; Fenical, W. Lobophorins A and B, new antiinflammatory macrolides produced by a tropical marine bacterium. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2003–2006. [CrossRef]

33. Niu, S.; Li, S.; Chen, Y.; Tian, X.; Zhang, H.; Zhang, G.; Zhang, W.; Yang, X.; Zhang, S.; Ju, J.; et al. Lobophorins e and F, new spirotetronate antibiotics from a South China Sea-derived Streptomyces sp. SCSIO 01127. *J. Antibiot. (Tokyo)* **2011**, *64*, 711–716. [CrossRef] [PubMed]
34. Pan, H.Q.; Zhang, S.Y.; Wang, N.; Li, Z.L.; Hua, H.M.; Hu, J.C.; Wang, S.J. New spirotetronate antibiotics, lobophorins H and I, from a South China Sea-derived streptomyces sp. 12A35. *Mar. Drugs* **2013**, *11*, 3891–3901. [CrossRef] [PubMed]
35. Lacoske, M.H.; Theodorakis, E.A. Spirotetronate polyketides as leads in drug discovery. *J. Nat. Prod.* **2015**, *78*, 562–575. [CrossRef] [PubMed]
36. Ramos, J.L.; Martinez-Bueno, M.; Molina-Henares, A.J.; Teran, W.; Watanabe, K.; Zhang, X.; Gallegos, M.T.; Brennan, R.; Tobes, R. The TetR Family of Transcriptional Repressors. *Microbiol. Mol. Biol. Rev.* **2005**, *69*, 326–356. [CrossRef] [PubMed]
37. Bister, B.; Bischoff, D.; Ströbele, M.; Riedlinger, J.; Reicke, A.; Wolter, F.; Bull, A.T.; Zähner, H.; Fiedler, H.P.; Süssmuth, R.D. Abyssomicin C—A polycyclic antibiotic from a marine Verrucosispora strain as an inhibitor of the p-aminobenzoic acid/tetrahydrofolate biosynthesis pathway. *Angew. Chem. Int. Ed.* **2004**, *43*, 2574–2576. [CrossRef] [PubMed]
38. Song, Y.; Huang, H.; Chen, Y.; Ding, J.; Zhang, Y.; Sun, A.; Zhang, W.; Ju, J. Cytotoxic and antibacterial marfuraquinocins from the deep south china sea-derived streptomyces niveus scsio 3406. *J. Nat. Prod.* **2013**, *76*, 2263–2268. [CrossRef] [PubMed]
39. Hohmann, C.; Schneider, K.; Bruntner, C.; Irran, E.; Nicholson, G.; Bull, A.T.; Jones, A.L.; Brown, R.; Stach, J.E.; Goodfellow, M.; et al. Caboxamycin, a new antibiotic of the benzoxazole family produced by the deep-sea strain Streptomyces sp. NTK 937. *J. Antibiot. (Tokyo)* **2009**, *62*, 99–104. [CrossRef] [PubMed]
40. Mahé, S.; Rédou, V.; Calvez, T.L.; Vandenkoornhuyse, P.; Burgaud, G. Fungi in Deep-Sea Environments and Metagenomics. In *The Ecological Genomics of Fungi*; Martin, F., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2013; pp. 325–354.
41. Bhatnagar, I.; Kim, S.K. Immense Essence of Excellence: Marine Microbial Bioactive Compounds. Immense essence of excellence: Marine microbial bioactive compounds. *Mar. Drugs* **2010**, *8*, 2673–2701. [CrossRef] [PubMed]
42. Okutani, K. Gliotoxin produced by a strain of *Aspergillus* isolated from marine mud. *Bull. Jpn. Soc. Sci. Fish.* **1977**, *43*, 995–1000. [CrossRef]
43. Blunt, J.; Copp, B.; Keyzers, R.; Munro, M.; Prinsep, M.R. Marine natural products. *Nat. Prod. Rep.* **2014**, *31*, 160–258. [CrossRef] [PubMed]
44. Zhang, X.Y.; Zhang, Y.; Xu, X.Y.; Qi, S.H. Diverse Deep-Sea Fungi from the South China Sea and Their Antimicrobial Activity. *Curr. Microbiol.* **2013**, *67*, 1–6. [CrossRef] [PubMed]
45. Pinto, M.M.M.; Sousa, M.E.; Nascimento, M.S.J. Xanthone derivatives: New insights in biological activities. *Curr. Med. Chem.* **2005**, *12*, 2517–2538. [CrossRef] [PubMed]
46. Pinto, M.M.M.; Castanheiro, R.A.P. Synthesis of Prenylated Xanthones: An Overview. *Curr. Org. Chem.* **2009**, *13*, 1215–1240. [CrossRef]
47. Fredimoses, M.; Zhou, X.; Lin, X.; Tian, X.; Ai, W.; Wang, J.; Liao, S.; Liu, J.; Yang, B.; Yang, X.; et al. New prenylxanthones from the deep-sea derived fungus *Emericella* sp. SCSIO 05240. *Mar. Drugs* **2014**, *12*, 3190–3202. [CrossRef] [PubMed]
48. Yao, Q.; Wang, J.; Zhang, X.; Nong, X.; Xu, X.; Qi, S. Cytotoxic polyketides from the deep-sea-derived fungus *Engyodontium album* DFFSCS021. *Mar. Drugs* **2014**, *12*, 5902–5915. [CrossRef] [PubMed]
49. Huang, K.J.; Lin, S.H.; Lin, M.R.; Ku, H.; Szkaradek, N.; Marona, H.; Hsu, A.; Shiuan, D. Xanthone derivatives could be potential antibiotics: Virtual screening for the inhibitors of enzyme i of bacterial phosphoenolpyruvate-dependent phosphotransferase system. *J. Antibiot. (Tokyo)* **2013**, *66*, 453–458. [CrossRef] [PubMed]
50. Niu, S.; Liu, D.; Hu, X.; Proksch, P.; Shao, Z.; Lin, W. Spiromastixones A-O, antibacterial chlorodepsidones from a deep-sea-derived spiromastix sp. fungus. *J. Nat. Prod.* **2014**, *77*, 1021–1030. [CrossRef] [PubMed]
51. Wang, W.; Liao, Y.; Chen, R.; Hou, Y.; Ke, W.; Zhang, B.; Gao, M.; Shao, Z.; Chen, J.; Li, F. Chlorinated Azaphilone Pigments with Antimicrobial and Cytotoxic Activities Isolated from the Deep Sea Derived Fungus *Chaetomium* sp. NA-S01-R1. *Mar. Drugs* **2018**, *16*, 61. [CrossRef] [PubMed]
52. Rateb, M.E.; Ebel, R. Secondary metabolites of fungi from marine habitats. *Nat Prod Rep.* **2011**, *28*, 290–344. [CrossRef] [PubMed]

53. Guo, W.; Zhang, Z.; Zhu, T.; Gu, Q.; Li, D. Penicyclones A-E, Antibacterial Polyketides from the Deep-Sea-Derived Fungus *Penicillium* sp. F23-2. *J. Nat. Prod.* **2015**, *78*, 2699–2703. [CrossRef] [PubMed]
54. Du, L.; Li, D.; Zhu, T.; Cai, S.; Wang, F.; Xiao, X.; Gu, G. New alkaloids and diterpenes from a deep ocean sediment derived fungus *Penicillium* sp. *Tetrahedron* **2009**, *65*, 1033–1039. [CrossRef]
55. Egan, S.T.; McCarthy, D.M.; Patching, J.W.; Fleming, G.T.A. An investigation of the physiology and potential role of components of the deep ocean bacterial community (of the NE Atlantic) by enrichments carried out under minimal environmental change. *Deep. Res. Part I Oceanogr. Res. Pap.* **2012**, *61*, 11–20. [CrossRef]
56. McCarthy, D.M.; Pearce, D.; Patching, J.W.; Fleming, G.T. Contrasting responses to nutrient enrichment of prokaryotic communities collected from deep sea sites in the southern ocean. *Biology* **2013**, *2*, 1165–1188. [CrossRef] [PubMed]
57. Nogi, Y.; Kato, C. Taxonomic studies of extremely barophilic bacteria isolated from the Mariana Trench and description of *Moritella yayanosii* sp. nov., a new barophilic bacterial isolate. *Extremophiles* **1999**, *3*, 71–77. [CrossRef] [PubMed]
58. Yanagibayashi, M.; Nogi, Y.; Li, L.; Kato, C. Changes in the microbial community in Japan Trench sediment from a depth of 6292 m during cultivation without decompression. *FEMS Microbiol. Lett.* **1999**, *170*, 271–279. [CrossRef] [PubMed]
59. Hohnber, H.-J.; Amann, H.; Abegg, F.; Mudrack, H.; Bohrmann, G.; Maggiulli, M. Pressurized Coring of Near-Surface Gas-Hydrate Sediments on Hydrate Ridge: The Multiple Autoclave Corer, and First Results from Pressure-Core X-ray CT Scans. In Proceedings of the EGS—AGU—EUG Joint Assembly, Nice, France, 6–11 April 2003.
60. Parkes, R.J.; Sellek, G.; Webster, G.; Martin, D.; Anders, E.; Weightman, A.J.; Sass, H. Culturable prokaryotic diversity of deep, gas hydrate sediments: First use of a continuous high-pressure, anaerobic, enrichment and isolation system for subseafloor sediments (DeepIsoBUG). *Environ. Microbiol.* **2009**, *11*, 3140–3153. [CrossRef] [PubMed]
61. Galloway, K.C.; Becker, K.P.; Phillips, B.; Kirby, J.; Licht, S.; Tchernov, D.; Wood, R.J.; Gruber, D.F. Soft Robotic Grippers for Biological Sampling on Deep Reefs. *Soft Robot.* **2016**, *3*, 23–33. [CrossRef] [PubMed]
62. Zobell, C.E.; Morita, R.Y. Barophilic bacteria in some deep sea sediments. *J. Bacteriol.* **1957**, *73*, 563–568. [PubMed]
63. Yayanos, A.A.; Dietz, A.S.; Van Boxtel, R. Isolation of a deep-sea barophilic bacterium and some of its growth characteristics. *Science* **1979**, *205*, 808–810. [CrossRef] [PubMed]
64. Sogin, M.L.; Morrison, H.G.; Huber, J.A.; Welch, D.M.; Huse, S.M.; Neal, P.R.; Arrieta, J.M.; Herndl, G.J. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 12115–12120. [CrossRef] [PubMed]
65. Yilmaz, P.; Yarza, P.; Rapp, J.Z.; Glöckner, F.O. Expanding the world of marine bacterial and archaeal clades. *Front. Microbiol.* **2016**, *6*, 1–29. [CrossRef] [PubMed]
66. Eloe, E.A.; Malfatti, F.; Gutierrez, J.; Hardy, K.; Schmidt, W.E.; Pogliano, K.; Azam, F.; Barlett, D.H. Isolation and characterization of a psychropiezophilic alphaproteobacterium. *Appl. Environ. Microbiol.* **2011**, *77*, 8145–8153. [CrossRef] [PubMed]
67. Takai, K.; Miyazaki, M.; Hirayama, H.; Nakagawa, S.; Querellou, J.; Godfroy, A. Isolation and physiological characterization of two novel, piezophilic, thermophilic chemolithoautotrophs from a deep-sea hydrothermal vent chimney. *Environ. Microbiol.* **2009**, *11*, 1983–1997. [CrossRef] [PubMed]
68. Takai, K.; Nakamura, K.; Toki, T.; Tsunogai, U.; Miyazaki, M.; Miyazaki, J.; Hirayama, H.; Nakagawa, S.; Nunoura, T.; Horikoshi, K. Cell proliferation at 122 °C and isotopically heavy CH_4 production by a hyperthermophilic methanogen under high-pressure cultivation. *Proc. Natl. Acad. Sci.* **2008**, *105*, 10949–10954. [CrossRef] [PubMed]
69. Aoki, M.; Ehara, M.; Saito, Y.; Yoshioka, H.; Miyazaki, M.; Saito, Y.; Miyashita, A.; Kawakami, S.; Yamaguchi, T.; Ohashi, A.; et al. A long-term cultivation of an anaerobic methane-oxidizing microbial community from deep-sea methane-seep sediment using a continuous-flow bioreactor. *PLoS ONE* **2014**, *9*, 1–14. [CrossRef] [PubMed]
70. Landreau, M.; Duthoit, F.; Roussel, E.; Schönherr, S.; Georges, M.; Godfroy, A.; Le Blay, G. Cultivation of an immobilized (hyper)thermophilic marine microbial community in a bioreactor. *FEMS Microbiol. Lett.* **2016**, *363*, 1–7. [CrossRef] [PubMed]

71. Barone, R.; De Santi, C.; Palma Esposito, F.; Tedesco, P.; Galati, F.; Visone, M.; Di Scala, A.; de Pascale, D. Marine metagenomics, a valuable tool for enzymes and bioactive compounds discovery. *Front. Mar. Sci.* **2014**, *1*, 1–6. [CrossRef]
72. Tangherlini, M.; Dell'Anno, A.; Zeigler Allen, L.; Riccioni, G.; Corinaldesi, C. Assessing viral taxonomic composition in benthic marine ecosystems: Reliability and efficiency of different bioinformatic tools for viral metagenomic analyses. *Sci. Rep.* **2016**, *6*, 1–10. [CrossRef] [PubMed]
73. Verma, P.; Raghavan, R.V.; Jeon, C.O.; Lee, H.J.; Priya, P.V.; Dharani, G.; Kirubagaran, R. Complex bacterial communities in the deep-sea sediments of the Bay of Bengal and volcanic Barren Island in the Andaman Sea. *Mar. Genom.* **2017**, *31*, 33–41. [CrossRef] [PubMed]
74. Fujita, M.J.; Kimura, N.; Yokose, H.; Otsuka, M. Heterologous production of bisucaberin using a biosynthetic gene cluster cloned from a deep sea metagenome. *Mol. BioSyst.* **2012**, *8*, 482–485. [CrossRef] [PubMed]
75. Chen, L.; Tang, X.X.; Zheng, M.; Yi, Z.W.; Xiao, X.; Qiu, Y.K.; Wu, Z. A novel indole alkaloid from deep-sea sediment metagenomic clone-derived *Escherichia coli* fermentation broth. *J. Asian Nat. Prod. Res.* **2011**, *13*, 444–448. [CrossRef] [PubMed]
76. Yan, X.; Tang, X.X.; Chen, L.; Yi, Z.W.; Fang, M.J.; Wu, Z.; Qui, Y.K. Two new cytotoxic indole alkaloids from a deep-sea sediment derived metagenomic clone. *Mar. Drugs* **2014**, *12*, 2156–2163. [CrossRef] [PubMed]
77. Yu, Z.C.; Zhao, D.L.; Ran, L.Y.; Mi, Z.H.; Wu, Z.Y.; Pang, X.; Zhang, X.Y.; Su, H.N.; Shi, M.; Song, X.Y.; et al. Development of a genetic system for the deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913. *Microb. Cell Fact.* **2014**, *13*. [CrossRef] [PubMed]
78. Yang, X.W.; Jian, H.H.; Wang, F.P. pSW2, a novel low-temperature-inducible gene expression vector based on a filamentous phage of the deep-sea bacterium *Shewanella piezotolerans* WP3. *Appl. Environ. Microbiol.* **2015**, *81*, 5519–5526. [CrossRef] [PubMed]
79. Gomez-Escribano, J.; Alt, S.; Bibb, M. Next Generation Sequencing of Actinobacteria for the Discovery of Novel Natural Products. *Mar. Drugs* **2016**, *14*, 78. [CrossRef] [PubMed]
80. Borchert, E.; Jackson, S.A.; O'Gara, F.; Dobson, A.D.W. Diversity of natural product biosynthetic genes in the microbiome of the deep sea sponges *Inflatella pellicula*, *Poecillastra compressa*, and *Stelletta normani*. *Front. Microbiol.* **2016**, *7*, 1027. [CrossRef] [PubMed]
81. Skinnider, M.A.; Dejong, C.A.; Rees, P.N.; Johnston, C.W.; Li, H.; Webster, A.L.H.; Wyatt, M.A.; Magarvey, N.A. Genomes to natural products PRediction Informatics for Secondary Metabolomes (PRISM). *Nucleic Acids Res.* **2015**, *43*, 9645–9662. [CrossRef] [PubMed]
82. Weber, T.; Blin, K.; Duddela, S.; Krug, D.; Kim, H.U.; Bruccoleri, R.; Lee, S.Y.; Fischbach, M.A.; Muller, R.; Wohlleben, W.; et al. AntiSMASH 3.0-A comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* **2015**, *43*, W237–W243. [CrossRef] [PubMed]
83. de Jong, A.; van Hijum, S.A.; Bijlsma, J.J.; Kok, J.; Kuipers, O.P. BAGEL: A web-based bacteriocin genome mining tool. *Nucl. Acids. Res.* **2006**, *34*, W273–W279. [CrossRef] [PubMed]
84. van Heel, A.J.; de Jong, A.; Montalban-Lopez, M.; Kok, J.; Kuipers, O.P. BAGEL3: Automated identification of genes encoding bacteriocins and (non-) bactericidal posttranslationally modified peptides. *Nucl. Acids. Res.* **2013**, *41*, W448–W453. [CrossRef] [PubMed]
85. Jackson, S.; Crossman, L.; Almeida, E.; Margassery, L.; Kennedy, J.; Dobson, A. Diverse and Abundant Secondary Metabolism Biosynthetic Gene Clusters in the Genomes of Marine Sponge Derived *Streptomyces* spp. Isolates. *Mar. Drugs* **2018**, *16*, 67. [CrossRef] [PubMed]
86. Wu, C.; Choi, Y.H.; van Wezel, G.P. Metabolic profiling as a tool for prioritizing antimicrobial compounds. *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 299–312. [CrossRef] [PubMed]

Marine Biotechnology (2018) 20:502–511
https://doi.org/10.1007/s10126-018-9821-9

ORIGINAL ARTICLE

Identification of a Sorbicillinoid-Producing *Aspergillus* Strain with Antimicrobial Activity Against *Staphylococcus aureus*: a New Polyextremophilic Marine Fungus from Barents Sea

Paulina Corral[1,2] · Fortunato Palma Esposito[1] · Pietro Tedesco[1] · Angela Falco[1] · Emiliana Tortorella[1] · Luciana Tartaglione[3] · Carmen Festa[3] · Maria Valeria D'Auria[3] · Giorgio Gnavi[4] · Giovanna Cristina Varese[4] · Donatella de Pascale[1]

Received: 18 October 2017 / Accepted: 26 March 2018 / Published online: 12 April 2018

Abstract

The exploration of poorly studied areas of Earth can highly increase the possibility to discover novel bioactive compounds. In this study, the cultivable fraction of fungi and bacteria from Barents Sea sediments has been studied to mine new bioactive molecules with antibacterial activity against a panel of human pathogens. We isolated diverse strains of psychrophilic and halophilic bacteria and fungi from a collection of nine samples from sea sediment. Following a full bioassay-guided approach, we isolated a new promising polyextremophilic marine fungus strain 8Na, identified as *Aspergillus protuberus* MUT 3638, possessing the potential to produce antimicrobial agents. This fungus, isolated from cold seawater, was able to grow in a wide range of salinity, pH and temperatures. The growth conditions were optimised and scaled to fermentation, and its produced extract was subjected to chemical analysis. The active component was identified as bisvertinolone, a member of sorbicillonoid family that was found to display significant activity against *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) of 30 μg/mL.

Keywords Sediments · Marine fungi · *Aspergillus protuberus* · Bisvertinolone · Antimicrobial activity · MDR

Introduction

Antimicrobial resistance has spread dramatically in the last 30 years, leading to an increase in the number of deaths due to infectious diseases. The excessive and often inappropriate use of antimicrobial drugs has affected the development of a new group of microorganisms, the multidrug-resistant (MDR) bacteria, which show resistance towards the most common antibiotics (Muller et al. 2017). Currently, there is a pressing need to discover novel and effective antimicrobial drugs to counteract this dramatic emergence of MDR infections. Nature has always been the main source of new molecular scaffolds; a prime example was the discovery of penicillin. Henceforth, bioprospecting of marine macro- and microorganisms for new natural drug candidates still represents the best opportunity for the discovery of new bioactive compounds. In particular, extreme environments constitute an unexplored reservoir of biodiversity (Poli et al. 2017). The use of different strategies of adaptation by organisms and microorganisms allowed the colonisation of extreme habitats, which are characterised by low nutrient concentration, extremes of pH, low temperature, high pressure and salinity. Some of the most extreme marine habitats known like Mediterranean deep hypersaline anoxic basins (DHABs; water depth ~ 3500 m) are nearly saturated with salt, from these sediments were retrieved different halotolerant/halophilic fungal groups that might have adapted to different local environments

Paulina Corral, Fortunato Palma Esposito and Pietro Tedesco contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10126-018-9821-9) contains supplementary material, which is available to authorized users.

✉ Donatella de Pascale
d.depascale@ibp.cnr.it

[1] National Research Council of Italy (CNR)-Institute of Protein Biochemistry (IBP), Naples, Italy

[2] Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, USA

[3] Department of Pharmacy, University of Naples Federico II, Naples, Italy

[4] Department of Life Science and Systems Biology, University of Torino, Turin, Italy

(Bernhard et al. 2014). Currently, particular attention has been focused on marine fungi (Zhao et al. 2016; Saleem et al. 2007), since they are considered an interesting untapped resource of biodiversity and biotechnological potential for production of secondary metabolites (Ebada and Proksch 2015).

The ability to grow in different ranges of salt concentration is probably one of the most remarkable features of some species of marine fungi that could confer the potential to synthesise new bioactive metabolites (Gostincar et al. 2010). Particularly, under hypersaline conditions, the cell keeps its stability and integrity accumulating compatible solutes in its interior to overcome osmotic stress and to prevent lysis (Delgado-Jarana et al. 2006). This condition induces changes in the membrane composition that effectively increases the sterol to phospholipid ratio, and the fatty acid unsaturation in turn increases the membrane fluidity (Turk et al. 2004). Among all marine fungi, *Penicillium* and *Aspergillus* are a very powerful source of new bioactive compounds (Blunt et al. 2015). The genus *Aspergillus* is widespread all over the marine ecosystem. *Aspergillus* sp. fungi are heterotroph and polyextremophiles. This significant nutritional and physiological versatility is complemented by the metabolic capacity to produce numerous secondary metabolites, which is believed to be important in ecological signalling (Baker and Bennett 2007). Genomic studies are revealing putative biosynthetic genes of *Aspergillus* strains; however, the biocomposites described in the literature are still limited. The prediction of genes exceeds the number of new molecules obtained so far, leaving a large number of compounds yet to be discovered (Rateb and Ebel 2011).

Despite the fact that natural products from some fungal genera have been often and intensively studied, in particular, *Aspergillus* and *Penicillium*, there is still a great potential of secondary metabolites produced by these fungi, which have not as yet been fully and adequately explored.

Barents Sea is part of Arctic Continental Shelf, and it is also the deepest of the Arctic shelf seas. The most distinctive oceanographic feature of the Barents Sea is the influx of the salty Atlantic waters from the southwest that meets and mixes with Arctic water masses along the Polar Front (Loeng 1991). This condition makes the Barents Sea warmer in comparison with other areas of the same latitude, but the oceanographic conditions are highly variable. Barents Sea is a poorly explored environment, and therefore it is an appealing prospect within the biodiscovery pipeline.

Herein, we report the isolation of 14 fungi from marine sediments collected in the Barents Sea and their taxonomic characterisation. Furthermore, we also report the identification of a sorbicillonoid-producing strain of *Aspergillus protuberus* MUT 3638, a species recently described belonging to the *Aspergillus* section *Versicolores* (Jurjevic et al. 2012), and the isolation of bisvertinolone as an active antimicrobial component.

Materials and Methods

Sediment Collection

The strains used in this work were isolated from sub-sea sediments collected from the Barents Sea (Fig. 1), during the Barkut expedition in June 2015, in collaboration with Prof. Bjarne Landfald of the University of Tromsø. Sediments were collected from nine different sampling sites and depths (Table 1) using a multicorer drill device. Several layers were cut and then aseptically placed into 50-mL conical tubes and kept at 4 °C during the shipments and stored at − 80 °C after their arrival to the laboratory.

Culture Media

Specific culture media were designed for the isolation of different groups of psychrophilic and halotolerant/halophilic microorganisms present in extreme marine environments. Each medium was prepared with natural seawater and artificial seawater in a range of six salinities [3, 5, 10, 15, 20 and 25% (*w*/*v*) of total salts] and three different pH conditions. Natural seawater was used to prepare media with a basal salt concentration in order to mimic the marine environment (3–3.5% of total salinity) while a stock solution of artificial seawater SW30 (Subov's salt solution 30% (*w*/*v*)) was used to prepare other media with higher salt concentrations.

All media except LB were adjusted to three different pH values (4.0, 7.0 and 9.0) and sterilised by autoclaving at 121 °C for 15 min. The names and formula of each medium are given in grams per litre, and for solid media, 20 g/L of agar was added.

Artificial seawater (SW30): 234.0 g NaCl, 39.0 g $MgCl_2 \cdot 6H_2O$, 61.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g $^*CaCl_2$; 6.0 g KCl, 0.2 g $NaHCO_3$, 0.7 g NaBr. This medium was prepared with distilled water up to 1000 mL. $^*CaCl_2$ was dissolved separately in 20 mL of distilled water to prevent the formation of insoluble complexes of $CaCO_3$.

The following media were prepared dissolving the compounds in natural seawater. For media with higher salinities, the artificial seawater SW30 diluted in distilled water was used to reach the salt concentration required.

Seawater minimal (SWM): 1.0 g casamino acids, 1.0 g yeast extract, 1.0 g peptone, 1.0 g glucose.

Casein yeast chitin (CYC): 5.0 g casein, 0.5 g yeast extract, 5.0 g chitin, 0.5 g K_2HPO_4, 5.0 g malt extract, 0.1 g sodium pyruvate salt, 500 μL glycerol.

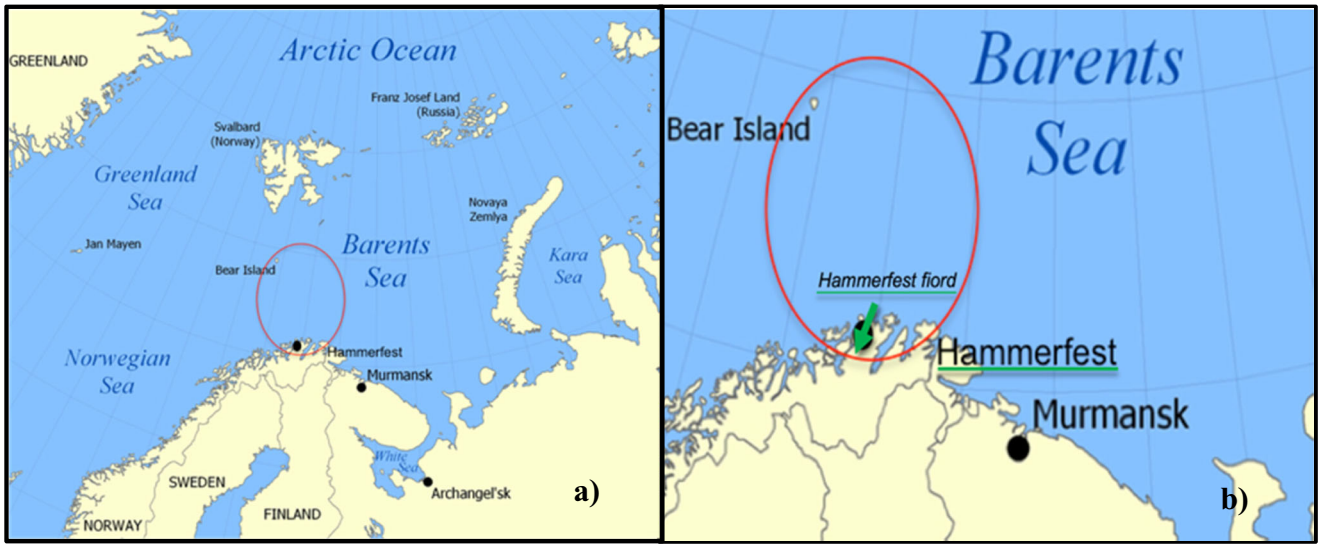


Fig. 1 Geographic localisation of the Barents Sea. **a** The area where sampling was performed is circled. **b** The city and the fiord of Hammerfest and final leg of the mission are highlighted.

Tryptone yeast starch (TYS): 10.0 g tryptone, 5.0 g yeast extract, 0.5 g starch, 10.0 g NaCl, 0.5 g malt extract, 0.1 g sucrose, 500 μL glycerol.
Malt yeast extract (MY): 10.0 g malt extract, 4.0 g yeast extract, 4.0 g glucose.
Luria Bertani (LB): 10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl, pH 7 (20.0 g agar for solid media). This medium was prepared with distilled water up to 1000 mL.

Isolation of Microorganisms

Standard protocols for air quality controls were performed to discriminate contaminations. The bacterial and mould tests consisted of leaving opened for 1 h a Petri dish with LB agar for bacteria and Sabouraud agar for moulds, then closed and room temperature incubated for 7 to 14 days. Besides bacteria and mould testing, controls were always included with all media used in this work and incubated with the rest of samples at the temperature and time programmed.

Further of standard disinfection, all microbiological procedures: culture, isolation and screenings were rigorously performed under a biosafety cabinet class II with a laminar flow to reduce contamination by airborne spores.

For the isolation of microorganisms, two different growth media were used: SWM for bacteria and CYC for actinobacteria and fungi. Both media with increasing salt concentrations ranging from 3% to 25% (*w*/*v*) for the isolation of halotolerant and moderate to halophilic microorganisms.

In order to isolate microorganisms, 1.0 g of sediment was mixed with 9 mL of filtered and sterilised natural seawater (NSW) in a 15-mL conical tube and gently mixed. The supernatant was then serially diluted tenfold until 10^{-6} dilution (i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}), and 100 μL of the serial dilution was spread plated on sterile SWM and CYC Petri dishes.

The inoculated plates and controls were incubated for 5 to 20 days at two different temperatures, 4 and 15 °C. Then, several colonies were picked using as criteria the morphology, size and pigments and were then incubated for 5–6 days at 20 °C with agitation in liquid medium SWM and CYC, respectively. All the microorganisms isolated were suspended in glycerol (10% (*v*/*v*) for fungal strains, and 20% for the bacterial strains, of the final volume) and stored at − 80 °C in cryogenic vials.

Table 1 Coordinates and depth of several sampling sites from Barents Sea

Sampling location	Latitude and longitude	Depth (m)
Bjørnøyenna	72° 20′ 07″ N; 18° 09′ 50″ E	364
Salina	72° 00′ 97″ N; 20° 03′ 46″ E	345
Well 7119	71° 12′ 87″ N; 20° 03′ 46″ E	204
Well 7120-1	71° 07′ 65″ N; 20° 48′ 97″ E	163
Well 7120-2	71° 12′ 87″ N; 19° 56′ 59″ E	165
Bønna	70° 50′ 05″ N; 16° 33′ 20″ E	1365
Goliat	71° 17′ 09″ N; 22° 19′ 38″ E	380
Repparfjord	70° 27′ 59″ N; 24° 17′ 17″ E	47
HammerFest	70° 39′ 69″ N; 23° 40′ 12″ E	46

Identification of Fungal Strains

The isolated fungi were identified with a polyphasic approach, which couples morpho-physiological features with molecular studies. After determination of the genera according to the macroscopic and microscopic features (Domsch et al. 1980),

the fungal strains were transferred to the media recommended by the authors of a selected genus monographs for species identification. For molecular identification, genomic DNA of all strains was extracted from approximately 100 mg of mycelium, scraped from MY Petri dishes, using a NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to the manufacturer instructions. The quality and quantity of DNA samples were measured with the ND-1000 Spectrophotometer NanoDropH (Thermo Scientific, Wilmington, Germany). DNA extracts were stored at − 20 °C. The ITS sequences were amplified using the primer pair ITS1/ITS4 (White et al. 1990). For those strains morphologically identified as *Aspergillus* spp. and *Penicillium* spp., amplification of the β-tubulin gene was performed using primers Bt2a/Bt2b (Glass and Donaldson 1995; Geiser et al. 1998). Reaction mixtures consisted of 30 ng genomic DNA, 1 μM each primer, 1 U Taq DNA Polymerase (Qiagen, Chatsworth, CA, USA), 1× reaction buffer and 200 μM of dNTPs. DNA amplifications were performed using a T-Gradient thermal cycler (Biometra, Göttingen, Germany) with the following profile: 95 °C for 5 min; 35 cycles: 95 °C for 40 s, 55 °C (58 °C for Bt2a/Bt2b) for 45 s, 72 °C for 50 s; 72 °C for 8 min. PCR products were purified and sequenced at Macrogen Europe Laboratory (Amsterdam, The Netherlands).

All strains were taxonomically identified at the species level. The partial sequences obtained were verified and analysed by querying with the Blastn algorithm (default setting; mismatch 1/−2; gap cost linear) hosted at NCBI, the specific sequences against the GenBank (http://www.ncbi.nlm.nih.gov). Pairwise alignments were also performed using the sequence database of the Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl/).

Finally, dereplication of the isolates belonging to the same species was performed applying the microsatellite screening by using the core sequence of the microsatellite M-13 as a primer (Abd-Elsalam et al. 2010). Amplicons were separated on 1.5% (*w*/*v*) agarose gels stained with 5 μL 100 mL^{-1} ethidium bromide and electrophoresed alongside a GelPilot 1 kb plus DNA Ladder; images were acquired with a Gel Doc 1000 System (Bio-Rad, Hercules, CA, USA), and fingerprints were analysed using Bionumerics 7.1 software.

Pathogenic Strains

The following human pathogens were used in this work: *Pseudomonas aeruginosa* PA01 (Alonso et al. 1999), *Staphylococcus aureus* 6538P (Lima et al. 2014), *Klebsiella pneumoniae* DF12SA (Shahi et al. 2013), *Acinetobacter baumannii* Ab13 (Poirel et al. 2008), *Burkholderia cenocepacia* LMG 16656 and *Burkholderia metallica* LMG 24068 (Soriano et al. 2009). The strains were routinely grown at 37 °C in LB medium.

Primary Screening for Antagonistic Activity

Preliminary screening for antimicrobial activity was performed by the cross-streaking method against a panel of human pathogens ("Pathogenic Strains"). This method allows the observation of antagonistic interactions among a tester strain and several target strains. To overcome the limitation of the assay that the pathogen and the tested strain 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 marine bacterial or fungal isolates (SWM or TYS), and the other half of the plate was filled with LB medium, suitable for the growing of pathogenic bacteria. The tester strains were selected on the basis of different morphologies, pigments and shapes and were streaked onto one side of the plate with their appropriate medium and then incubated at 20 °C for 10 days. Thereafter, the pathogenic strains were streaked perpendicularly to the initial streak and incubated at 37 °C for 24 h. A control plate using the pathogens was also maintained without inoculating the marine fungal strains, to assess the normal growth of pathogenic bacteria.

Preparation of Crude Extracts

A pre-inoculum was prepared from the active fungal strains. A small piece of mycelium was inoculated in 3 mL of liquid medium TYS and incubated for 5 days at 20 °C. This pre-inoculum was used to inoculate 250 mL of the same medium in 500-mL flasks and incubated at different temperatures and days in a static condition. Thereafter, exhausted culture broths were centrifuged at 7000 rpm for 30 min at 4 °C. For the extracellular extraction, the supernatant was mixed and vigorously shaken five times with three volumes of ethyl acetate using a separator funnel. This mixing facilitated the transfer of a dissolved compound from one solvent layer to another. Finally, the solvent was dried using a Laborota 4000 Rotary Evaporator (Heidolph, Schwabach, Germany). The extract was weighed and dissolved in DMSO (100%) at a final concentration of 100 mg/mL.

Liquid Inhibition Assay

The produced crude extracts were checked for their ability to inhibit the growth of a selected panel of human pathogens, using a liquid inhibition assay in 96-well plates. The different extracts were placed into each well at an initial concentration of 2 mg/mL and were serially diluted twofold using the appropriate medium. Wells that contained no compounds represented the negative control. DMSO (2% (*v*/*v*)) was also used as control to determine the effect of the solvent on bacterial growth. A single colony of each pathogenic strain was used to inoculate 3 mL of LB broth in sterile bacteriological tube. After 5–8 h of incubation, the growth was measured by

monitoring the absorbance at 600 nm. The cells were then diluted using OD/CFU correlation growth curves, and about 40,000 CFU were dispensed into each well containing the dilutions of the compound. Wells not containing any compounds were used as negative control. Plates were incubated at 37 °C overnight, and growth was measured using a VICTOR X Multilabel Plate Reader (PerkinElmer, Waltham, MA) by monitoring the absorbance at 600 nm.

Purification of Active Compounds from the Newly Isolated *A. protuberus* MUT 3638

The crude extract obtained as described ("Compound Identification") 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. The fractions obtained were subsequently screened again to identify the activity. The active fraction was further purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column. HPLC separations were carried out using a 5-μm Nucleodur reversed-phase HTec (C18, 250/10 mm, L × i.d.) column connected to an UltiMate 3000 series pump with an UltiMate photodiode array detector and in-line degasser (Dionex, Sunnyvale, CA). Detection was carried out at 220, 254, 280 and 320 nm. Elution was carried out at 2.00 mL/min with a 46-min linear gradient of acetonitrile:water:trifluoroacetic acid. The peaks were manually collected, dried and tested for bioactivity.

Liquid Chromatography High-Resolution Mass Spectrometry

The crude extract from the fungus MUT 3638 was subjected to preliminary chemical profiling.

The analyses were carried out on a Dionex Ultimate 3000 quaternary system coupled to a hybrid linear ion trap LTQ Orbitrap XLTM Fourier Transform MS (FTMS) equipped with an ESI ION MAXTM source (Thermo-Fisher, San José, CA, USA). Chromatographic separation was accomplished by using a Kinetex C18 column, 2.6 μm, 2.10 × 100 mm (Phenomenex, USA) maintained at room temperature and eluted at 0.2 mL min^{-1} with water (eluent A) and acetonitrile (eluent B), both containing 0.1% (*v*/*v*) formic acid. The following gradient elution was used: 20% B at $t = 0$, 95% B at $t = 30$ min and hold for 5 min; re-equilibration time was 9 min. Injection volume was 5 μL.

Nuclear Magnetic Resonance Method

Spectral data for bisvertinolone ^{1}H NMR spectrum of bisvertinolone was obtained on Varian Inova 500 NMR and recorded in $CDCl_3$ ($\delta_H = 7.26$ and $\delta_C = 77.0$ ppm) and DMSO ($\delta_H = 3.50$). High-resolution full MS experiments (positive ions) were acquired in the m/z 100–1000 range at a resolving power of 30,000. The following source settings were used: spray voltage = 4.5 kV, capillary temperature = 400 °C, capillary voltage = 29 V, sheath gas flow = 34 and auxiliary gas flow = 0 (arbitrary units) and tube lens voltage = 115 V. Thermo Scientific software Xcalibur has been used to obtain molecular formula. The calibration procedure was carried out using Thermo Scientific positive calibration solution composed by caffeine, Met-Arg-Phe-Ala (MRFA) and Ultramark.

Bisvertonolone Light yellow amorphous powder; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.58 (1H, dd, $J = 14.8$, 9.7 Hz, H-12), 7.42 (1H, d, $J = 14.8$ Hz, H-11), 7.32 (1H, dd, $J = 14.6$, 11.6 Hz, H-21), 6.40 (1H, d, $J = 14.6$ Hz, H-20), 6.37 (3H, ovl, H-13, H-14 and H-22), 6.14 (1H, m, H-23), 3.75 (1H, s, H-9a), 1.93 (3H, d, $J = 4.8$ Hz, H_3–15), 1.89 (3H, d, $J = 6.5$ Hz, H_3–24), 1.49 (3H, s, H_3-C7), 1.47 (3H, s, H_3-C4), 1.45 (3H, s, H_3-C5a), 1.38 (3H, s, H_3-C9b) (see Fig. S4 supplemental material). HRESIMS: calculated for $C_{28}H_{33}O_9$ 513.2119, found 513.2124 $[M + H]^+$ (see Fig. S3 supplemental material). HRESI MSMS (major fragmentation peaks) 495.2012 ($C_{28}H_{31}O_8$), 477.1905 ($C_{28}H_{29}O_7$), 265.1070 ($C_{14}H_{17}O_5$), 249.1121 ($C_{14}H_{17}O_4$), 207.0651 ($C_{11}H_{11}O_4$), 95.0489 (C_6H_7O) (see Fig. S5 supplemental material).

Results and Discussion

Isolation of Marine Microorganisms

From a total of 9 sea sediment samples, 85 microorganisms were isolated; specifically, 75 of them were identified as bacteria and the remaining 14 were identified as fungi. No bacteria or Achaea were isolated using media from 15 to 25% (*w*/*v*) of total salts, while the total population of fungi was isolated by diluting the sediments in growth media at varying salt concentrations.

Identification of Fungal Strains

All the isolated fungi were identified using a polyphasic approach, according to their macroscopic and microscopic features, as well as by molecular analysis. The 14 isolated strains belong to the Ascomycota phylum: *Aspergillus protuberus* (seven strains), *Penicillium rubens* (three strains), *Penicillium chrysogenum* (two strains), *Aspergillus sydowii* and *Microascus trigonosporus* (one strain). Sequences related to the fungi isolated in this study were deposited at the NCBI database. GenBank accession numbers are reported in Table 2. The dereplication analysis showed that only two strains were isogenic within the same species (see Fig. S1, Supplementary materials), demonstrating that the isolation procedure using

Table 2 Identification of the isolated fungi preserved in MUT culture collection

Isolated strain	Sampling site	Final identification (taxa)	Isolation media	MUT code	GenBank acc. #
1Na	Goliat	*Penicillium rubens*	CYC 15%	3588	MF574999
1Nb	Goliat	*Penicillium chrysogenum*	CYC 20%	3589	MF575000
2Aa	Repparfjord	*Penicillium chrysogenum*	CYC 20%	3596	MF575001
3Na	HammerFest	*Penicillium rubens*	CYC 20%	3597	MF575002
4Na	Salina	*Aspergillus sydowii*	CYC 20%	3610	MF575003
4Nb	Salina	*Microascus trigonosporus*	CYC 5%	3612	MF509599
5Ea	Bjørnøyenna	*Aspergillus protuberus*	CYC 20%	3613	MF575004
5Na	Bjørnøyenna	*Aspergillus protuberus*	CYC 5%	3614	MF575005
5Nb* 5Nc*	Bjørnøyenna	*Aspergillus protuberus*	CYC 15%	3615	MF575006
6Na	Bønna	*Penicillium rubens*	CYC 3%	3616	MF575007
7Na	Bønna	*Aspergillus protuberus*	CYC 10%	3617	MF575008
7Nb	Bønna	*Aspergillus protuberus*	CYC 10%	3618	MF575009
7Nc	Bønna	*Aspergillus protuberus*	CYC 10%	3632	MF575010
8Na	Bønna	*Aspergillus protuberus*	CYC 15%	3638	MF150910

*Strains 5Nb and 5Nc were considered isogenic because of genetic dereplication; for this reason, only one strain was used for the following experiments

different media and different salinity is a powerful tool to retrieve more strains. All identified isolates were stored and preserved in the fungal collection bank, Mycotheca Universitatis Taurinensis (MUT) from the University of Turin. The 14 strains deposited were assigned with the culture collection code MUT. The main features of identification are reported in Table 2.

Antimicrobial Screening

A preliminary screening by cross-streaking (CT) revealed that only the fungus MUT 3638, identified as *Aspergillus protuberus*, was able to inhibit completely the growth of four pathogens from the panel tested: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinectocater baumanii* and *Burkholderia metallica* with the exception of *Pseudomonas aeruginosa* that was not inhibited by the fungal strains. The qualitative interpretation is shown in Fig. S2.

As a fact, the anticancer, antimicrobial and antiviral potentials of marine fungi especially of *Aspergillus* and *Penicillium* are well known (Xu et al. 2015). Among these candidates, fungal strain MUT 3638 was selected, since it showed the best inhibitory activity compared to the other tested strains. The antimicrobial activity of all fungal strains is summarised in Table 3. Therefore, MUT 3638 was selected for up-scaled fermentation, growth optimisation and purification of the molecule involved in the antimicrobial activity.

Growth Optimisation for Antimicrobial Production

MUT 3638 was initially isolated at 15% (*w*/*v*) of salinity in media CYC. In order to optimise the growth conditions for the

Table 3 Growth analysis of fungal strains in cross-streaking experiments carried out using Petri dishes

Target strains	Fungal strains													
	Culture collection codes MUT													
	3588	3589	3596	3597	3610	3612	3613	3614	3615	3616	3617	3618	3632	3638
Staphylococcus aureus 6538P	±	+	+	±	+	+	+	±	±	+	±	+	+	−
Pseudomonas aeruginosa PA01	+	+	±	±	+	±	+	+	+	±	+	+	±	+
Klebsiella pneumoniae DF12SA	+	+	±	+	±	+	±	+	±	±	±	±	±	−
Acinetobacter baumannii Ab13	±	±	+	+	+	±	+	+	+	+	+	+	±	−
Burkholderia metallica LMG 24068	±	+	+	+	±	+	+	±	±	±	+	±	+	−

Symbols: +, non-inhibition, ±, partial inhibition, −, full inhibition

Table 4 Crude extract yield and antimicrobial activity against *S. aureus* of *A. protuberus* MUT 3638 species by optimising growth conditions

Conditions tested			Yield (mg) of crude extract from 500 mL exhausted broth	MIC (mg/mL) against *S. aureus* crude extract
pH	Incubation Time	Temperature (°C)		
pH 4.0	10 days	10	7	1
		25	10	1
		37	12	1
	25 days	10	15	0.5
		25	25	0.06
		37	34	0.06
pH 9.0	10 days	10	7	1
		25	9	1
		37	12	1
	25 days	10	14	0.25
		25	30	0.06
		37	38	0.06

secondary metabolite production, a series of parameters was changed.

The growth medium used for the first isolation (CYC) was replaced with one richer medium (TYS) maintaining the same concentration of salt (15%), in static conditions. In order to evaluate the antimicrobial activity of crude extracts, liquid inhibition assays were carried out against selected pathogenic bacteria. Despite that MUT 3638 was able to inhibit the vast major of pathogens (except *P. aeruginosa*) tested in the initial screening, after the liquid screening of crude extracts, the highest antimicrobial effect was detected towards *Staphylococcus aureus* 6538P with a minimal inhibitory concentration (MIC) of 0.06 mg/mL.

MUT 3638 extract showed antimicrobial activity towards all the tested strains with the exception of *P. aeruginosa* PA01. MIC values were 0.12 mg/mL against both *B. cenocepacia* strains and 0.5 mg/mL against *K. pneumoniae* and *A. baumannii Ab13*.

The impact of NaCl concentration on the growth and antimicrobial activity was then evaluated. The strain MUT 3638 was inoculated in TYS medium with varying NaCl concentrations from 3 to 25% (*w*/*v*), and the inoculum was incubated in the flask at 20 °C for 10 days. Interestingly, considering that the sediments do not come from waters with high salinities, MUT 3638 was able to grow from natural seawater (about 3% of salinity) until 25% (*w*/*v*) of NaCl. Concerning the production of metabolites, the best yield was obtained by adding NaCl at a concentration of 10–15% (*w*/*v*). In fact, from 250 mL of culture yielded 17 mg of crude extracts in presence of 10 and 15% of NaCl after 20 days of incubation. However, the activity of extracts was identical for all conditions tested (i.e. 60 μg/mL as per MIC results); therefore, the concentration of 10% NaCl was selected for growth optimisation.

The effects of incubation time, pH and temperature were subsequently evaluated. To this aim, the fungus was inoculated in TYS medium with 10% (*w*/*v*) NaCl, varying pH (from 4.0 to 9.0) and temperature (10, 25 and 37 °C). The length of incubation was 10 days and a second set for 25 days. After the incubation, the antimicrobial activity was evaluated and results are summarised in Table 4.

Most fungi are mesophilic; however, few fungal species can grow at low or high temperature (Deacon 2006). The *A. protuberus* strain MUT 3638 has shown higher versatility, with the ability to grow at low and high temperatures, and in a wide range of salt concentrations and diverse pH range. This particular behaviour makes this fungus a promising candidate for biotechnological applications.

Extracts from growth at pH 4.0 and pH 9.0 displayed identical antimicrobial activities, while temperature proved to be a key factor. The increase of temperature (25 and 37 °C) resulted in a higher yield and activity of the extract compared to the one obtained from the growth at 10 °C. Moreover, prolonged time of incubation also significantly affected the yield of the extracts, but not an increase of its biomass. Extracts from 25 days of incubation weighed up to three times more than their 10-day counterparts. This finding might suggest that the production of the antimicrobial compounds is triggered by the

Table 5 The inhibitory activity of fractions produced from the active extract of *A. protuberus* MUT 3638 reported as percentage of inhibition of target *S. aureus* 6538P

% Solvent	Concentration [mg/mL]						
	1	0.5	0.25	0.12	0.06	0.03	0.015
25% MeOH	20	20	10	10	10	10	10
50% MeOH	30	30	20	20	20	10	10
100% MeOH	100	100	100	90	70	40	20
100% MetOH + 0.1% TFA	30	30	20	10	10	10	10

Table 6 Metabolite profiling for sorbicillonoid components in the culture broth of *A. protuberus* MUT 3638 by LC-HRMS analysis of its crude extract

Compound	Formula	$[M+H]^+$ (calculated)	Retention time (RT) (min)
Sorbicillin	$C_{14}H_{16}O_3$	233.1165	15.07
Sorbicillinol	$C_{14}H_{16}O_4$	249.1113	21.9
Dihydrosorbicillinol	$C_{14}H_{18}O_4$	251.1278	19.43
Oxosorbicillinol	$C_{14}H_{16}O_5$	265.1071	21.31
Sorbicillactone A	$C_{21}H_{23}NO_8$	418.1502	10.60
Bisvertinol	$C_{28}H_{34}O_8$	499.2319	17.86
Bisvertinolone	$C_{28}H_{32}O_9$	513.2119	21.26
Dihydrobisvertinolone	$C_{28}H_{34}O_9$	515.2276	21.26

activation of the organism's secondary metabolism, in stressed conditions, such as starvation.

Bioactivity-Guided Purification of Aspergillus Bioactive Compounds

Once the best conditions for the production of antimicrobials by *A. protuberus* MUT 3638 to counteract *S. aureus* 6538P were established, an up-scaled fermentation was set up to 1.5 L. After 25 days of incubation, the exhausted broth was extracted, yielding 250 mg of crude extract. The crude extract was subsequently re-suspended in methanol and fractionated using solid-phase extraction (SPE) C-18 column cartridge, by using a water-methanol system as described in "Materials and Methods" ("Purification of Active Compounds from the Newly Isolated *A. protuberus* MUT 3638").

Four fractions were collected, dried and used to perform MIC assays against the pathogens. As reported in Table 5, the fraction eluted with 100% MeOH displayed a strong antimicrobial activity, compared to the other samples with a MIC between 0.25 and 0.112 mg/mL.

Subsequently, the 100% methanol fraction (88 mg) was subjected to purification by reverse phase HPLC by using a C-18 with an ACN/water linear gradient (as specified in "Materials and Methods," "Purification of Active Compounds from the Newly Isolated *A. protuberus* MUT 3638"). The assay against *S. aureus* showed that the peak # 9 is responsible of the activity. The peak # 9 has a retention time (RT) at minute = 26.534 and possesses a MIC of 30 μg/mL and a yield of 3.6 mg.

Preliminary Metabolic Profiling by LC-HRMS Analysis

The metabolic profile of the crude 100% methanol fraction by MUT 3638 strain was analysed by LC/HRMS analysis. The molecular formulas were deduced by HRESI analysis, and the corresponding structures were tentatively assigned by comparison with literature data (Harned and Volp 2011; Meng et al. 2016). The results of this analysis were reported in Table 6. The crude extract contained a complex mixture of monomeric and dimeric members of sorbicillinoid family This growing family of polyketide natural products is comprised of 90 members that have been mainly isolated from fungi of terrestrial origin (*Acremonium*, *Aspergillus*, *Clonostachys*, *Emericella*, *Penicillium*, *Phaeoacremonium*, *Scytalidium*, *Trichoderma* and *Verticillium* genera) and in few marine species (*Paecilomyces*, *Penicillium*, *Phialocephala*, *Trichoderma* and *Trichothecium* genera).

Compound Identification

Bioassay-guided HPLC fractionation of the crude 100% methanol extract revealed the antimicrobial activity exclusively associated to peak # 9. Combined HRESI MS, MS^n and ^1H NMR analysis indicated that peak 9 is a pure compound. An $[M+H]^+$ quasi-molecular ion detected in the HRESIMS spectrum at *m/z* 513.2124 indicated the molecular formula $C_{28}H_{32}O_9$ (calculated for $C_{28}H_{33}O_9$ 513.2119) (see Fig. S3 supplemental material). The presence of a Na adducts at *m/z* 535.1939 was also observed.

The ^1H NMR spectrum (see Fig. S4 supplemental material and "Material and Methods") of peak 9 showed signals superimposable to those reported for bisvertinolone (Trifonov et al. 1986). Indeed, the HRESI MS^2 (see Fig. S5 supplemental material) fragmentation pattern of peak 9 (precursor ion at *m/z* 513.2) matched with the backbone of bisvertinolone and furthermore was in good agreement with $EIMS^2$ data—acquired at unit resolution—reported by Trifonov et al. for

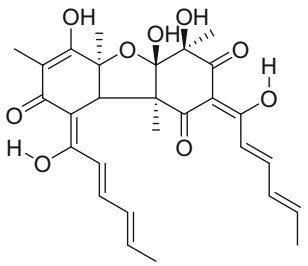


Fig. 2 Structure of the bisvertinolone compound isolated from *A. protuberus* MUT 3638

bisvertinolone. These data led to confirm the identity of peak 9 as bivertinolone.

Bisvertinolone was obtained as a light yellow amorphous powder. An $[M + H]^+$ HRESI MS quasi-molecular ion detected at m/z 513.2124 suggested the molecular $C_{28}H_{32}O_9$ (calculated for $C_{28}H_{33}O_9$ 513.2119) (see Fig. S3 supplemental material).

The compound was identified as bisvertinolone (Fig. 2) by comparison of its optical and NMR data.

Bisvertinolone was firstly detected from the culture medium of *Verticillium intertextum* (Trifonov et al. 1986) and later described as the first β-1,6-glucan biosynthesis inhibitor from the fungus *Acremonium strictum* (Kontani et al. 1994). Several biological activities have been ascribed to members of this family, including radical scavenger activity and antiproliferative activity. Although the data relative to the antiproliferative activity of the sorbicillinoids are fragmentary (Liu et al. 2005), bisvertinolone represents one of the most active compounds. Preliminary results seem to suggest the importance of two unsaturated sorbyl side chain in a correct relative orientation for the observed cytostatic activity. As concerning the antimicrobial activity, there are very few reports in the literature dealing with the investigation of the antibacterial activity of members of sorbicillinoid family. Some monomeric or dimeric sorbicillinoids demonstrated to possess weak activity against *Staphylococcus aureus* and *Bacillus subtilis* (Maskey et al. 2005). Recently, two dimeric sorbicillinoids, dihydrotrichodimerol and tetrahydrotrichodimerol, were reported as potent antibacterial agents against *Bacillus megaterium* with MIC values of 25 μg and 12.5 μg/mL, respectively (Zhai et al. 2016).

Conclusions

By exploiting a bioassay-driven purification approach, the bioactive compound bisvertinolone with antimicrobial activity against *Staphyloccoccus aureus* 6538P was isolated from a new polyextremophilic fungus *Aspergillus protuberus* MUT 3638.

To the best of our knowledge, the antibacterial activity of the bisvertinolone was demonstrated for the first time. Future investigation will be focused on unveiling the mechanisms of action and the scale-up production of the active compound.

Furthermore, this fungus was isolated from cold seawater, but showed a great versatility to different environmental conditions. In particular, it was able to grow in a wide salinity range, as well as in varying ranges of pH and temperature. This capability makes the new strain *A. protuberus* MUT 3638 an ideal candidate for biotechnological applications.

Author Contributions P.C., F.P.E. and P.T. equally contributed to the work. P.C., F.P.E, A.F. and D.d.P. conceived and designed the experiments; P.C., F.P.E., P.T., A.F., E.T., L.T., M.V.D.A., G.G. and G.C.V. performed the experiments; P.C., F.P.E, P.T., A.F. and D.d.P. analysed the data; G.C.V., L.T., M.V.D.A. and D.d.P. contributed reagent/material/analysis tools; P.C., F.P.E., P.T. and D.d.P. wrote the paper.

Funding Information This work was supported by the EU FP7 KBBE2012-2016 project: "Pharmasea," Grant Agreement 312184, and by the H2020-MSCA-RISE: Ocean Medicines, Grant Agreement 690944.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

Abd-Elsalam KA, Almohimeed I, Moslem MA, Bahkali AH (2010) M13-microsatellite PCR and rDNA sequence markers for identification of *Trichoderma* (Hypocreaceae) species in Saudi Arabian soil. Genet Mol Res 9:2016–2024

Alonso A, Campanario E, Martinez JL (1999) Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. Microbiology 145:2857–2862

Baker SE, Bennett JW (2007) An overview of the genus *Aspergillus*, vol 26. In: Goldman GH, Osmani SA (eds) The Aspergilli: genomics, medical aspects, biotechnology, and research methods. CRC Press, New York

Bernhard JM, Kormas KA, Pachiadaki MG, Rocke E, Beaudoin DJ, Morrison C, Visscher PT, Cobban A, Starczak VR, Edgcomb VP (2014) Benthic protists and fungi of Mediterranean deep hypersaline anoxic basin redoxcline sediments. Front Microbiol 5:605

Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep MR (2015) Marine natural products. Nat Prod Rep 32:116–211

Deacon J (2006) Fungal growth. In: Deacon J (ed) Fungal biology. Blackwell Publishing Ltd, London, pp 67–84

Delgado-Jarana J, Sousa S, Gonzalez F, Rey M, Llobell A (2006) ThHog1 controls the hyperosmotic stress response in *Trichoderma harzianum*. Microbiology 152:1687–1700

Domsch KH, Gams W, Anderson TH (1980) Compendium of soil fungi. Academic Pres, London

Ebada SS, Proksch P (2015) Marine-derived fungal metabolites. In Hb25_Springer handbook of marine biotechnology. Springer, pp 759–788

Geiser DM, Frisvad JC, Taylor JW (1998) Evolutionary relationships in *Aspergillus* section Fumigati inferred from partial β-tubulin and hydrophobin DNA sequences. Mycologia 90:831–845

Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 61:1323–1330

Gostincar C, Grube M, de Hoog S, Zalar P, Gunde-Cimerman N (2010) Extremotolerance in fungi: evolution on the edge. FEMS Microbiol Ecol 71:2–11

Harned AM, Volp KA (2011) The sorbicillinoid family of natural products: isolation, biosynthesis, and synthetic studies. Nat Prod Rep 28: 1790–1810

Jurjevic Z, Peterson SW, Horn BW (2012) *Aspergillus* section Versicolores: nine new species and multilocus DNA sequence based phylogeny. IMA Fungus 3:59–79

Kontani M, Sakagami Y, Marumo S (1994) First β-1,6-glucan biosynthesis inhibitor, bisvertinolone isolated from fungus, *Acremonium*

strictum and its absolute stereochemistry. Tetrahedron Lett 35:2577–2580

Lima DB, Torres AF, Mello CP, de Menezes RR, Sampaio TL, Canuto JA, da Silva JJ, Freire VN, Quinet YP, Havt A, Monteiro HS, Nogueira NA, Martins AM (2014) Antimicrobial effect of *Dinoponera quadriceps* (Hymenoptera: Formicidae) venom against *Staphylococcus aureus* strains. J Appl Microbiol 117:390–396

Liu W, Gu Q, Zhu W, Cui C, Fan G (2005) Dihydrotrichodimerol and tetrahydrotrichodimerol, two new bisorbicillinoids, from a marine-derived *Penicillium terrestre*. J Antibiot (Tokyo) 58:621–624

Loeng H (1991) Features of the physical oceanographic conditions of the Barents Sea. Polar Res 10:5–18

Maskey RP, Grun-Wollny I, Laatsch H (2005) Sorbicillin analogues and related dimeric compounds from *Penicillium notatum*. J Nat Prod 68:865–870

Meng J, Wang X, Xu D, Fu X, Zhang X, Lai D, Zhou L, Zhang G (2016) Sorbicillinoids from Fungi and their bioactivities. Molecules 21(6): 715

Muller M, Lehmann P, Willy C (2017) Antibiotic stewardship: a programmatic approach to improved antimicrobial management. Unfallchirurg 120:540–548

Poirel L, Figueiredo S, Cattoir V, Carattoli A, Nordmann P (2008) *Acinetobacter radioresistens* as a silent source of carbapenem resistance for Acinetobacter spp. Antimicrob Agents Chemother 52: 1252–1256

Poli A, Finore I, Romano I, Gioiello A, Lama L, Nicolaus B (2017) Microbial diversity in extreme marine habitats and their biomolecules. Microorganisms 5(2):25

Rateb ME, Ebel R (2011) Secondary metabolites of fungi from marine habitats. Nat Prod Rep 28:290–344

Saleem M, Ali MS, Hussain S, Jabbar A, Ashraf M, Lee YS (2007) Marine natural products of fungal origin. Nat Prod Rep 24:1142–1152

Shahi SK, Singh VK, Kumar A, Gupta SK, Singh SK (2013) Interaction of dihydrofolate reductase and aminoglycoside adenyltransferase enzyme from *Klebsiella pneumoniae* multidrug resistant strain DF12SA with clindamycin: a molecular modelling and docking study. J Mol Model 19:973–983

Soriano F, Huelves L, Naves P, Rodriguez-Cerrato V, del Prado G, Ruiz V, Ponte C (2009) In vitro activity of ciprofloxacin, moxifloxacin, vancomycin and erythromycin against planktonic and biofilm forms of *Corynebacterium urealyticum*. J Antimicrob Chemother 63:353–356

Trifonov LS, Hilpert H, Floersheim P, Dreiding AS, Rast DM, Skrivanova R, Hoesch L (1986) Bisvertinols: a new group of dimeric vertinoids from Verticillium intertextum. Tetrahedron 42:3157–3179

Turk M, Mejanelle L, Sentjurc M, Grimalt JO, Gunde-Cimerman N, Plemenitas A (2004) Salt-induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi. Extremophiles 8:53–61

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR: protocols and applications - a laboratory manual. Academic Press, pp 315–322

Xu L, Meng W, Cao C, Wang J, Shan W, Wang Q (2015) Antibacterial and antifungal compounds from marine fungi. Mar Drugs 3:3479–3513

Zhai MM, Qi FM, Li J, Jiang CX, Hou Y, Shi YP, Di DL, Zhang JW, Wu QX (2016) Isolation of secondary metabolites from the soil-derived fungus *Clonostachys rosea* YRS-06, a biological control agent, and evaluation of antibacterial activity. J Agric Food Chem 64(11): 2298–2306

Zhao C, Liu H, Zhu W (2016) New natural products from the marine-derived *Aspergillus* fungi-a review. Wei Sheng Wu Xue Bao 56: 331–362

marine drugs

MDPI

Article

Antimicrobial Activity of Monoramnholipids Produced by Bacterial Strains Isolated from the Ross Sea (Antarctica) †

Pietro Tedesco [1], Isabel Maida [2], Fortunato Palma Esposito [1], Emiliana Tortorella [1], Karolina Subko [3], Chidinma Christiana Ezeofor [3], Ying Zhang [3], Jioji Tabudravu [3], Marcel Jaspars [3], Renato Fani [2] and Donatella de Pascale [1,*]

1 Institute of Protein Biochemistry, National Research Council, Via P. Castellino, 111, I-80131 Naples, Italy; p.tedesco@ibp.cnr.it (P.T.); f.palma@ibp.cnr.it (F.P.E.); e.tortorella@ibp.cnr.it (E.T.)

2 Department of Biology, University of Florence, Via Madonna del Piano 6, I-50019 Sesto Fiorentino (FI), Italy; isabel.maida@unifi.it (I.M.); renato.fani@unifi.it (R.F.)

3 Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Old Aberdeen, AB24 3UE Scotland, UK; k.subko.11@aberdeen.ac.uk (K.S.); chidinma.christiana.ezeofor.14@aberdeen.ac.uk (C.C.E.); ying.zhang.14@aberdeen.ac.uk (Y.Z.); j.tabudravu@abdn.ac.uk (J.T.); m.jaspars@abdn.ac.uk (M.J.)

* Correspondence: d.depascale@ibp.cnr.it; Tel.: +39-081-613-2314; Fax: +39-081-613-2277

† All the authors belong to the EU-FP7 PharmaSea project.

Academic Editor: Orazio Taglialatela-Scafati

Received: 12 December 2015; Accepted: 18 April 2016; Published: 26 April 2016

Abstract: Microorganisms living in extreme environments represent a huge reservoir of novel antimicrobial compounds and possibly of novel chemical families. Antarctica is one of the most extraordinary places on Earth and exhibits many distinctive features. Antarctic microorganisms are well known producers of valuable secondary metabolites. Specifically, several Antarctic strains have been reported to inhibit opportunistic human pathogens strains belonging to *Burkholderia cepacia* complex (Bcc). Herein, we applied a biodiscovery pipeline for the identification of anti-Bcc compounds. Antarctic sub-sea sediments were collected from the Ross Sea, and used to isolate 25 microorganisms, which were phylogenetically affiliated to three bacterial genera (*Psychrobacter*, *Arthrobacter*, and *Pseudomonas*) via sequencing and analysis of 16S rRNA genes. They were then subjected to a primary cell-based screening to determine their bioactivity against Bcc strains. Positive isolates were used to produce crude extracts from microbial spent culture media, to perform the secondary screening. Strain *Pseudomonas* BNT1 was then selected for bioassay-guided purification employing SPE and HPLC. Finally, LC-MS and NMR structurally resolved the purified bioactive compounds. With this strategy, we achieved the isolation of three rhamnolipids, two of which were new, endowed with high (MIC < 1 μg/mL) and unreported antimicrobial activity against Bcc strains.

Keywords: antimicrobials; ramnholipids; Antarctic; Bcc; microorganisms

1. Introduction

The alarming rise of Multi-Drug Resistance (MDR) bacteria in the last few decades has highlighted the need for novel antimicrobial compounds and for effective drug discovery approaches [1,2]. Natural products are the largest source of new antibiotic molecules, representing about two-thirds of new antibacterial therapies approved between 1980 and 2010 [3,4]. Bioprospecting for natural products from unexplored natural environments, such as the marine environment is considered a promising strategy to identify novel compounds. It is increasingly recognized that a huge number of natural products and novel chemical entities exist in these environments, but the overwhelming biological diversity of these environments has so far only been explored to a very limited extent [5,6]. The Antarctic environment,

as well as having incredibly low temperatures, possesses other diverse traits that may have helped to shape the unique way in which Antarctic bacteria have evolved. This extreme environment contains hyper-salinity that exists in sea ice brine channels, a lack of free water due to freezing temperatures, as well as low nutrient availability. Unique light conditions also exist due to the high latitude of the region. Several studies have shown that Antarctic bacteria harvested from Antarctic corals and sponges are a promising source of new antimicrobial compounds [7–14]. Specifically, several Antarctic strains belonging to the genus *Pseudoalteromonas*, *Psychrobacter*, *Pseudomonas*, and *Arthrobacter*, were able to inhibit the growth of several strains belonging to the *Burkholderia cepacia* complex (Bcc) [11,14]. Further studies demonstrated that the antimicrobial activity relies (at least in part) on the production of Volatile Organic Compounds (VOCs) [12,13,15]. The Bcc consists of at least 20 closely related species inhabiting different ecological niches such as water, soil, plants rizosphere, and plants and animals [16–18]. Bcc are also opportunistic human pathogens that cause lung infections in immune-compromised individuals, including cystic fibrosis (CF) patients [19]. In one-third of infected individuals, it causes the "cepacia syndrome"—a form of septic shock, which involves the lungs essentially shutting down, resulting in fatality [20–22]. Bcc bacteria have proven to be very resilient and incredibly difficult to combat as they are resistant to almost all known antimicrobial agents and can survive under the most extreme conditions [23]. In this publication, we report a complete biodiscovery pipeline aiming at the identification of novel anti-Bcc compounds, starting from the isolation of bacteria from Antarctic sub-sea sediments. Bacteria were tested for their antimicrobial potential and a bioassay-guided purification was performed that yielded three bioactive compounds active against Bcc. Structures were then elucidated and two compounds have not been reported previously.

2. Results and Discussion

2.1. Isolation of Bacteria, Typing and Phylogenetic Analysis

Psychrophilic Antarctic bacteria were isolated from sediments on PYG minimal medium. After 15 days of incubation at 4 °C, 25 visible colonies where picked and grown in liquid PYG at 15 °C for 48 h in agitation, and glycerol stab were stored at −80 °C.

In order to check whether the 25 bacterial isolates represented either the same or different strains, an RAPD analysis was carried out using the primers 1253 (5′-GTTTCCGCCC-3′) and AP5 (5′-TCACGCTGCG-3′). The RAPD profiles obtained were then compared among them; the comparative analysis obtained with primer 1253 revealed that the 25 Antarctic isolates were split into 18 different RAPD groups (hereinafter, RAPD haplotypes), most of which were represented by just one bacterial isolate as summarized in Table 1. Two groups embedding more than one isolate were identified: group 1 (RAPD halpotype 1) including strains BTN1, BTN6, BTN 7, BTN8, BTN9 and BTN10 and group 4 (embedding isolates BTN20A, BTN20B, and BTN24). These data were completely confirmed by the RAPD analysis performed with primer AP5.

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. Sequences were then aligned using the program ClustalW and the alignment was used to construct the phylogenetic trees shown in Figure S1, revealing that:

(i) As expected on the basis of the sharing of RAPD profiles, the six strains exhibiting the same RAPD profile (RAPD haplotype 1) share the same 16S rRNA gene sequence and were clustered together joining the species *Pseudomonas azotoformans*.

(ii) Strain BTN4 was affiliated to the genus *Arthrobacter*.

(iii) All the other strains were affiliated to the genus *Psychrobacter* and, according to the different RAPD profiles they exhibited, joined different *Psychrobacter* clades. The three strains (BTN20A, BTN24 and BTN 20B) sharing the same RAPD profile (RAPD haplotype 4), joined the same *Psychrobacter* cluster.

Table 1. List of the strains used in this work; for each strain, the genus and the RAPD haplotype are reported.

Genus	Strains	RAPD Profile	Accession Number
Pseudomonas	BTN1	1	KT989002
	BTN6		KT989003
	BTN7		KT989004
	BTN8		KT989005
	BTN9		KT989006
	BTN10		KT989007
Psychrobacter	BTN3	2	KT989009
	BTN19	3	KT989019
	BTN20B	4	KT989021
	BTN24		KT989022
	BTN21	5	KT989025
	BTN23	6	KT989024
	BTN2	7	KT989008
	BTN11	8	KT989011
	BTN5	9	KT989010
	BTN20A	4	KT989020
	BTN15	10	KT989015
	BTN13	11	KT989012
	BTN14	12	KT989013
	BTN17	13	KT989017
	BTN16	14	KT989016
	BTN18	15	KT989018
	BTN12	16	KT989014
	BTN22	17	KT989023
Arthrobacter	BTN4	18	KT989001

2.2. Cross-Streaking Experiments

In order to check the ability of Antarctic bacteria to inhibit the growth of Bcc strains, cross-streaking experiments were performed using representatives of each RAPD haplotype as test strains. We used as targets a panel of 84 different Bcc strains belonging to 17 known species (see Table S1). Most of the strains had a clinical origin. Data obtained are summarized in Table S1, revealing that all BTN strains are able to completely inhibit the growth of Bcc strains. In order to check whether this anti-Bcc activity was due to Volatile Organic Compounds (VOCs) synthesis, a further cross-streaking experiment was performed using Petri dishes with a central septum, which physically separates the tester (Antarctic) from the target strains. To perform this analysis, we selected the 17 Bcc type strains listed in Table S2, which are highlighted in red. Data obtained are reported in Table 2 and revealed that the inhibitory power of the BTN strains decreased in the presence of the central septum. This finding suggested that BTN strains synthesize a combination of volatile and soluble molecules and that the Bcc-inhibitory activity likely might rely principally on the soluble fraction. Thus, we decided to concentrate our efforts on the soluble molecules for this study.

Table 2. Growth of Bcc strains in cross-streaking experiments carried out using Petri dishes either with (W) or without (N) a central septum (S). Symbols: +, growth; ±, reduced growth; -, no growth.

Bcc Strain	S	BTN Strain																			
		1	**2**	**3**	**5**	**11**	**13**	**14**	**4**	**12**	**15**	**16**	**17**	**18**	**19**	**20 a**	**20 b**	**21**	**22**	**23**	**C+**
B. ambifaria LMG 19182	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. anthina LMG 20980	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. vietnamensis LMG10929	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. cenocepacia LMG 16656	W	±	-	±	±	-	±	-	-	-	-	-	±	±	-	±	±	-	-	-	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. cepacia LMG 1222	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. contaminas LMG 23361	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. diffusa LMG 24065	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. dolosa LMG 18943	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. lata LMG 22485	W	±	±	±	±	±	±	±	-	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. latens LMG 24064	W	-	-	±	±	±	-	-	-	-	±	±	±	±	-	±	±	-	-	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. metallica LMG 24068	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. multivorans LMG 13010	W	-	±	±	±	±	±	±	-	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 2. *Cont.*

Bcc Strain	S	BTN Strain																			
		1	**2**	**3**	**5**	**11**	**13**	**14**	**4**	**12**	**15**	**16**	**17**	**18**	**19**	**20 a**	**20 b**	**21**	**22**	**23**	**C+**
B. pseudomultivorans LMG 26883	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. pyrrocinia LMG 14191	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. seminalis LMG 24067	W	-	±	±	±	±	±	±	-	±	-	±	±	±	±	±	-	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. stabilis LMG 14294	W	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	-	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. uborrensis LMG 20358	W	-	-	±	±	±	±	±	-	±	±	±	±	±	-	±	±	±	±	±	+
	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

2.3. Extracts' Antimicrobial Assays

Eight of the most active Antarctic strains belonging to the three different genera (*Pseudomonas*, *Psychrobacter*, and *Arthrobacter*) were selected and used to produce extracts, which were then tested against a reduced panel of Bcc type-strains isolated from CF patients. The MIC assays were carried out as described in Materials and Methods. Table 3 reports the antimicrobial activity as percentage of Bcc growth inhibition in the presence of each extract at a concentration of 1 mg/mL.

Table 3. Antimicrobial activity of BTN cell extracts reported as % of inhibition of Bcc strains treated with 1 mg/mL of BTN extracts.

		Pseudomonas			*Psychrobacter*				*Arthrobacter*
Species	**Strain**	**BTN 1**	**BTN 2**	**BTN 15**	**BTN 3**	**BTN 19**	**BTN 21**	**BTN 5**	**BTN 4**
B. diffusa	LMG 24065	100 ± 0	75 ± 3	77 ± 3	43 ± 7	45 ± 11	70 ± 4	77 ± 9	63 ± 3
B. metallica	LMG 24068	92 ± 4	70 ± 5	71 ± 3	32 ± 2	30 ± 3	53 ± 5	77 ± 4	64 ± 9
B. cenocepacia	LMG 16656	100 ± 0	78 ± 2	87 ± 1	84 ± 6	64 ± 4	45 ± 1	84 ± 2	57 ± 1
B. latens	LMG 24064	100 ± 0	53 ± 11	75 ± 2	55 ± 6	43 ± 3	65 ± 2	56 ± 3	41 ± 2
B. seminalis	LMG 24067	100 ± 0	43 ± 6	67 ± 5	73 ± 8	45 ± 6	78 ± 11	40 ± 3	56 ± 3

Data obtained revealed that the extracts were differentially active against the selected Bcc strains. Three Antarctic bacterial strains, *i.e.*, BTN2, BTN15, and BTN5, were able to inhibit at least three of the five Bcc strains more than 70% of growth. However, the extract from *Pseudomonas* BTN1 exhibited the best anti-Bcc activity; indeed, it was able to almost completely inhibit the growth of all the target strains at the concentration used. For this reason, this strain was selected for further scale-up and extract purification.

2.4. Bioassay-Guided Purification of BTN1 Extract

Pseudomonas sp. BTN1 strain was grown in 3 L TYP medium for five days at 20 °C; then, the culture broth was extracted with ethyl acetate. Subsequently, the crude extract (1 g) was fractionated with an SPE C18 Cartridge. Elution was performed stepwise with an increasing methanol concentration. The four 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 against *B. cenocepacia* LMG 16656 with a MIC of 50 µg/mL and was subjected to HPLC separation. HPLC chromatograms extracted from 200 to 400 nm presented 11 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 promising inhibitory activity against *B. cenocepacia* strain LMG 16656 of three compounds, hereinafter referred to as Compound **1**, **2** and **3**, respectively.

2.5. Compound Structure Elucidation

Compounds structures are shown in Figure 1. The molecular formula of compound **1** was established as $C_{28}H_{52}O_9$ by HRESIMS (555.3514 Δ 0.92 ppm $[M + Na]^+$. Dereplication of this compound based on 1D, 2D NMR and LC-MS data indicated that it is a known rhamnolipid [24] containing two fully saturated lipid chains. The chain lengths of lipids A and B of compound **1** contained 10 and 12 carbons respectively were confirmed by analysis of MS/MS fragmentation data (Figure S19). The molecular formula of compound **2** was established as $C_{28}H_{50}O_9$ by high-resolution electrospray ionization mass spectrometry (HRESIMS) (553.3343 Δ −0.75 ppm $[M + Na]^+$) and subsequent dereplication suggested it was new. The molecular formula suggested four degrees of unsaturation.

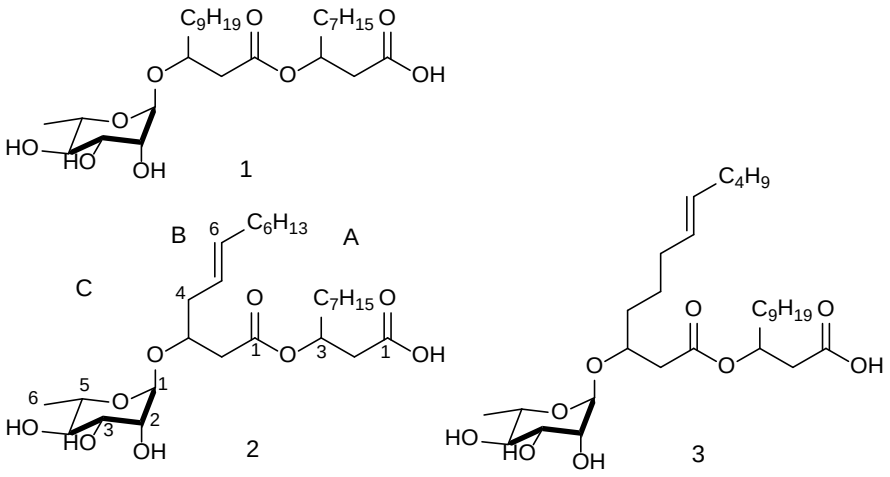


Figure 1. Structures of the three rhamnolipids isolated from *Pseudomonas* BTN1.

The ^{1}H, ^{13}C-NMR data (Table 1) in CD_3OD of **2** revealed one ester (δ_C 173.4 ppm), one carboxylic acid group (δ_C 171.40) ppm, two olefinic carbons (δ_C 132.8, 123.7 ppm), and an anomeric carbon (δ_C 98.47 ppm) of a sugar unit. This analysis accounted for three of the double bond equivalents, suggesting that the sugar unit was present as a ring. The structure of compound **2** was elucidated based on 2D NMR correlation experiments. Data clearly showed three distinctive spin systems. There were COSY correlations observed between the anomeric proton and the adjacent protons of the sugar unit. There was a strong observed COSY correlation between the methyl group at δ_H 1.27 ppm and the proton at δ_H 3.38 ppm placing the methyl group at position C5. The proposed structure was fully supported by COSY and HMBC correlations (Table 4 and Figure S10) indicating that compound **2** is a rhamnolipid with the A and B chains having 10 and 12 carbons respectively, and a single unsaturation at position B5. Further evidence supporting the structure came from careful interpretation of MS/MS fragmentation data (Figure S21). The relative orientation of the rhamnose moiety in compound **2** was identified as α based on detailed analysis of ROE data (2D ROESY), chemical shifts and proton coupling constants [24,25]. Analysis of ROESY data showed an ROE correlation between H-1 and H-2 suggesting that both occupied equatorial positions. This is in agreement with the observed small coupling constant ^{3}J(1,2) of about 1.4 Hz in CD_3OD and a broad singlet in DMSO-D_6. A ROE correlation between H-3 and H-5 suggested that both were in axial positions (Figure S13). In addition, ROE correlations were observed between H-3 and H-2 (axial-equatorial), H-6 and H-5 (equatorial-axial) and between H-6 and H-4 (equatorial-axial). This conformation is in agreement with observed coupling constants: ^{3}J(2,3, 3.5 Hz), ^{3}J(3,5, 9.5 Hz) and ^{3}J(4,5, 9.8 Hz). All the data is consistent with the rhamnose unit oriented in a α position. A 2D ROESY NMR spectrum is available in the supporting information (Figure S12).

The molecular formula of compound **3** was established as $C_{30}H_{54}O_9$ by HRESIMS 581.3649 Δ 1.72 ppm $[M + Na]^+$. Based on 1D, 2D NMR and LC-MS data compound **3** was similar to **2**, the difference being an additional C_2H_4 unit. However, careful interpretation of the data indicated that both the lipid chains A and B were C12 carbons with a single unsaturation at position B7 instead of C10 and C12 carbons and an unsaturation position at B5 in **2**. Further evidence of this structure came from MS/MS fragmentation data (Figure S23). The relative configuration of the rhamnose unit was similar to that of compounds **1** and **2** based on similarities of chemical shifts and proton coupling constants. In all three compounds, it is assumed that the rhamnose moieties have the normal L-configuration. In addition, the absolute stereochemistry of position H-3 in the lipid chains A and B have not been determined.

Table 4. NMR data of **2** and **3** in CD_3OD. [a] 150 MHz; [b] 600 MHz.

		2				**3**			
	Position	^{TM}C/ppm [a], m	^{TM}H/ppm (m, *J* in Hz) [b]	COSY ^1H–^1H	HMBC H→C	^{TM}C/ppm [a], m	^{TM}H/ppm (m, *J* in Hz) [b]	COSY ^1H–^1H	HMBC H→C
A	1	173.4, C				175.5, C			
	2	38.9, CH_2	2.58, m	A3	A1	40.9, CH_2	2.54, m	A3	A1
	3	71.1, CH	5.27, pentet, 6.4	A2, A3	A1, A2	72.7, CH	5.29, pentet, 6.5	A2, A4	A1, A2
	4	33.8, CH_2	1.64, m	A3	A3	34.9, CH_2	1.63, bm	A3	A3
	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	1.37, overlap		
	7	29.3, CH_2	1.31, overlap			30.1, CH_2	1.32, overlap		
	8	31. 6, CH_2	1.31, overlap			29.8, CH_2	1.33, overlap		
	9	22.3, CH_2	1.33, overlap	A10	A10	30.2, CH_2	1.36, overlap	A10	A10
	10	13.1, CH_3	0.92, m	A9	A9	32.7, CH_2	1.31, overlap	A9	A9
	11					23.4, CH_2	1.33, overlap		
	12					14.1, CH_3	0.92, m		
B	1	171.4, C				172.3, CH			
	2	39.5, CH_2	2.53, m	B3	B1	41.0, CH_2	A: 2.60, m B: 2.50, m	B3	B1
	3	72.9, CH	4.16, pentet, 5.8	B2, B4	B1, B5	74.8, CH	4.10, pentet, 5.9	B2, B4	B1, B5
	4	30.4, CH_2	A: 2.39, m B: 2.33, m	B3, B5	B3, B5	33.5, CH_2	1.58, bm	B3,B5	B3, B5
	5	123.7, CH	5.40, m	B4, B6	B3, B4, B6, B7	25.7, CH_2	1.43, overlap	B4, B6	
	6	132.8, CH	5.55, m	B5, B7	B5, B8	27.8, CH_2	2.08, overlap	B5, B7	
	7	27.1, CH_2	2.08, m	B6	B5, B6	130.0, CH	5.37, m	B6, B8	B8, B6, B9
	8	29.3, CH_2	1.31, overlap			131.2, CH	5.39, m	B7	B7
	9	28.9, CH_2	1.33, overlap	B7		32. 7, CH_2	1.31, overlap	B8	
	10	31.6, CH_2	1.31, overlap			32.7, CH_2	1.31, overlap		
	11	22.3, CH_2	1.33, overlap		B12	23.4, CH_2	1.33, overlap	B12	
	12	13.1, CH_3	0.92, m		B11	14.1, CH_3	0.92, m	B11	
C	1	98.5, CH	4.86, overlap	C2	B3, C2	100.0, CH	4.80, d, 1.4	C2	B3, C2
	2	71.2, CH	3.77, dd, 3.5, 1.4	C1, C3	C3, C4	72.4, CH	3.76, dd, 3.4, 1.4	C1, C3	C3, C4
	3	70.9, CH	3.64, dd, 9.5, 3.5	C2, C4	C5	71.9, CH	3.66, dd, 9.7, 3.4	C2, C4	C5
	4	72.7, CH	3.38, dd, 9.5, 9.8	C3,C5	C3	73.8, CH	3.35, dd, 9.7, 9.8	C3, C5	C3
	5	68.7, CH	3.67, m	C4, C6	C4, C6	69.8, CH	3.68, m	C4, C6	C4, C6
	6	16.6, CH_3	1.27, d, 6.2	C5	C5	17.6, CH_3	1.27, d, 6.3	C5	C5

2.6. Antimicrobial Activity of BTN1 Pure Compounds

The three monorhamnolipids isolated from strain BTN1 were tested against a selected panel of Bcc strains isolated from CF patients and *S. aureus*. MIC and MBC values are reported in Table 5. It is worth noticing that the three compounds have identical MIC and MBC values indicating a bactericidal effect against the target bacteria, as reported for several natural biosurfactants [26,27]. Compounds **2** and **1** were the most active compounds as they were effective against all the tested stains, with the only exception of *B. diffusa*. Specifically, compounds **2** and **1** had the lowest MBC values against *B. cenocepacia* (3.12 µg/mL) and *S. aureus* (respectively, 3.12 and 1.56 µg/mL). Compound **3** had antimicrobial effect only against *S. aureus* with an MBC value of 100 µg/mL, while it resulted in being ineffective towards Bcc strains. Rhamnolipids (RLs) are well-known secondary metabolites synthesized by members of different Gram-negative species, particularly from bacteria belonging to the genus *Pseuedomonas*. They perform several potential functions in bacteria: as powerful biosurfactants, they are involved in the uptake and b tested stains, with the only exception of polymerase and 0.6 iodegradation of poorly soluble substrates and are essential for surface motility and biofilm development [28]. 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* [29–31]. RLs act like synthetic surfactants and their proposed mechanism of action consists of intercalation into biological membranes and destruction by their permeabilizing effect leading to cell death [32].

Table 5. MIC and MBC values of the 3 mono-rhamnolipids isolated in this study.

	Antimicrobial Activity (µg/mL)											
	B. cenocepacia LMG 16656		*B. metallica* LMG 24068		*B. seminalis* LMG 24067		*B. diffusa* LMG 24065		*B. latens* LMG 24064		*S. aureus* 6538P	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
C1	3.12	3.12	50	50	12.5	12.5	>200	>200	12.5	12.5	1.56	1.56
C2	3.12	3.12	25	25	3.12	3.12	200	200	12.5	12.5	3.12	3.12
C3	200	200	>200	>200	>200	>200	>200	>200	>200	>200	100	100

3. Experimental Section

3.1. Isolation of Bacterial Strains

The Antarctic bacterial strains used in this study were isolated from environmental samples collected at −20 m of depth (sub-sea sediments) near the Mario Zucchelli Station, Baia Terranova, Ross sea, Antarctica (74.6936° S, 164.1117° E). 1 gr of sediments was mixed with 20 mL of M9 salts solution (KH_2PO_4 3.0 g/L, $Na_2HPO_4$6.0 g/L, NaCl 0.5 g/L, NH_4Cl 1.0 g/L) in a 50 mL Falcon tube and gently mixed; the supernatant was serially diluted in sterile M9 buffer and plated on PYG medium (Peptone 5.0 g/L, Yeast extract 4.0 g/L, Glucose 1.0 g/L, $CaCl_2$ 0.2 g/L, $MgSO_4.7H_2O$ 0.4 g/L, K_2HPO_4 1.0 g/L, KH_2PO_4 1.0 g/L, $NaHCO_3$10.0 g/L NaCl 2.0 g/L and 17 g/L). After 15 days of incubation, 24 visible colonies were picked, grown in liquid PYG and stored at −80 °C.

3.2. Target Strains and Growth Conditions

Bcc strains used in this work are listed in Table 2 and Table S1. Bcc and *S. aureus* 6538P were routinely grown on Luria-Bertani broth (LB) (Tryptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L) at 37 °C. BTN isolated Antactic strains were routinely grown in TYP medium (Bacto-tryptone 16 g/L, 16 g/L Yeast extract, 10 g/L NaCl) and Marine Broth (MB) at 21 °C. To allow bacterial growth on solid media, 17 g/L of bacteriological agar were added to each medium.

3.3. RAPD Analysis

Typing of bacterial isolates was performed using the Random Amplified Polymorphic DNA (RAPD) technique performed on cell lysates [33–35]; to this purpose, Antarctic bacterial colonies grown overnight at 21 °C on MA plates were suspended in 25 µL of sterile distilled water, heated to 95 °C for 10 min, and cooled on ice for 5 min. RAPD analysis was carried out in a total volume of 25 µL containing 1× Reaction Buffer, 300 µM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 0.5 U of Polytaq DNA polymerase (Polymed, Florence, Italy), 10 µM of primer 1253 (5′GTTTCCGCCC3′) or primer AP5 (5′TCACGCTGCG3′) and 2 µL of lysate cell suspension [35]. PCR were performed using MasterCycle Personal Thermal Cycler (Eppendorf, Hamburg, Germany). After incubation at 90 °C for 1 min and 95 °C for 1.5 min, the reaction mixtures were cycled 45 times through the following temperature profile: 95 °C for 30 s, 36 °C for 1 min, and 75 °C for 1 min. Samples were then incubated at 60 °C for 10 min, and finally at 5 °C for 10 min. Amplification products were then stored at −20 °C. Reaction products were analyzed by agarose (2.5 % *w*/*v*) gel electrophoresis in TAE buffer containing 0.5 µg/mL (*w*/*v*) of ethidium bromide.

3.4. Phylogenetic Affiliation of BTN Strains

Two µL of each cell lysate were used for the amplification via PCR of 16S rRNA genes. PCR was carried out in a total volume of 50 µL containing 1X Reaction Buffer, 150 µM $MgCl_2$, 250 µM of each deoxynucleoside triphosphate, and 2.0 U of Polytaq DNA polymerase and 0.6 µM of primer P0 (5′GAGAGTTTGATCCTGGCTCAG3′) and P6 (5′CTACGGCTACCTTGTTACGA3′) [36]. The reaction conditions used were: one cycle (95 °C for 90 s), 30 cycles (95 °C 30 s, 50 °C 30 s, and 72 °C 1 min), with a final extension of 10 min at 72 °C. Amplicons corresponding to the 16S rRNA genes (observed under UV light, 312 nm) were excised from the gel and purified using the "QIAquick" gel extraction kit (QIAGEN, Chatsworth, CA, USA) according to manufacturer's instructions. Direct sequencing was performed on both DNA strands using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA) and the chemical dye terminator [37]. Each 16S rRNA gene sequence was submitted to GenBank and assigned the accession number shown in Table 1. BLAST probing of DNA databases was performed with the BLASTn option of the BLAST program using default parameters [38]. Nucleotide sequences were retrieved from RDP databases. The ClustalW program was used to align the 16S rRNA gene sequences obtained with the most similar ones retrieved from the databases [39]. Each alignment was checked manually, corrected, and then analyzed. The evolutionary history was inferred using the Neighbor-Joining method according to the model of Kimura two-parameter distances [40,41]. The percentage of replicate trees where the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [42].

3.5. Cross-Streaking

Cross-streaking experiments were carried out as previously described [11]. Petri dishes with or without a septum separating two hemi-cycles were used. Plates with a central septum allowed the growth of tester and target strains without any physical contact. Antarctic strains (tester strains) were grown on MA for four days at 21 °C; then, they were streaked on TYP and incubated at 21 °C for four days. Bcc strains (target strains) were perpendicularly streaked to the initial streak and plates were further incubated at 21 °C for two days and at 37 °C for two additional days. The experiments were conducted in parallel with a positive control to verify the viability of Bcc cells.

3.6. Extract Preparation

A single colony of a bacterial isolate was used to inoculate 3 mL of liquid TYP media in sterile bacteriological tubes. After 48 h of incubation at 21 °C at 200 rpm, the pre-inoculum was used to inoculate 100 mL of TYP 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 21 °C at 200 rpm. The cultures were then centrifuged

at 6800× *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 three volumes of ethyl acetate in a 500 mL separatory funnel. The organic phase collected was evaporated using a Laborota 4000 rotary evaporator (Heidolph, Schwabach, Germany), and the extracts were weight, dissolved in 100% DMSO at 50 or 100 mg/mL and stored at −20 °C.

3.7. Antimicrobial Assays

3.7.1. Minimal Inhibitory Concentration Assay (MIC)

To evaluate the antimicrobial potential of Antarctic 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. Wells containing no compound represented the negative control. DMSO was used as control to determine the effect of solvent on cell growth. A single colony of a Bcc strain was used to inoculate 3 mL of liquid LB media in sterile bacteriological tube. 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.

3.7.2. Minimal Bactericidal Concentration (MBC) Assay

To determine the MBC, the dilution representing the MIC and two of the more concentrated test product dilutions were plated on LB agar plates and enumerated to determine CFU/mL. An aliquot of the positive control was plated and used to establish a baseline concentration of the microorganism used.

3.8. Purification of Ethyl-Acetate Crude Extract

Crude extract of 3 L BTN1 fermentation, prepared as described above, was subjected to fractionation using Chromabond SPE C18 column cartridges (Macherey-Nagel, Duren, Germany). HPLC separations were carried out using a VP 250/10 Nucleodur C18 HTec, 5 µm, (Macherey-Nagel Duren, Germany) connected to a Ultimate 3000 HPLC Chromatograph with a Ultimate 3000 Diode Array detector and in-line degasser (Dionex, Sunnyvale, CA, USA). Detection was achieved on-line through a scan of wavelengths from 200 to 400 nm. This process yielded 4.8 mg of **1**, 5.3 mg of **2** and 5.7 mg of **3**.

Compound 2. $[\alpha]_D$ −53.4° (*c* 0.001 MeOH; UV(MeOH) λ_{max} (log ε) 202 (3.55) nm; IR (film) υmax 3361, 2925, 2855, 1735, 1671, 1575, 1455, 1380, 1314, 1207, 1161, 1126, 1037, 983, cm^{-1}; ^1H, ^{13}C, HMBC NMR data see Table 1; HRESIMS *m*/*z* 553.3343 Δ −0.75 ppm $[M + Na]^+$ calculated for $C_{28}H_{50}O_9$.

Compound 3. $[\alpha]_D$ +49.3° (*c* 0.001 MeOH UV(MeOH) λ_{max} (log ε) 202 (3.78) nm; IR (film) υmax 3387, 2926, 2855, 1667, 1587, 1402, 1316, 1204, 1130, 1072, 1049, 983 cm^{-1}; ^1H, ^{13}C, HMBC NMR data see Table 1; HRESIMS *m*/*z* 581.3649 Δ 1.72 ppm $[M + Na]^+$ calculated for $C_{30}H_{54}O_9$.

3.9. NMR–LCMS Experiments

NMR data, both 1D and 2D were recorded on a spectrometer (Bruker, Billerica, MA, USA) at 600 and 150 MHz for ^1H and ^{13}C, respectively, using an ID cryoprobe in methanol-d_4 as solvent. Chemical shifts are reported in parts per million (δ/ppm) downfield relative to residual CD_3OD at 3.31 ppm for protons and 49.0 ppm for carbons. High-resolution mass spectrometry and fragmentation data were recorded using an LTQ Orbitrap system (ThermoScientific, Whaltman, MA, USA) coupled to an 1290 Infinity HPLC system (Agilent, Santa Clara, CA, USA). The following conditions were used: capillary voltage 45 V, capillary temperature 320 °C, auxiliary gas flow rate 10–20 arbitrary units, sheath gas flow rate 40–50 arbitrary units, spray voltage 4.5 kV, mass range 100–2000 amu (maximum

resolution 30,000). Optical rotation measurements were recorded using a Perkin Elmer, Model 343 Polarimeter at 589 nm (Perkin Elmer, Whaltman, MA, USA). The UV spectrum was recorded on a UV-Vis spectrophotometer model S10 (Spectromlab, Barcelona, Spain). The IR was recorded on a PerkinElmer FTIR Spectrum Two instrument (Perkin Elmer, Whaltman, MA, USA).

4. Conclusions

Exploiting a bioassay-driven purification approach, three RLs (one of which was novel) with antimicrobial activity against Bcc strains, were isolated from *Pseudomonas* sp. BTN1, recovered from Antarctic sediments. RLs represent a promising class of biosurfactants as antimicrobials or in combination with antibiotics. A recent study suggested the use of RLs as an additive in the formulation of antibiotic and other antimicrobial agents for enhancing the effectiveness of chemotherapeutics [43]. Moreover, the possibility of RL production by the fermentation of organic waste (such us waste oils), makes these products economically appealing [44]. To the best of our knowledge, this is the first report of antimicrobial activity of RLs against Bcc strains, and it prompts future studies aimed at RL exploitation as drugs to counteract these hazardous opportunistic human pathogens.

Acknowledgments: This work was supported by the EU FP7 KBBE 2012–2016 project PharmaSea, grant N° 312184 and from the Italian Cystic Fibrosis Research foundation (Grant FFC#12/2011).

Author Contributions: P.T., I.M., R.F., J.T., M.J. and D.D.P. conceived and designed the experiments; P.T., I.M., F.P.E., E.T., J.T., K.S., C.C.E., and Y.Z. performed the experiments; P.T., I.M., F.P.E., K.S., C.C.E., Y.Z. R.F., J.T., and D.D.P. analyzed the data; R.F., M.J. and D.D.P. contributed reagents/materials/analysis tools; P.T., I.M., F.P.E., J.T., R.F., M.J. and D.D.P. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Berdy, J. Thoughts and facts about antibiotics: Where we are now and where we are heading. *J. Antibiot.* **2012**, *65*, 385–395. [CrossRef] [PubMed]
2. Tegos, G.P.; Hamblin, M.R. Disruptive innovations: New anti-infectives in the age of resistance. *Curr. Opin. Pharmacol.* **2013**, *13*, 673–677. [CrossRef] [PubMed]
3. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **2012**, *75*, 311–335. [CrossRef] [PubMed]
4. Bologa, C.G.; Ursu, O.; Oprea, T.I.; Melancon, C.E., 3rd; Tegos, G.P. Emerging trends in the discovery of natural product antibacterials. *Curr. Opin. Pharmacol.* **2013**, *13*, 678–687. [CrossRef] [PubMed]
5. Zhu, F.; Ma, X.H.; Qin, C.; Tao, L.; Liu, X.; Shi, Z.; Zhang, C.L.; Tan, C.Y.; Chen, Y.Z.; Jiang, Y.Y. Drug discovery prospect from untapped species: Indications from approved natural product drugs. *PLoS ONE* **2012**, *7*, e39782. [CrossRef] [PubMed]
6. Jaspars, M.; Challis, G. Microbiology: A talented genus. *Nature* **2014**, *506*, 38–39. [CrossRef] [PubMed]
7. Von Salm, J.L.; Wilson, N.G.; Vesely, B.A.; Kyle, D.E.; Cuce, J.; Baker, B.J. Shagenes A and B, new tricyclic sesquiterpenes produced by an undescribed Antarctic octocoral. *Org. Lett.* **2014**, *16*, 2630–2633. [CrossRef] [PubMed]
8. Godinho, V.M.; Goncalves, V.N.; Santiago, I.F.; Figueredo, H.M.; Vitoreli, G.A.; Schaefer, C.E.; Barbosa, E.C.; Oliveira, J.G.; Alves, T.M.; Zani, C.L.; *et al.* Diversity and bioprospection of fungal community present in oligotrophic soil of continental Antarctica. *Extremophiles* **2015**, *19*, 585–596. [CrossRef] [PubMed]
9. Lee, L.H.; Cheah, Y.K.; Nurul Syakima, A.M.; Shiran, M.S.; Tang, Y.L.; Lin, H.P.; Hong, K. Analysis of Antarctic proteobacteria by PCR fingerprinting and screening for antimicrobial secondary metabolites. *Genet. Mol. Res.* **2012**, *11*, 1627–1641. [CrossRef] [PubMed]
10. Papa, R.; Parrilli, E.; Sannino, F.; Barbato, G.; Tutino, M.L.; Artini, M.; Selan, L. Anti-biofilm activity of the Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125. *Res. Microbiol.* **2013**, *164*, 450–456. [CrossRef] [PubMed]
11. Papaleo, M.C.; Fondi, M.; Maida, I.; Perrin, E.; Lo Giudice, A.; Michaud, L.; Mangano, S.; Bartolucci, G.; Romoli, R.; Fani, R. Sponge-associated microbial Antarctic communities exhibiting antimicrobial activity against *Burkholderia cepacia* complex bacteria. *Biotechnol. Adv.* **2012**, *30*, 272–293. [CrossRef] [PubMed]

12. Papaleo, M.C.; Romoli, R.; Bartolucci, G.; Maida, I.; Perrin, E.; Fondi, M.; Orlandini, V.; Mengoni, A.; Emiliani, G.; Tutino, M.L.; *et al.* Bioactive volatile organic compounds from Antarctic (sponges) bacteria. *N. Biotechnol.* **2013**, *30*, 824–838. [CrossRef] [PubMed]
13. Romoli, R.; Papaleo, M.C.; de Pascale, D.; Tutino, M.L.; Michaud, L.; LoGiudice, A.; Fani, R.; Bartolucci, G. Characterization of the volatile profile of Antarctic bacteria by using solid-phase microextraction-gas chromatography-mass spectrometry. *J. Mass Spectrom.* **2011**, *46*, 1051–1059. [CrossRef] [PubMed]
14. Maida I., B.E.; Fondi, M.; Perrin, E.; Orlandini, V.; Papaleo, M.C.; Mengoni, A.; de Pascale, D.; Tutino, M.L.; Michaud, L.; Lo Giudice, A.; *et al.* Antimicrobial activity of *Pseudoalteromonas* strains isolated from the ross sea (Antarctica) *vs.* cystic fibrosis opportunistic pathogens. *Hydrobiologia* **2015**. in press.
15. Romoli, R.; Papaleo, M.C.; de Pascale, D.; Tutino, M.L.; Michaud, L.; Lo Giudice, A.; Fani, R.; Bartolucci, G. GC-MS volatolomic approach to study the antimicrobial activity of the Antarctic bacterium *Pseudoalteromonas* sp.Tb41. *Metabolomics* **2013**, *10*, 42–51.
16. Coenye, T.; Vandamme, P. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ. Microbiol.* **2003**, *5*, 719–729. [CrossRef] [PubMed]
17. Compant, S.; Nowak, J.; Coenye, T.; Clement, C.; Ait Barka, E. Diversity and occurrence of *Burkholderia* spp. In the natural environment. *FEMS Microbiol. Rev.* **2008**, *32*, 607–626. [CrossRef] [PubMed]
18. De Smet, B.; Mayo, M.; Peeters, C.; Zlosnik, J.E.; Spilker, T.; Hird, T.J.; LiPuma, J.J.; Kidd, T.J.; Kaestli, M.; Ginther, J.L.; *et al.* *Burkholderia stagnalis* sp. Nov. And *Burkholderia territorii* sp. Nov., two novel *Burkholderia cepacia* complex species from environmental and human sources. *Int. J. Syst. Evol. Microbiol.* **2015**, *65*, 2265–2271. [CrossRef] [PubMed]
19. Drevinek, P.; Mahenthiralingam, E. *Burkholderia cenocepacia* in cystic fibrosis: Epidemiology and molecular mechanisms of virulence. *Clin. Microbiol. Infect.* **2010**, *16*, 821–830. [CrossRef] [PubMed]
20. Ramsay, K.A.; Butler, C.A.; Paynter, S.; Ware, R.S.; Kidd, T.J.; Wainwright, C.E.; Bell, S.C. Factors influencing acquisition of *Burkholderia cepacia* complex organisms in patients with cystic fibrosis. *J. Clin. Microbiol.* **2013**, *51*, 3975–3980. [CrossRef] [PubMed]
21. Rose, H.; Baldwin, A.; Dowson, C.G.; Mahenthiralingam, E. Biocide susceptibility of the *Burkholderia cepacia* complex. *J. Antimicrob. Chemother.* **2009**, *63*, 502–510. [CrossRef] [PubMed]
22. Nash, E.F.; Thomas, A.; Whitmill, R.; Rashid, R.; Barker, B.; Rayner, R.J.; Whitehouse, J.L.; Honeybourne, D. "Cepacia syndrome" associated with *Burkholderia cepacia* (Genomovar I) infection in an adolescent with cystic fibrosis. *Pediatr. Pulmonol.* **2010**, *46*, 512–514. [CrossRef] [PubMed]
23. Tegos, G.P.; Haynes, M.K.; Schweizer, H.P. Dissecting novel virulent determinants in the *Burkholderia cepacia* complex. *Virulence* **2012**, *3*, 234–237. [CrossRef] [PubMed]
24. Sharma, A.; Jansen, R.; Nimtz, M.; Johri, B.N.; Wray, V. Rhamnolipids from the rhizosphere bacterium *Pseudomonas* sp. Grp(3) that reduces damping-off disease in chilli and tomato nurseries. *J. Nat. Prod.* **2007**, *70*, 941–947. [CrossRef] [PubMed]
25. De Bruyn, A.; Anteunis, M. 1H-N.m.r. study of L-rhamnose, methyl alpha-L-rhamnopyranoside, and 4-*O*-beta-D-galactopranosyl-L-rhamnose in deuterium oxide. *Carbohydr. Res.* **1976**, *47*, 158–163. [CrossRef]
26. Das, P.; Mukherjee, S.; Sen, R. Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans*. *J. Appl. Microbiol.* **2008**, *104*, 1675–1684. [CrossRef] [PubMed]
27. Das, P.; Yang, X.P.; Ma, L.Z. Analysis of biosurfactants from industrially viable pseudomonas strain isolated from crude oil suggests how rhamnolipids congeners affect emulsification property and antimicrobial activity. *Front. Microbiol.* **2014**, *5*, 696. [CrossRef] [PubMed]
28. Abdel-Mawgoud, A.M.; Lepine, F.; Deziel, E. Rhamnolipids: Diversity of structures, microbial origins and roles. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1323–1336. [CrossRef] [PubMed]
29. Haba, E.; Pinazo, A.; Jauregui, O.; Espuny, M.J.; Infante, M.R.; Manresa, A. Physicochemical characterization and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044. *Biotechnol. Bioeng.* **2003**, *81*, 316–322. [CrossRef] [PubMed]
30. Benincasa, M.; Abalos, A.; Oliveira, I.; Manresa, A. Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* lBI from soapstock. *Antonie van Leeuwenhoek* **2004**, *85*, 1–8. [CrossRef] [PubMed]

31. Haba, E.; Bouhdid, S.; Torrego-Solana, N.; Marques, A.M.; Espuny, M.J.; Garcia-Celma, M.J.; Manresa, A. Rhamnolipids as emulsifying agents for essential oil formulations: Antimicrobial effect against *Candidaalbicans* and methicillin-resistant *Staphylococcus aureus*. *Int. J. Pharm.* **2014**, *476*, 134–141. [CrossRef] [PubMed]
32. Sotirova, A.V.; Spasova, D.I.; Galabova, D.N.; Karpenko, E.; Shulga, A. Rhamnolipid-biosurfactant permeabilizing effects on gram-positive and gram-negative bacterial strains. *Curr. Microbiol.* **2008**, *56*, 639–644. [CrossRef] [PubMed]
33. Williams, J.G.; Kubelik, A.R.; Livak, K.J.; Rafalski, J.A.; Tingey, S.V. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **1990**, *18*, 6531–6535. [CrossRef] [PubMed]
34. Welsh, J.; McClelland, M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **1990**, *18*, 7213–7218. [CrossRef] [PubMed]
35. Mori, E.; Lio, P.; Daly, S.; Damiani, G.; Perito, B.; Fani, R. Molecular nature of RAPD markers from *Haemophilus influenzae* Rd genome. *Res. Microbiol.* **1999**, *150*, 83–93. [CrossRef]
36. Grifoni, A.; Bazzicalupo, M.; Di Serio, C.; Fancelli, S.; Fani, R. Identification of *Azospirillum* strains by restriction fragment length polymorphism of the 16s rDNA and of the histidine operon. *FEMS Microbiol. Lett.* **1995**, *127*, 85–91. [CrossRef] [PubMed]
37. Sanger, F.; Nicklen, S.; Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 5463–5467. [CrossRef] [PubMed]
38. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [CrossRef] [PubMed]
39. Thompson, J.D.; Higgins, D.G.; Gibson, T.J. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **1994**, *22*, 4673–4680. [CrossRef] [PubMed]
40. Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [PubMed]
41. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]
42. Felsenstein, J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **1985**, *39*, 783–791. [CrossRef]
43. Bharali, P.; Saikia, J.P.; Ray, A.; Konwar, B.K. Rhamnolipid (RL) from *Pseudomonas aeruginosa* OBP1: A novel chemotaxis and antibacterial agent. *Colloids Surf. B Biointerfaces* **2013**, *103*, 502–509. [CrossRef] [PubMed]
44. Dobler, L.; Vilela, L.F.; Almeida, R.V.; Neves, B.C. Rhamnolipids in perspective: Gene regulatory pathways, metabolic engineering, production and technological forecasting. *N. Biotechnol.* **2016**, *33*, 123–135. [CrossRef] [PubMed]

BJD
British Journal of Dermatology

A new T helper 17 cytokine in hidradenitis suppurativa: antimicrobial and proinflammatory role of interleukin-26

E. Scala,[1] R. Di Caprio,[1] S. Cacciapuoti,[1] G. Caiazzo,[1] A. Fusco,[2] E. Tortorella,[3] G. Fabbrocini[1] and A. Balato[4]

[1]Department of Clinical Medicine and Surgery and [4]Department of Advanced Biomedical Sciences, University of Naples Federico II, Via Pansini 5, 80131, Naples, Italy
[2]Department of Experimental Medicine, University of Campania 'Luigi Vanvitelli', Naples, Italy
[3]Institute of Protein Biochemistry, National Research Council, Naples, Italy

Linked Comment: Giamarellos-Bourboulis. Br J Dermatol 2019; **181**:892–893.

Summary

Background Interleukin (IL)-26 is a signature T helper 17 cytokine described as a proinflammatory and antimicrobial mediator. So far, IL-26 has been reported in several immune-mediated inflammatory diseases, but its involvement in inflammatory skin disorders is poorly known.
Objectives To investigate the role of IL-26 in hidradenitis suppurativa (HS), through its involvement in antimicrobial activity.
Methods IL-26 was assessed in patients with HS through gene expression and protein analysis at skin and circulating levels. *Ex vivo* HS organ skin cultures, together with IL-26 antibody treatment, were performed to determine the IL-26 activity. Peripheral blood mononuclear cells (PBMCs) from patients with HS and healthy controls were either silenced or not with IL-26 small interfering (si)RNA in order to measure its antimicrobial, cytotoxic and phagocytic activities against *Staphylococcus aureus*.
Results Firstly, we observed that IL-26 is able to modulate the proinflammatory response at the immune cell level. IL-26 was increased in the plasma of patients with HS compared with healthy controls. Subsequently, we explored the bactericidal, cytotoxic and phagocytic activities of PBMCs against *S. aureus* in patients with HS and healthy controls. These activities were lower in patients with HS than in controls. Remarkably, the killing activities were reduced when healthy control PBMCs were transfected with IL-26 siRNA. However, the transfection did not affect the killing activity of HS PBMCs, supporting the idea that IL-26 lacks efficacy in HS.
Conclusions Our findings suggest that infection susceptibility in HS might be related to IL-26. Although the role of bacteria remains controversial in HS, this paper supports that there is a defect of antimicrobial response in these patients.

Correspondence
Anna Balato.
E-mail: *annabalato@yahoo.it*

Accepted for publication
28 February 2019

Funding sources
None.

Conflicts of interest
None to declare.

DOI 10.1111/bjd.17854

What's already known about this topic?

- Interleukin (IL)-26 is a T helper 17 cytokine described as an antimicrobial and proinflammatory mediator.
- IL-26 has been reported in immune-mediated inflammatory diseases, but its involvement in inflammatory skin disorders remains unclear.
- Hidradenitis suppurativa (HS) is a chronic inflammatory skin disorder characterized by deficiency of IL-20 and IL-22 (a close homologue of IL-26), which causes antimicrobial peptide pauperization leading to severe and recurrent skin infections.

What does this study add?

- IL-26 plasma levels are higher in patients with HS than in healthy control individuals.
- The antimicrobial activity of IL-26 might be ineffective in patients with HS.

What is the translational message?

- Cutaneous antimicrobial incompetence in HS could be related to IL-26.

Interleukin (IL)-26 is a member of the IL-10 cytokine family, which includes IL-10, interferon-λs (IL-28A/B and IL-29) and the IL-20 subfamily (IL-19, IL-20, IL-22, IL-24 and IL-26).[1–3] IL-26 is expressed mainly in T helper (Th)17 cells and plays an important role in enhancing the host defence against pathogens.[4–6] IL-26 is a proinflammatory cytokine that exerts an antimicrobial response producing direct bactericidal action by affecting the formation of membrane pores.[6] IL-26 acts through the IL-10R2–IL-20R1 heterodimeric receptor.[7,8] While IL-10R2 is broadly expressed, IL-20R1 is mainly expressed by epithelial cell types, including keratinocytes,[9,10] suggesting its possible role in the immune response of the skin.

To date, IL-26 has been implicated in psoriasis and allergic contact dermatitis (ACD).[11,12] In particular, we have previously shown that IL-26 contributes to the cytotoxic mechanism in ACD.[12] This mechanism is responsible for tissue injury in immune-mediated skin diseases, including hidradenitis suppurativa (HS).[13] HS is characterized by recurrent, painful nodules and abscesses that break, leading to the formation of sinus tracts and scarring.[14] Lesions usually affect apocrine gland-bearing anatomical areas of the body. This involves build-up of keratin within the hair follicle, which plugs the follicle causing the occlusion and subsequent rupture of the pilosebaceous unit.[15–18] Moreover, colonies of specialized bacteria (mainly staphylococci and streptococci) bind irreversibly to the sinus tract epithelium and hair follicles, further sustaining chronic inflammation.[19–23] Here we evaluate the role of IL-26 in HS, exploring its antimicrobial activity.

Patients and methods

Study population

The overall study population included 30 patients [eight affected by atopic dermatitis (AD), 12 affected by HS and 10 affected by psoriasis] and 30 age- and sex-matched healthy control volunteers. All participants were recruited from the database of patients attending the dermatology unit of the University of Naples Federico II, Italy. Inclusion criteria were diagnosis of moderate-to-severe AD (Scoring Atopic Dermatitis > 25), HS (Sartorius score > 30) or psoriasis (Psoriasis Area and Severity Index > 10); disease duration of at least 6 months; a topical and/or systemic treatment washout period of ≥ 3 weeks and age ≥ 18 years. Patients underwent lesional skin biopsies (3 mm) and blood extraction. For controls, blood and skin samples were taken from healthy donors. The inclusion criterion for healthy volunteers was age ≥ 18 years, and exclusion criteria were a present or past positive history of inflammatory skin disorders.

The experimental protocol was performed according to the current version of the Declaration of Helsinki, and each participant gave written informed consent before the onset of the study. The clinical and demographic characteristics of the patients with HS are summarized in Table 1. The disease severity of the HS population was defined according to Sartorius score and International Hidradenitis Suppurativa Severity Score System (IHS4) scale.[24,25] None of the recruited patients presented a Sartorius score below 50 or higher than 95. In addition, the patients had an IHS4 score ranging from 4 to 10 points. Thus, our study is focused on patients with moderate HS.

Peripheral blood mononuclear cell preparation and stimulation by toxic shock syndrome toxin-1

Blood samples were used for plasma and peripheral blood mononuclear cell (PBMC) isolation. IL-26 plasma levels from patients with HS (n = 12) and healthy controls (n = 12) were measured by enzyme-linked immunosorbent assay (ELISA) (Cusabio, Wuhan, China). PBMCs from healthy controls (n = 8) and patients with HS (n = 8) were separated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). For silencing experiments, PBMCs were transfected with 3 μmol L^{-1} Riboxx small interfering (si)RNA oligo (Qiagen, Venlo, the Netherlands) using electroporation (Amaxa Inc., Gaithersburg, MD, U.S.A.). IL-26 siRNA target sequence or control siRNA oligonucleotide with scrambled sequence was used. More than 80% of cells remained viable after the transfection procedures, as determined by trypan blue exclusion.

PBMCs silenced or not were placed in 12-well plates (1×10^6 per well) and cultured in Roswell Park Memorial Institute (RPMI) medium (Thermo Fisher Scientific Inc., Waltham, MA,

Table 1 Clinical and demographic characteristics of patients with hidradenitis suppurativa (n = 12)

Male, n (%)	3 (25)
Age (years)	27·3 ± 8·32
Disease duration (years)	7·42 ± 4·19
Family history (yes), n (%)	2 (17)
Body mass index (kg m^{-2})	28·4 ± 3·6
iHS4	6·25 ± 2·49
Sartorius score	61·1 ± 10·1

Data are given as the mean ± SD unless stated otherwise. iHS4, International Hidradenitis Suppurativa Severity Score System.

U.S.A.) supplemented with 10% fetal bovine serum in the presence or absence of toxic shock syndrome toxin (TSST)-1 (Sigma-Aldrich, Oakville, ON, Canada) at a final concentration of 100 ng mL^{-1}. Subsequently, mRNA was extracted from PBMCs for examination through quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) of selected genes. Culture supernatants were harvested and stored at −80 °C, and the presence of IL-26 was checked by ELISA and antimicrobial assay.

Antimicrobial assay

To evaluate the bactericidal effect of the collected supernatants, they were placed into a 96-well microtitre plate at an initial concentration of 4000 μg mL^{-1}. Then they were serially diluted using Luria Bertani (LB) medium (twofold dilution). The final concentrations of samples were 2000, 1000, 500, 250 and 125 μg mL^{-1}. A single colony of the selected pathogen strains, *Staphylococcus aureus* ATCC 6538 or *Pseudomonas aeruginosa* ATCC 27853, was used to inoculate 3 mL of liquid LB medium in a sterile bacteriological tube. Dimethyl sulfoxide (2% v/v) was used as a negative control. After 5 h of incubation, growth was measured by monitoring the absorbance at 600 nm, and about 0·04 optical density per mL in 100 μL was dispensed into each well of the prepared plate, reaching a final volume of 200 μL. Plates were incubated at 37 °C for 24 h and growth was measured with a Cytation3 Plate Reader (Biotek, Winoosky, VT, U.S.A.) by monitoring the absorbance at 600 nm. The antimicrobial activity of the samples is reported as the IC_{50}, the half maximal inhibitory concentration.

Cytotoxicity assay

HaCaT target cells (2500 per well) were plated in 96-well flat-bottom plates in the presence or absence of *S. aureus* as described previously.[26] They were cocultured with PBMCs (healthy controls, n = 8; HS, n = 8) transfected or not at various target–effector ratios for 5 h at 37 °C. Next, supernatants were checked for the presence of lactate dehydrogenase (Pierce LDH Cytotoxicity assay; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol.

Phagocytosis assay

PBMCs, transfected or not (healthy controls, n = 8; HS, n = 8), were cultured to adhere to 96-well plates maintaining the same effector ratios used for the cytotoxicity tests. After 30 min, nonadherent cells were removed by repeated washes in RPMI medium, as described previously.[27] Then, 10^8 colony-forming units (CFU) per mL of *S. aureus* was added to the plates before incubation at 37 °C for 1 h. A control test in the absence of PBMCs was carried out. Adherent cells were lysed with 0·1% Triton X-100. Solubilized bacteria were counted by spreading serial dilutions on trypticase soy agar plates and incubation at 37 °C overnight. The bacterial survival index was calculated with the equation: CFU number in the experimental test divided by CFU number in the control test. A survival index ≥ 1·0 represented bacteria that were not killed by PBMCs.

In vivo skin analysis

Skin biopsies (3 mm) from involved sites of patients affected by AD (n = 8), HS (n = 10) or psoriasis (n = 10), as well as from normal skin of healthy controls (n = 20), were used for mRNA examination through qRT-PCR (LightCycler; Roche, Indianapolis, IN, U.S.A.) of *IL26*, *DEFB4A* [encoding human β-defensin (HBD)2] and *DEFB103A* (encoding HBD3). The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of the 18S reference gene. Fold induction of gene expression was calculated using the ΔΔCT method.

Ex vivo skin organ cultures

Three skin biopsies (3 mm) obtained from involved sites of each patient with HS (n = 9), as well as from normal skin of healthy controls (n = 9), were placed in 12-well culture plates (Becton Dickinson Co., Franklin Lakes, NJ, U.S.A.) with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 2 mmol L^{-1} L-glutamine (all Thermo Fisher Scientific Inc.). Biopsies were treated in the presence or absence of IL-26-neutralizing antibodies or respective isotype IgG controls (R&D Systems Inc., Minneapolis, MN, U.S.A.) at a final concentration of 5, 10 or 15 μg mL^{-1}. Specimens were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. mRNA was extracted from each sample for qRT-PCR analysis of selected genes.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6·0 (GraphPad Software Inc., La Jolla, CA, U.S.A.). Data were analysed with Wilcoxon matched-pairs test or Mann–Whitney test to calculate statistical differences, and correlations were evaluated with Pearson's rho test. Values of $P < 0·05$ were considered significant. Data are expressed as the mean ± SD.

Results

Interleukin-26 acts as a proinflammatory and antimicrobial mediator in healthy controls

Healthy control PBMCs were stimulated with TSST-1, a superantigen secreted by *S. aureus*. This stimulation was able to induce an increase of IL-26 at both the gene and protein level in control PBMCs ($P < 0·05$ and $P < 0·001$, respectively) (Fig. S1; see Supporting Information). To confirm IL-26 induction after infection, healthy control PBMCs were transfected with *IL26* siRNA or empty vector and stimulated with TSST-1. The transfection was able to reverse IL-26 induction

(Fig. S1). Expression of the genes encoding IL-1β, IL-23 and IL-17A was significantly decreased in IL-26-transfected cells after TSST-1 stimulation compared to nontransfected cells ($P < 0{\cdot}05$) (Fig. S2; see Supporting Information). No significant changes were observed in terms of gene modulation when healthy control PBMCs were transfected with empty vector (data not shown).

Considering the bactericidal role of IL-26 against several bacteria,[6,11] increasing concentrations of supernatants (from 125 to 1000 μg mL^{-1}) from healthy control PBMCs in resting, TSST-1-stimulated and IL-26-silenced conditions were incubated with *P. aeruginosa* or *S. aureus*. The results shown in Table 2 demonstrate that supernatants were totally inactive against *P. aeruginosa*, whereas *S. aureus* growth was inhibited by supernatants of healthy control PBMCs previously stimulated with TSST-1 (IC_{50} value 125 μg mL^{-1}). A slight bioactivity against *P. aeruginosa* was observed with supernatants from stimulated healthy control PBMCs (at higher concentrations ranging from 2000 to 4000 μg mL^{-1}), but without reaching an IC_{50} value (data not shown). Supernatants from resting samples, and silenced healthy control PBMCs with or without TSST-1 stimulation showed no inhibitory effects on *S. aureus* growth (Table 2).

Table 2 Antimicrobial activity of supernatants from resting, silenced healthy control (HC) or hidradenitis suppurativa (HS) peripheral blood mononuclear cells with or without toxic shock syndrome toxin (TSST)-1 (100 ng mL^{-1}) stimulation against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

	IC_{50} value	
Samples	*P. aeruginosa*	*S. aureus*
HC resting	NI	NI
HC + TSST-1	NI	125 μg mL^{-1}
HC IL-26 siRNA	NI	NI
HC IL-26 siRNA + TSST-1	NI	NI
HS resting	NI	NI
HS + TSST-1	NI	NI
HS IL-26 siRNA	NI	NI
HS IL-26 siRNA + TSST-1	NI	NI

Bacterial growth was measured as the optical density at 600 nm per mL. IL, interleukin; NI, no inhibition; si, small interfering.

Interleukin-26 is increased in patients with hidradenitis suppurativa

We quantified IL-26 by ELISA in the plasma of patients with HS and healthy controls. IL-26 was significantly increased in patients with HS compared with controls ($P < 0{\cdot}001$) (Fig. 1a). As shown in Figure S3 (see Supporting Information), increasing IL-26 concentration significantly correlated with disease severity ($r = 0{\cdot}6$, $P = 0{\cdot}02$). Cultured PBMCs of patients with HS produced higher basal levels of IL-26 than PBMCs from healthy controls ($P < 0{\cdot}05$) (Fig. 1b). TSST-1 stimulation induced a comparable increase of IL-26 in both HS and control PBMCs (Fig. 1b). IL-26 silencing was able to shut down IL-26 production in HS PBMCs (TSST-1 stimulated or not) and in TSST-1-stimulated healthy control PBMCs (Fig. 1b).

Antimicrobial activity of interleukin-26 in hidradenitis suppurativa

P. aeruginosa or *S. aureus* were incubated with increasing concentrations of supernatants (from 125 to 1000 μg mL^{-1}) from HS PBMCs in resting, TSST-1-stimulated and IL-26-silenced conditions to assess IC_{50} values. The results in Table 2 show that supernatants from resting and silenced HS PBMCs were totally inactive for both bacterial strains. Likewise, HS PBMC supernatants previously stimulated with TSST-1 did not inhibit *S. aureus* growth as previously observed in healthy controls (Table 2). It is noteworthy that the results from silencing experiments performed with empty vector were comparable with those using unsilenced conditions (data not shown).

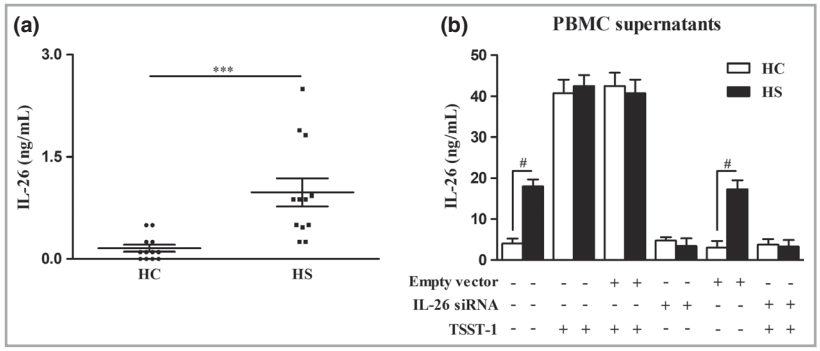


Fig 1. (a) Interleukin (IL)-26 plasma levels in 12 patients with hidradenitis suppurativa (HS) and 12 healthy controls (HC). Data are displayed as the mean ± SD. Statistical significance was assessed using the Mann–Whitney test. ***$P < 0{\cdot}001$. (b) IL-26 protein detection in supernatants derived from cultured HC ($n = 8$) and HS ($n = 8$) peripheral blood mononuclear cells (PBMCs) without or with *IL26* small interfering (si)RNA, empty vector or toxic shock syndrome toxin (TSST)-1 (100 ng mL^{-1}). Data are displayed as the mean ± SD. $^{\#}P < 0{\cdot}05$ calculated using the Mann–Whitney test.

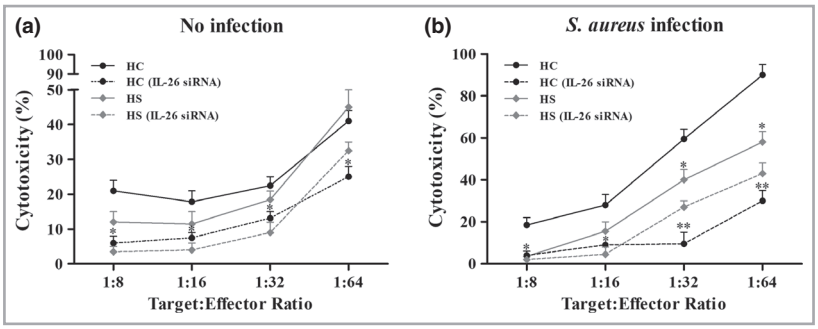


Fig 2. Lactate dehydrogenase release to measure the percentage of cytotoxicity of peripheral blood mononuclear cells (PBMCs) from healthy controls (HC) (n = 5) and patients with hidradenitis suppurativa (HS) (n = 5). PBMCs were transfected or not with *IL26* small interfering (si)RNA vs. HaCaT cells (a) without or (b) with *Staphylococcus aureus* infection. Data are displayed as the mean ± SD. Statistical significance was assessed using the Wilcoxon matched-pairs test (transfected vs. nontransfected) or Mann–Whitney test (HS vs. HC nontransfected). *P < 0·05, **P < 0·01.

Cytotoxic role of interleukin-26 in healthy controls and patients with hidradenitis suppurativa

Healthy control and HS PBMC cytotoxicity mechanisms were evaluated vs. HaCaT cells. The activity of healthy control PBMCs was comparable with that of HS PBMCs (Fig. 2a). IL-26 transfection was able to reduce significantly the capacity of healthy control PBMCs to kill the target cells (Fig. 2a). Conversely, no significant differences were detected in silenced HS PBMCs compared with unsilenced ones (Fig. 2a).

Cytotoxicity mechanisms in healthy control and HS PBMCs were evaluated also against HaCaT cells infected by *S. aureus*. HS PBMC cytotoxicity was significantly decreased compared with control PBMCs at almost all analysed ratios (Fig. 2b). Silenced control PBMCs dramatically lost the ability to kill target cells compared with unsilenced control PBMCs. On the other hand, silenced HS PBMCs showed no significant difference compared with HS unsilenced cells (Fig. 2b). No significant difference was observed between the cells receiving the empty vector and those not transfected (data not shown).

The killing activity of healthy control and HS PBMCs vs. *S. aureus* was thoroughly evaluated by phagocytosis tests. At a target–effector ratio of 1 : 8, HS PBMCs showed a lower phagocytic activity than PBMCs from healthy donors ($P < 0·05$) (Fig. 3). Interestingly, silencing experiments were able to reduce significantly the phagocytosis of control PBMCs ($P < 0·05$), whereas no significant differences were observed between silenced and unsilenced HS PBMCs (Fig. 3). The same outcomes were obtained at all examined target–effector ratios (data not shown). No difference was found between the cells receiving the empty vector and those not transfected (data not shown).

Involvement of interleukin-26 in hidradenitis suppurativa, psoriasis and atopic dermatitis skin

IL26 gene expression was evaluated in clinically different skin conditions to uncover its involvement in chronic inflammatory skin disorders. *IL26* expression was significantly enhanced in HS, psoriasis and AD lesional skin compared with healthy skin

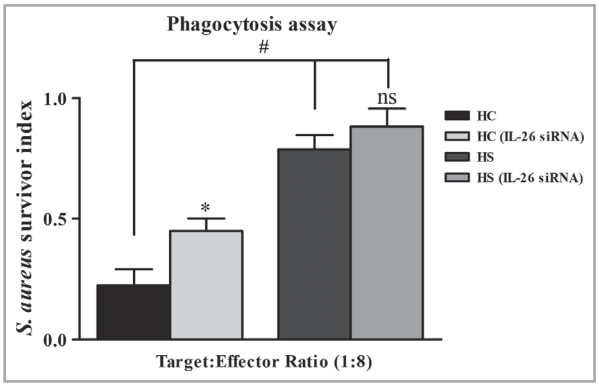


Fig 3. Survival index of *Staphylococcus aureus* in the presence of peripheral blood mononuclear cells (PBMCs) transfected or not with *IL26* small interfering (si)RNA from healthy controls (HC) (n = 5) and patients with hidradenitis suppurativa (HS) (n = 5). Data are displayed as the mean ± SD. Statistical significance was assessed using the Wilcoxon matched-pairs test (transfected vs. nontransfected) or Mann–Whitney test (HS vs. HC). *P < 0·05 (transfected vs. nontransfected), #P < 0·05 (HS vs. HC nontransfected). ns, not significant.

($P < 0·05$) (Fig. 4a). A slight increase, but not significant, of *IL26* expression was observed in HS compared with psoriasis and AD skin (Fig. 4a). *DEFB4A* and *DEFB103A* (encoding HBD2 and HBD3) were significantly enhanced in all diseases compared with healthy control skin (Fig. 4a). Interestingly, a relative significant decrease was found for both *DEFB4A* and *DEFB103A* in HS vs. psoriasis and AD skin ($P < 0·01$), whereas this was not detected for *IL26* (Fig. 4a).

Proinflammatory role of interleukin-26 in hidradenitis suppurativa skin

HS lesional skin was used to perform an *ex vivo* organ culture in the presence and absence of IL-26-neutralizing antibodies or IgG isotype (5, 10 or 15 μg mL^{-1}). Gene expression of *IL1B* and *IL6* was significantly decreased by treatment with IL-26 antibodies (15 μg mL^{-1}) ($P < 0·05$), whereas *IL23A* and

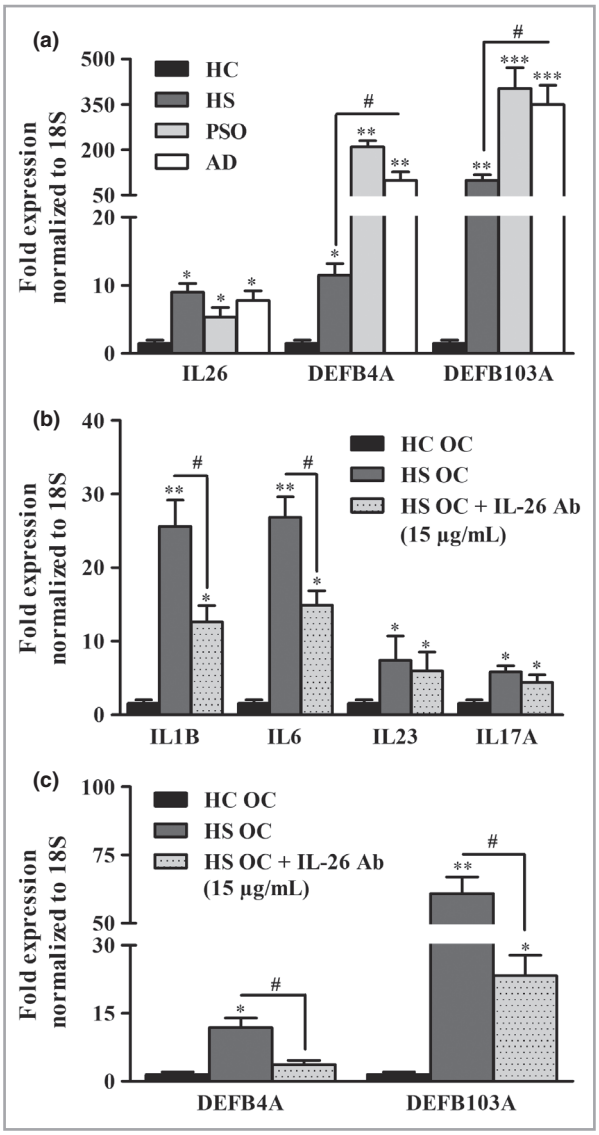


Fig 4. (a) Gene expression of IL26, DEFB4A and DEFB103A [encoding interleukin (IL)-26, human β-defensin (HBD)2 and HBD3, respectively] in patients with hidradenitis suppurativa (HS) (n = 10), psoriasis (PSO) (n = 10) and atopic dermatitis (AD) (n = 8) and in healthy controls (HC) (n = 20). Data are displayed as the mean ± SD. Statistical significance was assessed using the Mann–Whitney test. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ for disease vs. HC; $^{\#}P < 0{\cdot}05$ between PSO, AD and HS. (b, c) Gene expression of (b) IL-1β, IL6, IL23 and IL17A and (c) DEFB4A and DEFB103A in HS *ex vivo* skin organ culture (OC) treated or not with IL-26-neutralizing antibody (Ab) compared with HC OC. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ vs. HC OC, calculated using the Mann–Whitney test. $^{\#}P < 0{\cdot}05$ for IL-26 Ab treatment vs. nontreatment, calculated using the Wilcoxon matched-pairs test.

IL17A were not affected (Fig. 4b). Regarding antimicrobial peptides (AMPs), IL-26 inhibition was able to reduce significantly the expression of both *DEFB4A* and *DEFB103A* ($P < 0{\cdot}05$) (Fig. 4c). No significant differences were observed when HS skin biopsies were treated with IgG isotype controls or with lower concentrations of IL-26 antibodies (data not shown).

Discussion

IL-26 is a signature Th17 cytokine and a member of the IL-10 family, and has been described as both a potent antimicrobial and proinflammatory mediator. IL-26 has previously been reported in immune-mediated inflammatory diseases,[28–30] but its involvement in inflammatory skin disorders is unclear. Here we analysed IL-26 in HS, psoriasis and AD, paying particular attention to its involvement in HS. Our results showed that *IL26* gene expression was enhanced in HS lesional skin, although not significantly, with respect to psoriasis and AD skin, whereas *DEFB4A* and *DEFB103A* levels were lower in HS skin than in psoriasis and AD skin, as reported previously.[31,32] HS is characterized by a deficiency of IL-20 and IL-22 (a close homologue of IL-26), which could cause AMP pauperization by epithelial cells, leading to severe and recurrent infections of the skin.[31,32]

In line with other chronic inflammatory diseases such as rheumatoid arthritis,[28] we found a higher plasma concentration of IL-26 in patients with HS than in healthy controls. Increasing concentrations of IL-26 significantly correlated with disease severity, even if the number of patients was limited and we studied only patients with moderate HS. Upon these observations, we expanded the significance of IL-26 as an inducer of proinflammatory and antimicrobial mediators. Our results suggest that inhibition of IL-26 reduces cutaneous expression of proinflammatory mediators essential for T-cell differentiation, and the expression of AMPs in HS. For the first time, we identified the functional relevance of IL-26 in the positive regulation of HBD2 and HBD3 in HS. To date, other proinflammatory cytokines such as IL-1α, IL-20 and IL-22 have been described as strong inducers of AMPs.[31,33–35] Interestingly, previous neutralization studies on IL-1α have shown reduction of HBD2 in patients with HS.[33]

Going deeper into the antimicrobial activity of IL-26, we compared the behaviour of healthy control and HS PBMCs towards *S. aureus* and *P. aeruginosa*. Although both colonize HS skin, *S. aureus* infections are the most frequently reported.[19] No bactericidal activity against *P. aeruginosa* was observed, whereas *S. aureus* growth was inhibited only by healthy control PBMCs previously stimulated with TSST-1. Curiously, TSST-1-stimulated HS PBMCs were unable to kill *S. aureus*. This difference could be explained by the fact that HS is characterized by the absence or reduction of several cytokines whose cooperation is responsible for antimicrobial processes.[31,36] In particular, Wolk *et al.*[31] have reported that IL-22 and IL-20 downregulation is responsible for limited AMP levels in HS lesions.

We wondered, could these facts correlate with the attenuation of IL-26 function in HS? In view of this, we also explored the cytotoxic activity of IL-26 in HS. Similar to the antimicrobial results, cytotoxic activity during *S. aureus* infection was lower in patients with HS than in healthy volunteers. Interestingly, IL-26 silencing was able to reduce the cytotoxic activity in healthy controls but not in patients with HS, supporting the idea that the IL-26 cargo might be not efficacious in HS. Consistently with the bactericidal and cytotoxic activity,

phagocytosis against *S. aureus* was also lower in patients with HS than in healthy controls. Phagocytosis was strongly reduced by IL-26 silencing only in healthy controls.

Increasing evidence suggests that dysfunctional immune responses occur in HS, leading to the bacterial colonization seen in HS lesional skin.[37–39] Nowadays, it is still debated whether bacterial colonization is a primary or secondary event in HS lesion evolution. It has been speculated that bacterial invasion leads to a series of pathogen-associated molecular pathways, which may trigger the initiation of inflammasomes.[40] Alternatively, the deposition of keratin fragments into the dermis may be the trigger for bacterial colonization.[41] Consistent findings have been reported showing that the major bacterial species found in HS lesional skin are *S. aureus*, coagulase-negative staphylococci and *Corynebacterium* spp., which probably trigger local inflammation.[42,43] The efficacy of antibiotics like rifampicin, clindamycin or tetracycline in HS treatment further supports a microbial role in disease pathogenesis.[44,45]

Despite the role of bacteria in HS remaining controversial, this paper supports the theory that the antimicrobial response is defective in HS, shedding light on the possible involvement of IL-26 in this process along with inflammation.

Acknowledgments

We thank Donatella De Pascale from the National Research Council of Italy, and Giovanna Donnarumma from the Department of Microbiology and Clinical Microbiology, University of Campania 'Luigi Vanvitelli', Naples, Italy for their valuable support.

References

1 Knappe A, Hör S, Wittmann S *et al.* Induction of a novel cellular homolog of interleukin-10, AK155, by transformation of T lymphocytes with herpesvirus saimiri. *J Virol* 2000; **74**:3881–7.
2 Sabat R. IL-10 family of cytokines. *Cytokine Growth Factor Rev* 2010; **21**:315–24.
3 Ouyang W, Rutz S, Crellin NK *et al.* Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol* 2011; **29**:71–109.
4 Wolk K, Kunz S, Asadullah K *et al.* Cutting edge: immune cells as sources and targets of the IL-10 family members? *J Immunol* 2002; **168**:5397–402.
5 Wilson NJ, Boniface K, Chan JR *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 2007; **8**:950–7.
6 Stephen-Victor E, Fickenscher H, Bayry J. IL-26: an emerging proinflammatory member of the IL-10 cytokine family with multifaceted actions in antiviral, antimicrobial, and autoimmune responses. *PLOS Pathog* 2016; **12**:e1005624.
7 Hör S, Pirzer H, Dumoutier L *et al.* The T-cell lymphokine interleukin-26 targets epithelial cells through the interleukin-20 receptor 1 and interleukin-10 receptor 2 chains. *J Biol Chem* 2004; **279**:33343–51.
8 Sheikh F, Baurin VV, Lewis-Antes A *et al.* Cutting edge: IL-26 signals through a novel receptor complex composed of IL-20 receptor 1 and IL-10 receptor 2. *J Immunol* 2004; **172**:2006–10.
9 Nagalakshmi ML, Murphy E, McClanahan T *et al.* Expression patterns of IL-10 ligand and receptor gene families provide leads for biological characterization. *Int Immunopharmacol* 2004; **4**:577–92.
10 Dambacher J, Beigel F, Zitzmann K *et al.* The role of the novel Th17 cytokine IL-26 in intestinal inflammation. *Gut* 2009; **58**:1207–17.
11 Meller S, Di Domizio J, Voo KS *et al.* T_H17 cells promote microbial killing and innate immune sensing of DNA via interleukin 26. *Nat Immunol* 2015; **16**:970–9.
12 Caiazzo G, Di Caprio R, Lembo S *et al.* IL-26 in allergic contact dermatitis: resource in a state of readiness. *Exp Dermatol* 2017; **27**:681–4.
13 von Laffert M, Helmbold P, Wohlrab J *et al.* Hidradenitis suppurativa (acne inversa): early inflammatory events at terminal follicles and at interfollicular epidermis. *Exp Dermatol* 2010; **19**:533–7.
14 Alikhan A, Lynch PJ, Eisen DB. Hidradenitis suppurativa: a comprehensive review. *J Am Acad Dermatol* 2009; **60**:539–61.
15 von Laffert M, Stadie V, Wohlrab J *et al.* Hidradenitis suppurativa/acne inversa: bilocated epithelial hyperplasia with very different sequelae. *Br J Dermatol* 2011; **164**:367–71.
16 Marasca C, Scala E, Di Caprio R *et al.* Notch dysregulation and hidradenitis suppurativa, psoriasis, atopic dermatitis and lichen planus: let's talk about Numb. *Br J Dermatol* 2019; **180**:950–1.
17 Melnik BC, Plewig G. Impaired Notch–MKP-1 signalling in hidradenitis suppurativa: an approach to pathogenesis by evidence from translational biology. *Exp Dermatol* 2013; **22**:172–7.
18 Scala E, Balato A, Marasca C *et al.* New insights into mechanism of Notch signalling in hidradenitis suppurativa. *G Ital Dermatol Venereol* 2019; doi: 10.23736/S0392-0488.18.06083-2.
19 Ring HC, Riis Mikkelsen P, Miller IM *et al.* The bacteriology of hidradenitis suppurativa: a systematic review. *Exp Dermatol* 2015; **24**:727–31.
20 Monfrecola G, Balato A, Caiazzo G *et al.* Mammalian target of rapamycin, insulin resistance and hidradenitis suppurativa: a possible metabolic loop. *J Eur Acad Dermatol Venereol* 2016; **30**:1631–3.
21 Balato A, Caiazzo G, Annunziata MC *et al.* Anti-TNFα therapy modulates mTORC1 signalling in hidradenitis suppurativa. *J Eur Acad Dermatol Venereol* 2019; **33**:e43–5.
22 Schlapbach C, Hänni T, Yawalkar N *et al.* Expression of the IL-23/Th17 pathway in lesions of hidradenitis suppurativa. *J Am Acad Dermatol* 2011; **65**:790–8.
23 Balato A, Scala E, Balato N *et al.* Biologics that inhibit the Th17 pathway and related cytokines to treat inflammatory disorders. *Expert Opin Biol Ther* 2017; **17**:1363–74.
24 Sartorius K, Lapins J, Emtestam L *et al.* Suggestions for uniform outcome variables when reporting treatment effects in hidradenitis suppurativa. *Br J Dermatol* 2003; **149**:211–13.
25 Zouboulis CC, Tzellos T, Kyrgidis A *et al.* Development and validation of the International Hidradenitis Suppurativa Severity Score System (IHS4), a novel dynamic scoring system to assess HS severity. *Br J Dermatol* 2017; **177**:1401–9.
26 Fusco A, Coretti L, Savio V *et al.* Biofilm formation and immunomodulatory activity of *Proteus mirabilis* clinically isolated strains. *Int J Mol Sci* 2017; **18**:E414.
27 Wang L, Shen D, Wu H *et al.* Resistance of hypervirulent *Klebsiella pneumoniae* to both intracellular and extracellular killing of neutrophils. *PLOS ONE* 2017; **12**:e0173638.
28 Corvaisier M, Delneste Y, Jeanvoine H *et al.* IL-26 is overexpressed in rheumatoid arthritis and induces proinflammatory cytokine production and Th17 cell generation. *PLOS Biol* 2012; **10**:e1001395.
29 Fujii M, Nishida A, Imaeda H *et al.* Expression of interleukin-26 is upregulated in inflammatory bowel disease. *World J Gastroenterol* 2017; **23**:5519–29.

30 Kaabachi W, Bouali E, Berraïes A *et al.* Interleukin-26 is overexpressed in Behçet's disease and enhances Th17 related cytokines. *Immunol Lett* 2017; **190**:177–84.
31 Wolk K, Warszawska K, Hoeflich C *et al.* Deficiency of IL-22 contributes to a chronic inflammatory disease: pathogenetic mechanisms in acne inversa. *J Immunol* 2011; **186**:1228–39.
32 Hofmann SC, Saborowski V, Lange S *et al.* Expression of innate defense antimicrobial peptides in hidradenitis suppurativa. *J Am Acad Dermatol* 2012; **66**:966–74.
33 Kanni T, Argyropoulou M, Spyridopoulos T *et al.* MABp1 targeting IL-1α for moderate to severe hidradenitis suppurativa not eligible for adalimumab: a randomized study. *J Invest Dermatol* 2018; **138**:795–801.
34 Liang SC, Tan XY, Luxenberg DP *et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 2006; **203**:2271–9.
35 Eyerich S, Eyerich K, Cavani A *et al.* IL-17 and IL-22: siblings, not twins. *Trends Immunol* 2010; **31**:354–61.
36 Eyerich S, Wagener J, Wenzel V *et al.* IL-22 and TNF-α represent a key cytokine combination for epidermal integrity during infection with *Candida albicans*. *Eur J Immunol* 2011; **41**:1894–901.
37 Giamarellos-Bourboulis EJ, Antonopoulou A, Petropoulou C *et al.* Altered innate and adaptive immune responses in patients with hidradenitis suppurativa. *Br J Dermatol* 2007; **156**:51–6.
38 Melnik BC, John SM, Chen W, Plewig G. T helper 17 cell/regulatory T-cell imbalance in hidradenitis suppurativa/acne inversa: the link to hair follicle dissection, obesity, smoking and autoimmune comorbidities. *Br J Dermatol* 2018; **179**:260–72.
39 van der Zee HH, de Ruiter L, Boer J *et al.* Alterations in leucocyte subsets and histomorphology in normal-appearing perilesional skin and early and chronic hidradenitis suppurativa lesions. *Br J Dermatol* 2012; **166**:98–106.
40 Matusiak Ł, Bieniek A, Szepietowski JC. Bacteriology of hidradenitis suppurativa – which antibiotics are the treatment of choice? *Acta Derm Venereol* 2014; **94**:699–702.
41 van der Zee HH, Laman JD, Boer J *et al.* Hidradenitis suppurativa: viewpoint on clinical phenotyping, pathogenesis and novel treatments. *Exp Dermatol* 2012; **21**:735–9.
42 Sartorius K, Killasli H, Oprica C *et al.* Bacteriology of hidradenitis suppurativa exacerbations and deep tissue cultures obtained during carbon dioxide laser treatment. *Br J Dermatol* 2012; **166**:879–83.
43 Guet-Revillet H, Coignard-Biehler H, Jais JP *et al.* Bacterial pathogens associated with hidradenitis suppurativa. *France. Emerg Infect Dis* 2014; **20**:1990–8.
44 Gener G, Canoui-Poitrine F, Revuz JE *et al.* Combination therapy with clindamycin and rifampicin for hidradenitis suppurativa: a series of 116 consecutive patients. *Dermatology* 2009; **219**:148–54.
45 van der Zee HH, Boer J, Prens EP *et al.* The effect of combined treatment with oral clindamycin and oral rifampicin in patients with hidradenitis suppurativa. *Dermatology* 2009; **219**:143–7.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig S1. Interleukin-26 is increased by toxic shock syndrome toxin-1 stimulus.

Fig S2. Toxic shock syndrome toxin-1 stimulus enhances the production of inflammatory cytokines via interleukin-26 induction in peripheral blood mononuclear cells.

Fig S3. Interleukin-26 plasma levels of 12 patients with hidradenitis suppurativa correlated with Sartorius score.