Microbiota of the lichen holobiont Rhizocarpon geographicum : study of diversity and biotechnological applications

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Microbiote de l'holobionte lichénique *Rhizocarpon geographicum* : étude de la diversité et applications biotechnologiques

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Ilargian diren aingerueri,

Famili maiteari,

Lagun goxoeri.

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Liste des abréviations

BGC	Biosynthetic Gene Cluster
BLAST	Basic Local Alignment Search Tool
BS	Boîte Séparée
CC	Co-culture
CCM	Chromatographie sur Couche Mince
CMI	Concentration Minimale Inhibitrice
COSY	^{1}H-^{1}H COrrelation SpectroscopY
COV	Composé Organique Volatil
COrInt	Chimie Organique et Interfaces
DAD	Diode Array Detector
DCE	Dichroïsme Circulaire Electronique
ESI	Electrospray Ionization
GY	Glucose Yeast extract medium
GYM	Gym Streptomyces Medium
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High Pressure Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HSQC	^{1}H-^{13}C Heteronuclear Single-Quantum Coherence
ISP2	International Streptomyces Project 2
ITS	Internal Transcribed Spacers
LC	Liquid Chromatography
MC	Mono-culture
MEP	Malt Extract Peptone medium
MS	Mass spectroscopy
MYP	Mannitol Yeast extract Peptone
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect SpectroscopY
OSMAC	One Strain Many Compounds
P6P	Plaque 6 Puits
RMN	Résonance Magnétique Nucléaire
SMART	Small Molecule Accurate Recognition Technology
SPE	Solid Phase Extraction

TD-DFT	Time-Dependent Density Functional Theory
TOCSY	^{1}H-^{1}H TOtal Correlated SpectroscopY
Tr	Temps de rétention
TY	Tryptone Yeast extract medium
UHPLC	Ultra-High-Pressure Liquid Chromatography
UV	Ultraviolet
VIH	Virus de l'Immunodéficience Humaine
YS	Yeast Starch medium
ZC	Zone de Confrontation

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INTRODUCTION

GENERALE

INTRODUCTION GENERALE

Les symbioses représentent une association heureuse et pérenne et les lichens en sont le plus classique des exemples. La symbiose lichénique est l'intime interaction d'un champignon, le mycobionte, et d'une algue verte, le plus souvent, parfois remplacée ou accompagnée d'une cyanobactérie, le photobionte (Ahmadjian, 1993). Il y a plusieurs millions d'années, cette complexe synergie, formant un thalle, a contribué à des transitions majeures dans l'histoire de l'évolution en réussissant la conquête terrestre (Grube and Wedin, 2016; Keller et al., 2022; Yuan et al., 2005). Récemment, il a été admis qu'un troisième partenaire, la communauté microbienne associée au lichen, faisait partie intégrante de cet écosystème extrêmement adaptable et résilient (Hawksworth and Grube, 2020). Ce microbiome, constitué de bactéries (Bates et al., 2011), de champignons (Suryanarayanan and Thirunavukkarasu, 2017) et même de virus (Merges et al., 2021), a permis d'approcher l'étude des lichens d'une façon plus holistique et de le reconsidérer comme un superorganisme hautement organisé désigné sous le terme d'« holobionte » (Margulis et al., 1991; Simon et al., 2019). Ce mode de vie singulier, stable et durable ne serait pas possible sans un arsenal de défense chimique constitué de métabolites spécialisés uniques dans le monde du vivant (Calcott, 2018), qui pour certains d'entre eux présentent des bioactivités variées et significatives (Boustie and Grube, 2005).

C'est dans ce contexte que le groupe Produits Naturels de l'équipe Chimie Organique et Interfaces, COrInt - UMR 6226, s'intéresse aux lichens et à sa microflore comme source originale de métabolites pouvant être valorisés à des fins cosmétiques ou thérapeutiques. Les recherches s'y articulent autour de trois axes :

- L'isolement, l'identification et la synthèse de molécules présentant une diversité structurale,
- L'évaluation de leurs activités biologiques,
- La détermination des rôles joués par les métabolites spécialisés de chaque partenaire au sein de l'association symbiotique et sur leur environnement grâce à des approches d'écologie chimique.

Dans la cadre de la thématique du laboratoire, l'objectif de ce travail transversal était de contribuer, modestement, à la mise en lumière de l'incroyable diversité, encore trop secrètement gardée, demeurant au sein de l'holobionte lichénique. Cette diversité se reflète non seulement dans l'abondance microbienne arborée au sein de l'association mycobionte/photobionte mais également dans la richesse de l'arsenal chimique recélé par les bactéries et les champignons vivant étroitement au cœur du lichen crustacé et peu étudié qu'est *Rhizocarpon geographicum*. Ainsi, le travail réalisé s'est porté sur deux axes principaux : le premier a consisté en **l'étude de la flore fongique et bactérienne** abritée par le lichen *R. geographicum* grâce à des méthodes cultures-dépendantes alors que le second a consisté à **la valorisation de la souchothèque** d'un point de vue biotechnologique par l'étude d'une bactérie et de trois couples de champignons.

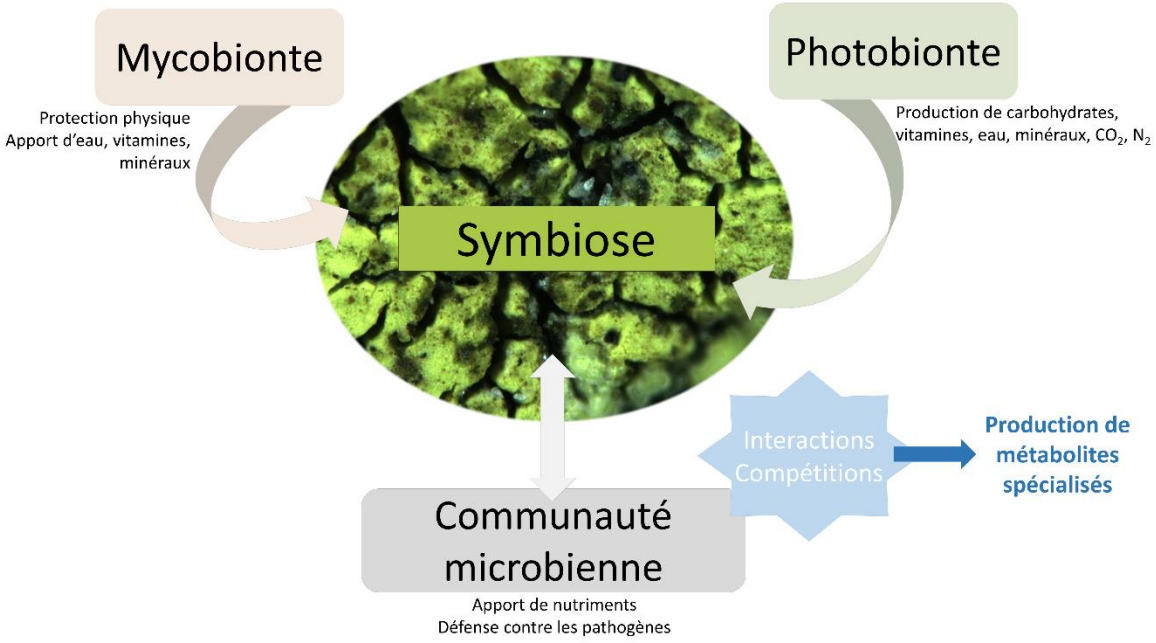
A travers le **Chapitre I**, un état de l'art des données bibliographiques disponibles sur la microflore bactérienne et fongique associée aux lichens sera présenté ainsi que la chimiodiversité issue de ces microorganismes et leurs potentielles bioactivités. Dans un premier temps, cette étude bibliographique portera sur la description de la microflore lichénique par des approches culture-indépendantes. Ensuite, les résultats des études réalisées par des méthodes culture-dépendantes seront synthétisés pour terminer sur une présentation synthétique des métabolites spécialisés et de leurs acitivités qui ont été isolés à partir de cultures de bactéries ou de champignons issus de lichens.

Dans le **Chapitre II**, une étude de la communauté microbienne de *Rhizocarpon geographicum* par des approches culture-dépendantes sera présentée suivant des méthodologies et des collaborations différentes. Le premier volet de ce chapitre consistera en la présentation des travaux menés sur un isolement classique de bactéries et champignons issus du lichen *R. geographicum* échantillonné sur les côtes bretonnes alors que le second volet présentera une approche d'isolement bactérien de haut débit à partir de différents échantillons provenant aussi bien d'environnements côtiers que terrestres.

Enfin, le dernier volet, le **Chapitre III**, de ce manuscrit consistera en l'étude de plusieurs microorganismes isolés de *R. geographicum*. D'une part, nous détaillerons l'étude d'une souche bactérienne, *Paenibacillus etheri,* pour une application dans le domaine du biocontrôle, et d'autre part l'étude chimique de cultures solides fongiques. Dans cette sous-partie, les résultats de tests biologiques des molécules isolées de ces cultures fongiques seront inclus.

Pour terminer, ce manuscrit s'achèvera par une discussion et une ouverture vers de nouvelles perspectives.

L'HOLOBIONTE LICHENIQUE : FOYER INTARISSABLE DE DIVERSITE MICROBIENNE ET CHIMIQUE



CHAPITRE 1 : L'HOLOBIONTE LICHENIQUE : FOYER INTARISSABLE DE DIVERSITE MICROBIENNE ET CHIMIQUE

Les lichens ont longtemps été peu et mal considérés jusqu'à ce qu'en 1867, Schwendener et De Bary, décrivent véritablement leur nature symbiotique (Perru, 2006). Depuis les années 2000, cette symbiose a suscité un intérêt grandissant et les études visant à mieux comprendre cette interaction se sont multipliées permettant même la redéfinition du lichen comme étant un holobionte, c'est-à-dire un ensemble constitué d'un hôte et son microbiote, ce dernier ayant lui aussi un rôle central dans la biologie, l'écologie et l'évolution du premier. Si certains de ces travaux se focalisent plutôt sur les différents acteurs et le contexte écologique de ce mutualisme grâce à l'essor des approches moléculaires de visualisation *in situ*, techniques dites culture-indépendantes, d'autres déploient des méthodes culture-dépendantes permettant l'isolement de souches bactériennes et fongiques. Isoler et cultiver des microorganismes permettent non seulement la description de nouvelles espèces mais également de valider certaines fonctions prédites grâce aux méthodes dites « omics » en étudiant leur métabolome spécialisé pour de potentielles valorisations en médecine humaine, animale voire végétale.

Cette étude approfondie de la bibliographie qui traite de la communauté microbienne associée aux lichens a été synthétisée sous la forme d'un article de revue qui sera prochainement soumis à *Biotechnological Advances* sous le titre « Lichen holobiont: an untapped source of surprising microorganisms with high chemodiversity ».

Lichen holobiont: an untapped source of surprising microorganisms with high chemodiversity

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Introduction

In the past, lichens were the most misunderstood and poorly appreciated organisms in the biological world, as called by Carl Linnaeus in 1775 the "poor trash of vegetation" for the reason that they did not fit anywhere [1]. The nature of the lichen life-form is an evolving story for at least 400 million years [2], and like mosses, they are not plants and not even fungi [3]. Lichens are classically defined as self-sufficient, symbiotic life-forms where algal or cyanobacterial partners, the photobionts, and fungal partners, the mycobiont or the dominant lichen-forming fungi, demonstrate harmony for coexistence [4], [5]. Because of their ubiquity due to their ability to grow on a number of diverse substrates and their worldwide distribution among various extreme environments, as for example rocky coasts, polluted sites, arctic tundra, hot deserts, the lichen thalli are well adapted to a wide range of ecological niches and can even survive to space conditions [6]. To overcome these harshest conditions, lichens produce unique and specific secondary metabolites, approximately 1000 of them were discovered so far [7], with considerable biological activities [7]–[24]. Few years ago, it was admitted that the symbiotic concept of lichen needed to be reconsidered as an example of holobiont [25], a complex network defined as a "self-sustaining ecosystem formed by the interaction of an exhabitant fungus and an extracellular arrangement of one or more photosynthetic partners and an indeterminate number of other microscopic organisms" [26]. Thus, as self-supporting ecosystems, lichens constitute a stable shelter for other microorganisms such as fungi [27], microalgae [28], bacteria [29], yeasts [30] and viruses [31] as an additional and integral component of the symbiosis with potential ecological and biological roles [32], nicely exemplified with *Lobaria pulmonaria* as a model [33]. Indeed, in 1983, it was estimated that millions of bacteria cells per gram could colonize a lichen thallus [34]. For a long time, their isolation was culture-dependent and their identification only based on phenotypical and physiological characters [35], [36]. However, recent emergence of sophisticated molecular technologies, referred to "omics" technologies [33], revealed more of the diversity and abundance of this microbiome [29], [37]–[41]. Through the bioinformatic tools improvement, a broader perspective of the lichenic holobiont is allowed. The functional implications of the symbiosis' microbiota to the host and its resulting phenotypic and fitness affects need to be further investigated and are a real playground for the study of the underlying mechanisms at the community level in terms of biochemical interactions and contributions toward the production of specialized metabolites. Indeed, the first surveys [42] reporting evidence on microbial metabolites production and their potential roles seem to comfort this hypothesis.

Herein, we will provide an in-depth assessment of lichen-associated microbial communities, focusing on endolichenic fungi and bacteria residing inside the lichen thalli. We will firstly

describe their biodiversity based on culture-independent and culture-dependent approaches. Then we will focus on their specialized bioactive metabolites as well as their biological activities potentially relevant in biotechnological applications.

Culture-independent investigations

Studies have applied various molecular technologies [33] as fingerprint of rRNA genes which is an array-based method that allows extensive analysis of microbial community composition [43], the fluorescence *in situ* hybridization (FISH), the assay of choice for localization of specific nucleic acids sequences in native context [44], and molecular cloning approaches. These molecular technologies have first displayed the tremendous diversity of lichen-associated microorganisms by providing an unequalled opportunity, simultaneously, sequence tons of DNA fragments for only a part of the cost per base pair of traditional Sanger approach [45]. They also permitted to increase the rate and scope at which microbial taxa can be detected and characterized in environmental samples [46] therefore, allowing a quick evolution of the microbial ecology knowledge [47], [48]. Nowadays, the improvement of the sequencing methods and bioinformatic tools allows an even more precise and sharp vision by using the new "omics" technologies [33].

Lichen-associated fungi

Metagenomic analyses based on high-throughput sequencing technology have been little employed to study the endolichenic fungi (Tab. 1). Indeed, molecular methods as pyrosequencing, can provide new insights into the diversity and distribution of fungal populations but can also skew this community richness due to a lack of differentiation between true information about fungal biodiversity and methodological artefacts as an excessive photo- and mycobiont sequences amplification [40], [49]–[52]. Thus, in 2016, a 454-pyrosequencing-based study - DNA sequencing technique based on DNA amplification by emulsion PCR and pyrosequencing allowing the sequencing of a large number of bases in short time [53] - was used to investigate fungal diversity and richness of 742 lichens from High Arctic, depending on the regions of sampling, and as a result, the lichen mycobionts sequences occupied an important part of the obtained data. Except for the high proportion of mycobiont sequences, this study showed a high diversity of endolichenic fungi belonging to 370 operational taxonomic units (OTUs), which is an operational definition used to classify groups of closely related individuals [54], most of them closely related to fungi from various cold habitats such as arctic, antarctic and alpine regions. Of these OTUs, 294 belonged to Ascomycota, 54 to Basidiomycota, 2 to Zygomycota, and 20 to unknown fungi. Leotiomycetes, Dothideomycetes, and Eurotiomycetes were the major classes, whereas the dominant orders were Helotiales, Capnodiales, and Chaetothyriales [55]. Studying 12 lichen samples from Barton and Weaver Peninsulas in King George Island, Antarctic, Park et al. also noted that the major OTU for each sample was related to the mycobiont amplicons. Moreover, depending on the sample, 26-66 fungal OTUs were identified either as Ascomycota (Arthoniomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes, and Sordariomycetes) or Basidiomycota (Cystobasidiomycetes and Tremellomycetes) [56]. To overcome the incidence of lichen mycobiont amplicons and to improve amplification of endolichenic fungi, a positive control designed primers should be used on each pyrosequencing run to control for errors in sample

handling, processing, sequencing or clustering [49]. In 2019, Gueidan et al. [48] proposed a PacBio amplicon sequencing approach for the sequencing of the fungal ITS barcode of recent lichen herbarium specimens as a promising approach not only for the generation of reference sequences for lichenized fungi but also for the characterization of lichen-associated fungal communities. The limited culture-independent studies treating the lichen-associated fungi outlined that fungal communities varied according to the host species [57] – [59], seasonal changes of environmental conditions [57], geographic location and altitude [51].

Table 1 Identification of endolichenic fungi by molecular methods.

Host species	Season	Region	Used method	Most abundant identified fungi class	Reference
Amandinea coniops	-	Antarctic	Pyrosequencing of partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA	Arthoniomycetes	[53]
Buellia granulosa	-	Antarctic	Pyrosequencing of partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA	Arthoniomycetes	[53]
Cetrariella delisei	Summer	High Arctic	454 amplicon pyrosequencing	Leotiomycetes	[52]
Cladonia arbuscula	Autumn	Southern Finland	Amplicon sequencing (ITS2), shotgun metagenomics	Lecanoromycetes	[40]
	Summer	High Arctic	454 amplicon pyrosequencing	Leotiomycetes	[52]
Cladonia borealis	Summer	High Arctic	454 amplicon pyrosequencing	Leotiomycetes	[52]
	-	Antarctic	Pyrosequencing of partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA	Arthoniomycetes	[53]
Cladonia gracilis	-	Antarctic	Pyrosequencing of partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA	Arthoniomycetes	[53]
Cladonia pocillum	Summer	High Arctic	454 amplicon pyrosequencing	Leotiomycetes	[52]
Cladonia rangiferina	Autumn	Southern Finland	Amplicon sequencing (ITS2), shotgun metagenomics	Lecanoromycetes	[40]
Cladonia squamosa	Winter	Antarctic	454 amplicon pyrosequencing and PacBio	Lecanoromycetes	[57]
Cladonia stellaris	Autumn	Southern Finland	Amplicon sequencing (ITS2), shotgun metagenomics	Lecanoromycetes	[40]
Cladonia uncialis	Autumn	Southern Finland	Amplicon sequencing (ITS2), shotgun metagenomics	Lecanoromycetes	[40]
Crustose lichens	-	Eastern Alpine Austria	454 amplicon pyrosequencing (ITS1)	Dothideomycetes	[55]
	-	Eastern Alpine Austria	ITS2 metabarcoding	Dothideomycetes	[56]
Flavocetraria nivalis	Summer	High Arctic	454 amplicon pyrosequencing	Leotiomycetes	[52]

Hypogymnia hypotrypa	-	Central and Southwestern China	IlluminaMiSeq sequencing	Dothideomycetes	[49]
Ochrolechia frigida	Summer	High Arctic	454 amplicon pyrosequencing	Leotiomycetes	[52]
Ochrolechia parella	-	Antarctic	Pyrosequencing of partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA	Arthoniomycetes	[53]
Peltigera canina	Summer	High Arctic	454 amplicon pyrosequencing	Leotiomycetes	[52]
Peltigera praetextata	Spring	Southeastern Arizona (USA)	454 amplicon pyrosequencing	Unclassified	[47]
Physconia distorta	Winter and autumn	Germany	PCR amplification of SSU_h35F and ITS4 rRNA	-	[54]
Rhizoplaca chrysoleuca	-	Northern Colorado (USA)	PCR amplification of 18S rRNA and barcoded pyrosequencing	Dothideomycetes	[48]
Umbilicaria americana	-	Northern Colorado (USA)	PCR amplification of 18S rRNA and barcoded pyrosequencing	Dothideomycetes	[48]
Umbilicaria antarctica	-	Antarctic	Pyrosequencing of partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA	Arthoniomycetes	[53]
Umbilicaria cylindrica	-	Eastern Alpine Austria	ITS2 metabarcoding	Dothideomycetes	[56]
Umbilicaria phaea	-	Northern Colorado (USA)	PCR amplification of 18S rRNA and barcoded pyrosequencing	Dothideomycetes	[48]
Usnea aurantico-atra	-	Antarctic	Pyrosequencing of partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA	Arthoniomycetes	[53]
Xanthoria parietina	Winter and autumn	Germany	PCR amplification of SSU_h35F and ITS4 rRNA	-	[54]

Lichen-associated bacteria

Unlike lichen-associated fungal consortia, bacteria sheltered by lichens have been broadly studied through molecular tools [29], [37], [41], [60]–[79]. The most significant aspects that next generation sequencing and metagenomics have brought to the study of lichens are the identification of a much higher diversity, a much deeper perception of the taxonomic distributions and potential contributions of bacterial communities to the lichen thallus [32], [64], [68], [71]. According to Grube et al., bacterial compositions in lichens are influenced by host species [37], observation strengthened by Bates et al. two years later [29]. Besides, it was reported that prevalent bioactive secondary metabolites from lichens such as usnic acid, abundant in some lichen species and displaying multiple biological activities such as antibacterial, cytotoxic, and antifungal activities [80]–[82], only influenced the bacterial diversity but not its abundance. Accordingly, bacterial communities associated with lichens producing usnic acid were more specific and more resistant to this metabolite [62], [68], [69], [83] suggesting a closer relationship between the different partners through their metabolite production (Fig.1). This diversity pattern of lichen-associated bacteria are influenced by various parameters such as extrinsic factors including sun exposure [71], substrate type and location [84], [85], and intrinsic factors like lichen species, photoautotrophic symbiont and growth form [84], age and part of lichen thallus [61] and chemical composition of lichen. These results imply that microbial compositions in lichen thalli are affected by surrounding biotic and abiotic factors, therefore, different microenvironment within the thallus may produce different lichen microbiome [86] [87]. It is also supposed that inoculation of different microbiota from the air or surrounding soil also affect the microbial community and could be recruited, at least in part, from the substrates where lichens grow [64], [86], [87] (Fig. 1).

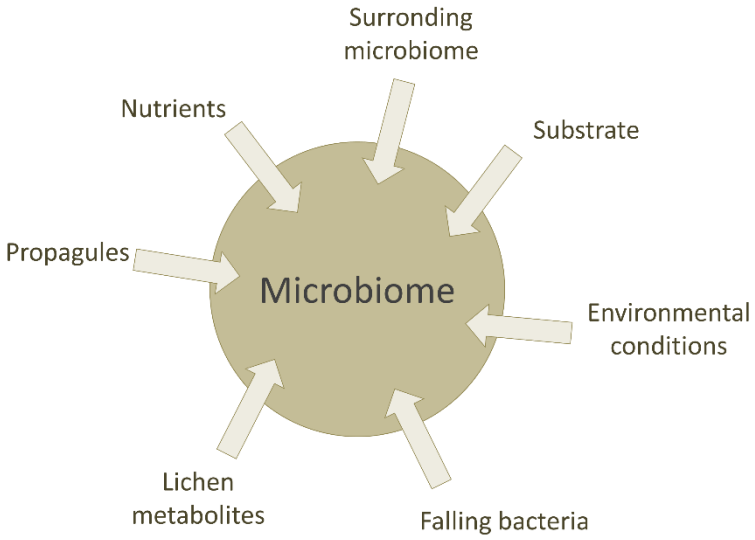

Figure 1 The main factors shaping the microbiome composition in lichens (adapted from Grimm et Grube 2021 [33]).

The inconsistencies in defining which of these factors would be the most significant on determining the structure of the microbial communities associated with lichens, are mainly due to the fact that most studies compare the bacterial communities between different lichen species and/or with different photobiont types (algae vs. cyanobacteria). Comparing two different sampling sites of the same lichen species, the presence of a relatively stable core microbiome

can be suggested [88] but comparing different lichen species even in close spatial proximity underscore dissimilar bacterial communities, showing that the lichen species itself appears to be the strongest predictor of community composition [29]. By applying next generation Illumina sequencing to characterize the bacterial communities associated to strict marine cyanolichens, maritime chlorolichens occupying different areas of the littoral zone and inland terrestrial cyanolichens, West et al. share the view that lichen species plays a crucial role in the bacterial specificity [79]. Indeed, the strictly marine lichens *Lichina confinis* and *L. pygmea* communities, dominated by the Bacteroidetes phylum, were significantly different from those of the maritime *Xanthoria aureola* (located closer to the marine species cited above) and *X. parietina* lichens considered as maritime because higher up located on the littoral zone, dominated by Alphaproteobacteria. These latter communities were shown to be more alike to those in the inland terrestrial lichens. This discrepancy could be discussed considering the possibility that the bacterial microbiome composition and functionality change according to ecological and climatic variations and could lead to an increase in the adaptability of the holobiont. Indeed, host-associated microbiota play an important role in the health and persistence of complex organisms and can adapt its community as a response to abiotic stress. For example, the bacterial community of three lichen samples collected from three different locations subjected to different degrees of arsenic pollution was shaped by acquisition of specific resistances [89].
To this date, the functional implications of these associated bacteria in the lichen symbiosis remain not enough explained and explored but some nonexclusive hypotheses have been proposed and/or reported. This includes essential functions such as i) nutrient supply by uptake and/or assimilation of e.g. nitrogen, phosphorus, iron, sulfur, amino acids, dipeptides, sugar and xylose, ii) resistance against abiotic factors and toxic compound protection, iii) fungal and algal growth support by the provision of hormones, iv) resistance against biotic stress factors by the production of bioactive metabolites, v) support of photosynthesis by the provision of vitamin B12, vi) recycling nutrients, vii) detoxification of metabolites, viii) degradation of older parts of the lichen thallus [33], [90]–[92].

The development of culture-independent techniques has revolutionized the understanding of microbial ecology especially through the illustration of the vast gap between the environmentally abundant microbial diversity and the cultivable part. However, the largest metagenomic efforts still sample only a small fraction of the metagenomes of microbial communities [49], [93] and metagenomics libraries are dominated by gene sequences with unknown functions, limiting the gained knowledge from these community genomes [94]. Thus, culture-based approaches are not only crucial for understanding the evolutionary, metabolic and ecological system of microbial diversity but also for the development of novel biotechnological applications [95]. Pure cultures of microorganisms and obtention of DNA sequences that cannot be attributed to known species remains basic, essential and a prerequisite for drug discovery from natural sources. Thus, culturing is indispensable and improvements in culturing techniques are needed [96] in order to recover a wider range of microbial community members living in natural environments to go further into metacommunities interactions and their functions in such complex systems as holobionts and even more in the lichen holobiont. The following part will discuss about the culturable microbiota.

Culturable microbiota

Since the discovery of microorganisms, *in vitro* cultivation and isolation of bacteria in pure cultures has represented one of the key pillars in the science of microbiology [97]. A great part of environmental microbial diversity (> 99%) resists cultivation in the laboratory [98]. Called as the "Great Plate Count Anomaly" [99] or the "Microbial Dark Matter" [100], this uncultivated microbial majority offers exciting opportunities for microbial discovery and represents approximatively 90 bacterial candidate phyla [97]. The Great Plate Count Anomaly being a consequence and manifestation of microbial growth strategies in nature assuming all species grow in nature, the cultivation of this "dark matter" might be possible via a reasonable imitation of conducive natural conditions [99]. Interestingly, all the specific culture-dependent strategies are addressed to the bacterial recovery whereas it also could be adapted to fungal isolation. Fungal strains also represent potential producers of novel bioactive compounds with wide-ranging biological activities but the use of standard media and classic cultivation methods only allow the recovery of a minor fraction. By using diverse *in vitro* growth conditions, as for the bacterial consortia, the fraction of endolichenic culturable fungi could be extended and better investigated.

Endolichenic fungi

In 1997, Hawksworth and Rossman identified three methodologies to isolate undescribed fungi. One of them was the unexplored habitats including lichens [101]. While culturing the mycobionts [33], [102]–[108] is well established, as the study of lichenicolous fungi [109]–[117] defined as obligate associate with lichens either as saprotrophs that colonize dead lichen thalli or parasites that obtain fixed carbon from living lichen hosts [115], the isolation of additional cryptically occurring fungi is not properly explored but gets more and more attention over time [36], [118]–[122]. The majority of the studies on endolichenic fungi relate to their specialized metabolite spectrum because of the beneficial properties exhibited by these metabolites [118], [123]–[144]. Although similar to the endophytic fungi of higher plants, the function of endolichenic fungi in maintaining the complex lichen association is not known.

The endolichenic fungi were shown to be distinct from the lichen mycobiont and the lichenicolous fungi and phylogenetically related, in terms of higher taxonomy, to the endophytic fungi harbored by plants [145]–[148]. During a large-scale survey of endophytic and endolichenic fungal communities associated with locally replicated and phylogenetically diverse sets of 23 plant and lichen host taxa, in five sites across North America, U'Ren et al. [36], isolated 4791 endophytic and endolichenic fungi indicating that the incidence, diversity and composition of symbiotrophic fungi reflect the interplay of climatic patterns, geographic localisation, host type [149] and host lineage [150]. The majority of these fungi belonged to the phylum Ascomycota and to the same five classes of Pezizomycotina, but a small number of Saccharomycotina and Basidiomycota were also found. These results converged with a previous research, also conducted by U'Ren et al., in which five classes and approximately 16 orders, 32 families, and 65 genera of Pezizomycotina were represented. Pezizomycetes were most common, followed by Sordariomycetes, Leotiomycetes, Dothideomycetes and Eurotiomycetes. Besides, the five classes of Pezizomycotina were equitably distributed among endophytes and endolichenic fungi [147].

The isolation of endolichenic fungi also depends on the isolation process. Indeed, surface sterilization procedure, sample size and growth media play a significant role in the isolation frequency and diversity [151], [152] (Table 2). Testing these four variables, Yang et al. isolated 1885 endolichenic fungi belonging to 104 species representing 2 phyla, 7 classes, 23 orders, 38 families, and 60 genera from five individual foliose thalli of *Parmotrema tinctorum*. Ascomycota was the dominant phylum (1736 isolates, 92.10%). In Ascomycota, the most abundant class was Sordariomycetes, followed by Eurotiomycetes and Dothideomycetes. Besides, among these four variables, they showed that thallus fragment size significantly influenced the recovered fungal diversity with the highest recovery rate for the smallest thallus fragment (1 mm^2) [137].

Comparing all the studies on the isolation of endolichenic fungi [119], [124], [132], [145], [152]–[157], most of the species belong to Ascomycota. However, depending on the country, continent or climate zone, the pattern differs: in North America, U'Ren et al. underscored the most abundant class as being Sordariomycetes and Pezizomycetes [36], in China for Yang et al. it was Sordariomycetes [151] as in South Korea [153] or France [154], Eurotiomycetes in Austria [27] and Leotiomycetes in Antarctica [155]. These results emphasize, once again, with the results obtained by Chagnon et al. [158] the generalist nature of endolichenic fungi compared to endophytic fungi living in the plants.

Table 2 Identification of endolichenic fungi by culture-dependent methods

Host species	Region	Surface treatment	Size of segments	Media used	Incubation	Most abundant identified fungi class	Reference
Xanthoria mandschurica, Cladonia coniocraea, Dermatocarpon miniatum, Melanelia sorediata, Parmelia sp., Punctelia borreri, Ramalina sinensis	Baihuan mountain of Beijing, China	1 min in 75 % EtOH, 3 min in 2 % NaOCl, 30 s in 75 % EtOH	0.5 cm^2	Malt extract agar supplemented with Rose bengal and streptomycin sulphate	2 months at 25°C	Sordariomycetes	[119]
Dermatocarpon spp., Flavopunctelia praesignis, Punctelia hypoleucites, Usnea hirta, Pseudevernia intensa, Xanthoparmelia viriduloumbrina, Lecidea tessellata, Physcia caesia, Peltigera spp., Diploschistes muscorum, Lecanora orenoides, Cladonia subtenuis, F. caperata, P. consocians, Parmotrema reticulatum, Usnea spp., X. conspersa, Lasallia spp., P. praetextata, Sticta beauvoisii, Diploschistes scruposus, C. evansii, C. leporina, Cladonia spp., C. subtenuis, P. perforatum, P. rampoddense, P. tinctorum, Usnea spp., Herpothallon rubrocinctum, Pyxine eschweileri, Alectoria ochroleuca, A. nigricans, Flavocetraria cucullata, C. mitis, Dactylina arctica, Masonhalea richardsonii, Arctoparmelia separata, Peltgera spp., R. geographicum, O. ventosa, Umbilicaria spp., Bryoria spp., F. cucullata, Cetraria spp., C. stellaris, H. physodes, P. omphalodes, Stereocaulon paschale, Amygdalaria panaeola, Umbilicaria proboscidea, U. phaea	SE Arizona, WN Carolina, Florida, W Alaska, E central Alaska	30 s running tap H_2O then 30 s in 95 % EtOH, 2 min in 0,5 % NaOCl, 2 min in 70 % EtOh	2 cm^2	Malt extract agar	Up to 1 year at room temperature (ca. 21.5°C)	Sordariomycetes and Pezizomycetes	[36]

Aspicilia calcarea, Caloplaca oasis, Lecanora albescens ; A. caesiocinerea, C. crenulatella, Diploschistes actinostomus, L. muralis, Neofuscelia verruculifera, Tephromela grumosa, C. aurantina, C. lactea, Xanthoria calcicola	Northern Israel	Running tap H_2O, immersion for 1 min in 75 % EtOH, 3 min in 2 % NaOCl,30 s in 75% EtOH	1 mm²	Malt extract agar	10 – 14 days in darkness at 25°C	Dothideomycetes	[157]
Parmotrema tinctorum	Jeju Island, South Korea	Running tap H_2O, 70 % EtOH, 60 - 90 or 120 s in 0,4 % NaOCl, rinsed with sterilized distilled H_2O for 120 s	100 ; 25 and 1 mm²	Potato dextrose agar, malt and yeast extract agar, Lysogeny broth and Bold's basal medium	More than 2 months at room temperature (ca. 21.5°C)	Sordariomycetes	[151]
Usnia antarctica, Cladonia borealis, Psylolechia lucida	Barton Peninsula, King George Island, Antarctic	Washing for 3h in streaming H_2O, 1 min in EtOH 75 %, 3 min in 2%NaOCl, 30 s in 75 % EtOH, rinsed with sterilized distilled H_2O for 30 s	nd	Potato dextrose agar supplemented with 0.01% streptomycin	15°C	Leotiomycetes	[155]
Umbilicaria cylindrica, Lecanora polytropa, Tephromela atra	Koralpe mountain, Austria	Washing 3 times for 15 min with distilled sterile H_2O, 30 min with 500µL Tween 80 (diluted 1:10), twice for 15 min with sterile H_2O	2 mm²	Lily & Barnett, Trebouxia, malt yeast agar, Sabouraud, potato dextrose agar, dichloran/glycerol agar	Growing chamber at 20°C with a light-dark regime of 14:10, controlled light intensity and 60% of humidity	Eurotiomycetes	[27]
Nephroma laevigatum	Nouvelle-Aquitaine, France	Consecutive immersions for 2 min in 70 % EtOH 2 min in 0.5 % NaOCl and 2 min in 70 % EtOH, washing in sterile distilled H_2O	0.5 cm²	Malt extract agar supplemented with Rose Bengal and streptomycin sulphate	Until observation of emergence of fungal colonies at room temperature (ca. 22°C)	Sordariomycetes	[132]

Evernia prunastri, Ramalina fastigata, Pleurosticta acetabulum	Nouvelle-Aquitaine, France	Consecutive immersions for 2 min in 70 % EtOH 2 min in 0.5 % NaOCl and 2 min in 70 % EtOH, washing in sterile distilled H_2O	0.5 cm²	Malt extract agar and potato dextrose agar supplemented with Rose Bengal and streptomycin sulphate	Up to 2 months	Sordariomycetes and Dothideomycetes	[154]
Usnea baileyi, U. bismolliuscula, U. pectinata	Mountain Province, Phillipines	Washing in H_2O then successive immersions in 70 – 80 – 85 – 90 % EtOH for 1min then rinsing with sterile distilled H_2O for 30 s inbetween each EtOH % then dipped in 10% NaOCl for 30 s then immersion in 95% EtOH for30 s finally rinsing with H_2O for 30 s	2 mm²	Malt extract agar	14 days under light for 12h atroom temperature (ca. 26°C)	Sordariomycetes and Eurotiomycetes	[159]
Peltigera neopolydactyla, Umbilicaria mammulata, Lobaria scrobiculata, P. aphthosa, P. leucophlebia, P. malacea, P. scabrosa, Nephroma arcticum	Artic, boreal, temperate and tropical sites	Washing in H_2O followed by immersion in 96 % EtOH for 10 s, immersion in 0,5 % NaOCl and 70 % EtOH for 10 - 30 or 120 s each or 120 or 240 s respectively	2 mm²	Malt extract agar	Up to one year at room temperature under ambient light	Sordariomycetes	[145]
Flavoparmelia caperata, Peltigera dilacerata	Forest of Sugadaira research Station, Japan	Washing under running tap H_2O then ten surface-washings by stirring in sterile H_2O containing 0.5 % of Aerosol OT for 1 min then in sterile water - non-surface-sterilization treatment: fragments immersed in sterile H_2O under agitation for 2 min - surface sterilization: immersion in 70 % EtOH for 2 min or immersion in 0.5 % NaOCl under agitation for 2 min or immersion in 20 % H_2O_2 under agitation for 2 min - after sterilization treatments rinsing twice with sterile water	0.25 cm²	Malt extract agar	Up to 3 months at room temperature (ca. 25°C)	Sordariomycetes	[152]

Anaptychia isidiza, Heterodermia isidiophora, Phaeophyscia erythrocardia, P. exornatula, P. imbricata, Cladonia symphycarpia, Collema japonicum, C. subflaccidum, Leptogium saturninum, Lobaria discolor, L. japonica, Peltigera degenii, P. didactyla, P. horizontalis, P. leucophlebia, P. neopolydactyla, P. polydactylon, Parmelia adaugescens, Leptogium pedicellatum, Cladonia sp., Parmotrema cristiferum, Punctelia subrudecta, Stereocaulon japonicum, C, pyxidata, C. rei, Stereocaulon japonicum, C. scabriuscula, P. cetratum, P. perlatum, Myelochroa entotheiochroa, Myelochroa indica, Parmotrema sp., C. mongolica, Phaeophyscia sp. 1, Cladonia sp. 2, Leptogium pedicellatum, Myelochroa aurulenta, P. praesorediosum, Physcia orientalis, P. austrosinense, P. dilatatum, P. tinctorum, P. reticulatum, P. defectum, C. kurokawae, Dirinaria applanata,	Jeju Island, South Korea	Washing under running tap H_2O then 30 s in 95 % EtOH, 2 min in 0,5 % NaOCl, 30 s in 70 % EtOH, rinsing 3 times with sterile distilled H_2O	1 cm²	Potato dextrose agar	up to 12 weeks at 25°C	Sordariomycetes	[153]
Acarospora molybdina, Allantoparmelia alpicola, Cetrariella delisei, Cladonia borealis, C. arbuscula, C. pocillum, Flavocetraria nivalis, Ochrolechia frigida, Peltigera canina, Placynthium asperellum, Pseudephebe pubescens, Stereocaulon alpinum, S. botryosum, S. vesuvianum, Umbilicaria aprina, U. arctica, U. torrefacta	High Arctic	Immersion for 1 min in 75 % EtOH, 2 min in 1 % NaOCl, 30 s in 75 % EtOH than rinsing for 30 s with sterile H_2O	1 - 5 mm²	Potato dextrose agar supplemented with tetracycline and streptomycin sulphate	1 month at 12°C	Leotiomycetes and Sordariomycetes	[156]
Arthonia antillarum, Opegrapha medusulina, Pyxine cocoes, Rocella montagnei	Sri Lanka	Washing under running tap H_2O then immersion for 10 s in 70 % EtOH, 3 min in 0,5 % NaOCl and washing in sterilized H_2O 3 times	9 mm²	Malt extract agar supplemented with 0.01 % streptomycin	14 days at room temperature (ca. 30°C)	Sordariomycetes	[124]

Lichen-associated bacteria

Plethora of lichen-associated bacteria studies have been published, often by culture-independent techniques, since bacteria clearly appeared to be a full participant of the lichen symbiosis and the taxonomic diversity has been relatively well established. Culturing bacteria, as fungi, is crucial to unravel and exploit their biotechnological potential and for the description of new taxa [160], [161] or full genomic sequencing [162]–[164]. In 2016, studies dealing with isolations of lichen-associated bacteria were summarized [32] and the review rightly highlighted that a number of these studies have focused on individual isolates or species with a keen interest for targeting specific class of bacteria including biotechnologically relevant strains [40], [70], [164]–[178]. The spectrum of the biotechnological potentiality of these lichen-associated isolated bacteria appears to be very wide from antimicrobial activities [42], [166], [179]–[181] to biofertilizers for agricultural crops [181]. Given the differences in objectives and methods used for bacterial isolation, i.e. no standardized methodologies, results are very heterogenous. Selective cultivation approaches, as much as surface sterilization [37], [182] or washing [42], [177], [183], use of selective media [42], [65], [169] or even lichen supplemented media [184], [185], antifungal supplementation [42], [169], [184], different incubation temperature and length, permit to target more classes than others. Indeed, Proteobacteria are the most represented isolates in several studies [37], [65], [87], [164], [166], [173], [177], [179]–[181], [186], [187] while Actinobacteria are the most abundant in other ones [42], [169], [177], [183], [188]–[190]. However, a few groups are more difficult to cultivate under laboratory conditions, Acidobacteria could be cited for instance. Pankratov et al. demonstrated in 2012 that their unculturability was a consequence of their low growth rate and their substrate specificity [191].

The heterogeneity in microbial populations could be also due to quickly growing "weeds" [192] which could prohibit – or mask – *in vitro* growth of other perfectly cultivable species that are either rare, do not grow as quickly, or both. Although serial dilutions are used in isolation protocols [37], [42], [169], [177], [184], cultivation by dilution to extinction have never been described for the isolation of lichen-associated bacteria. This tool could be an interesting way to eliminate the influence of such fast growing strains in order to increase microbial recovery [193], [194] and to get remarkable isolates [195]. In the same way, the low similarity of OTUs identified from the same marine lichen species by culture-dependent and culture-independent approaches [79] shed on the light the importance to use these different strategies, which could also be combined to the search for genes clusters of interest by metagenomics [196], to improve the bioprospection of lichen microflora.

Specialized metabolism of the lichen-associated microbiota

In addition to the isolation and/or identification of the lichen-associated microorganisms at the species level to characterize the biodiversity of these communities and the networking between them and their hosts, studies have also been conducted from a metabolic perspective.

Lichens can tolerate extreme conditions [197], [198] and can accumulate toxic compounds, heavy metals [199], [200] or radionuclides [201]. In order to sustain and protect the symbiotic association from these stress factors, lichens synthetize specialized metabolites, e.g. phenolic compounds, dibenzofurans derivatives such as usnic acid, depsides, depsidones, depsones,

lactones, quinones, and pulvic acid derivatives [7], [8]. Because of this rich secondary metabolism, and the resulting biological activities, lichens have been used for various purposes across the ages, in particular as dyes, perfumes and remedies in folk medicines as Ayurveda in India [81]. For the purpose of a better knowledge of the metabolites involved, the "omic" molecules-centered approach, called metabolomics, permits dereplication and a better understanding of the studied superorganisms' metabolomes, likely including a wide variety of molecules arising from the associated microorganisms. A thesis work dealing with the metabolomic study of 299 lichen samples permitted to detect about 8000 different molecules while the classical studies of isolation and characterization of lichen compounds only allow a limited number [202]. In parallel, the hypothesis of the presence of a wide range of compounds produced by the microflora present in lichens has been emphasized in a recent study. Indeed, Garg et al. used UHPLC-MS/MS based molecular networking to assess secondary metabolite production from scrapes of a *Peltigera hymenina* sample. The data from *P. hymenina* was compared with data collected from bacteria and fungi isolated from the sample, as well as data generated from fungal and freshwater cyanobacterial extracts. Based on this comparison, it was found that 15.5% of the compounds were fungal in origin, 17.6% were bacterial in origin, 7.6% were similar to fungal and bacterial compounds [203].

Specialized metabolism in lichen-associated fungi

Functionally advantageous endolichenic fungi constitute abundant taxa belonging to diverse classes, orders and family within Ascomycota [118], [122], [145], [149]. As described above, this diversity has been extensively studied during the last decade and the bioactivity of some of their specialized metabolites has been largely evaluated as summarized in 2017 by Kellogg et Raja [123] and more recently, in the last two years, by Agrawal et al. [125], by Wethalawe et al. [204] and Zheng et al. [205]. In addition to the biological activities cited, i.e. cytotoxic, antibacterial, antifungal, antioxidant, UV skin protector, anticancer, anti-inflammatory, antiviral [159], [206], in the last reviews, nematicidal activity could also be reported. Indeed, the fungus *Xylaria grammica* isolated from the lichen *Menegazzia primaria* produces grammicin, a polyketide showing a strong activity against the root-knot nematode *Meloidogyne incognita* [207]. Still in the field of agricultural application, in 2021, Lin et al. described two asperglaucins compounds, isolated from the fungus *Aspergillus chevalieri*, as potential promising candidates for lead compounds of agrochemical bactericides [208]. Showing moderate acetylcholinesterase inhibitory effects, very valuable for the treatment of Alzheimer disease, ophiosphaerellins polyketide compounds were isolated from the endolichenic fungus *Ophiosphaerella korrae* [209]. Beside the wide biological activities permitted by the lichen-associated fungi's secondary metabolites, it is worthy to note that their enzymatic machinery is also very promising in terms of bioremediative potential as suggested by Ting et al. in their study dealing with the dye decolorization potential of two endolichenic fungi [210].

Specialized metabolites production from lichen-associated bacteria

If for lichen-associated fungi, number of studies about their chemodiversity noticeably increased the last few years, lichen-associated bacteria have been extensively studied in order to understand their functional role within the lichen symbiosis [90]. Thus, the high diversity of lichen-associated bacterial communities, which comprises millions of bacterial cells per gram

of lichen thallus [29], [37], [60], appears to be potentially very interesting in terms of specialized metabolite production for further biotechnological applications [211]. However, lichen-associated bacterial chemodiversity have been poorly studied and focused on their biosynthetic potential analysis by using comparative genomics [42], [175] whereas few others also carried out antimicrobial activity assays [177], [212]–[214]. Seldom reviews summarize the scarce bacterial characterized chemodiversity [14], [32], [33], [215] reporting, for most of them, antimicrobial and cytotoxic bioactivities [216]–[220]. Recent studies highlighted the chemodiversity produced by several lichen-associated bacteria through the production of unusual brominated diketopiperazines [174], of the cytotoxic 6-methoxy-2-methyl-3-heptylprodiginin [220] or of the potent cytotoxic *N*-methyldactinomycin along with the known usnic acid [221]. In the same manner, an Actinomycete strain associated to an inland lichen produced new actinofuranones analogues with significant anti-inflammatory properties [170]. Moreover, it has been shown that a new echinosporin derivative, amycolasporin C, isolated from the lichen *Lepiolostroma* sp. associated *Amycolatopsis hippodrome,* exhibited antioxidant activity [222]. Another strain of *Amycolatopsis* isolated from the lichen *Squamarina* sp. has been described as a producer of antimicrobial compounds [167], among them two new compounds such as amycophthalazinone A and an isoflavonoid glycoside named 7-*O*-methyl-5-*O*-α-L-rhamnopyrano sylgenestein. A focus on strains belonging to Actinomycetes is not surprising as they have been long known as prolific producers of specialized metabolites and especially antibiotics [223], and indeed, as reported here, the last works dealing with lichen-associated bacterial chemodiversity studied actinobacteria [170], [224]. As reviewed for the lichen-associated fungi, an actinomycete, *Agromyces alli*, isolated from the lichen *Flavoparmelia caperata*, produced nematicidal metabolites (e.g. 2-((5-nitrofuran-2-carboxamido)oxy)propanoic acid) against *M. incognita* [225]. Moreover, a Fimircutes strain, *Paenibacillus etheri*, isolated from the lichen *Rhizocarpon geographicum*, produced microbial volatile organic compounds (specially isoamyl acetate) exhibiting *in vitro* nematicidal activity against two different plant-parasitic cyst nematodes (article in preparation).

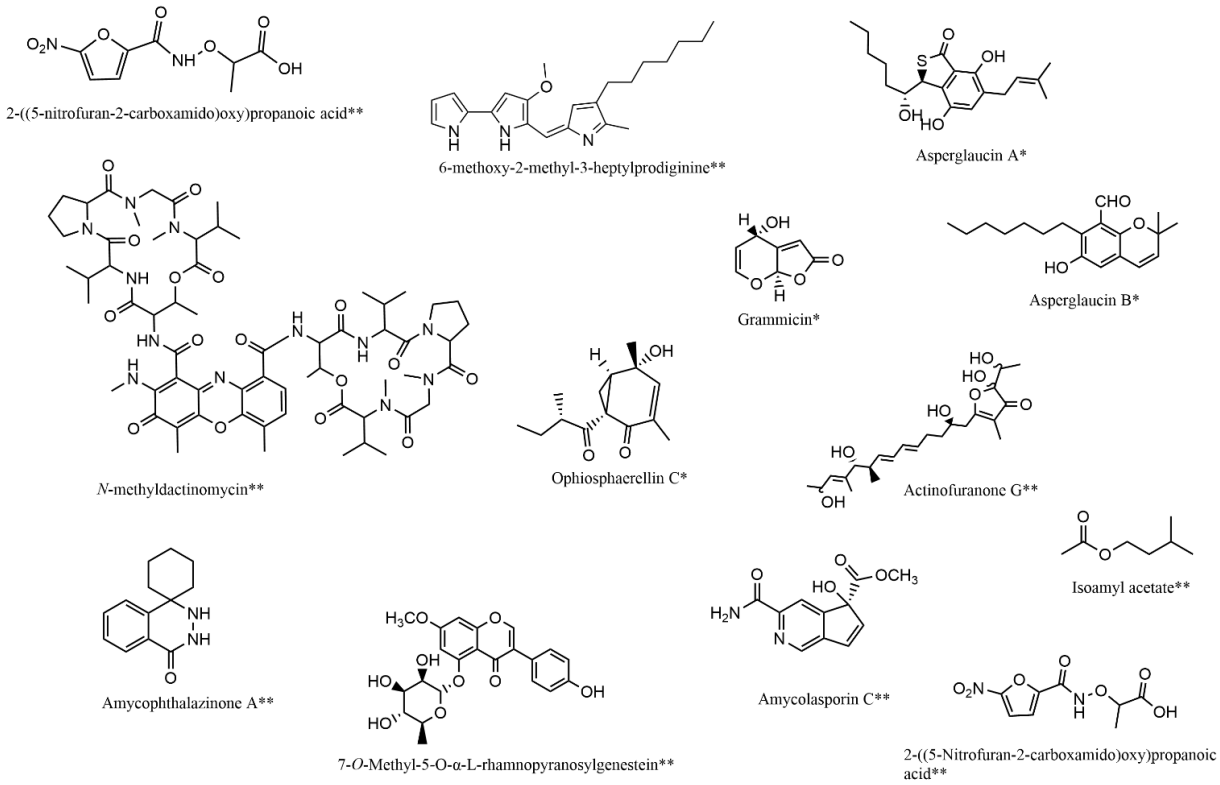

Figure 2 Some examples of bioactive compounds isolated from lichen-associated fungi (*) or from bacteria (**).

Way to enhance metabolites chemodiversity

Lichens are, without a doubt, one of the most propitious niches for the discovery of novel fungal and bacterial strains with tremendous abilities for original metabolites production. Unfortunately, some of these metabolites may have been left out from detection, isolation and characterization of their biological activities because of the low abundance of active compounds present in the extracts. In order to be able to recover these substantial amounts of novel and unknown active metabolites, mass cultures, up to 100 L of culture, are required [131], [133], [170], [216], [219], [222], [226]–[229]. However, as more and more natural compounds are discovered, the rate of discovering new metabolites decreases. To address this issue, different options could be considered. OSMAC, One Strain Many (Active) Compounds, and co-cultivation are two techniques, extensively used, and that can be applied to uncover the lichen-associated culturable microflora metabolome by activating cryptic secondary metabolites pathways [230]–[250]. As described for the recovering of higher microbial diversity during isolation steps, varying culture media conditions could disclose the unseen chemical diversity of this microbiota. For example, the secondary metabolites of a lichen-associated *Amycolatopsis* sp. were investigated by cultivating the bacterial strain using three different media. A total of 21 metabolites were characterized, only 5 of them were recovered from all the media and none of them were already described as produced by this actinobacteria strain [251].

Conclusion

With increased metagenomic sampling and analysis, taxonomic boundaries and nomenclature are constantly being reassessed [97]. Meanwhile, scientists have realized that bacterial and

archaeal phyla without a single cultivated representative constitute the majority of life's current diversity [252], [253]. Despite more than 130 years of microbial cultivation studies, many microorganisms remain recalcitrant to traditional cultivation approaches. Unraveling the mysteries of these candidate phyla, not only the substantial roles played by microorganisms in the function of the biosphere but also their reservoir of novel bioactive compounds, is a great challenge in microbiology and is especially important in habitats where they are abundant, including some extreme environments and low-energy ecosystems [254] and that's what makes the unculturable lichen-associated bacteria or fungi particularly interesting. The last decade, novel *in situ* cultivation techniques, for example diffusion chambers [57], iChip [60], I-Tip [61] and even a diffusion bioreactor [257], have been introduced to mimic natural conditions and provide access to critical growth factors found in the environment and/or supplied by neighboring species, allowing the recovery of otherwise recalcitrant species [258]. Wells from these innovative technologies allow an unimpeded growth and the possibility to conduct long incubations, e.g. 1.5 years, leading to a 700-fold increase in cultivability and a significant difference in novelty of slow growing isolated microorganisms [259], [260]. While, pure cultures were maintained thanks to diffusion chambers, it was suggested that growth synergy occurring during co-cultures might involve specific signals originating from their neighbors, also defined as appropriate "helpers" [261]. However, it is noteworthy that some of the most interesting isolates appeared to have acquired the ability to grow *in vitro* after at least 3 transfers through the diffusion chamber, leaving the exact nature of "dosmestication" progress mysterious. Besides *in situ* technologies, culturomics, a high-throughput method that multiplies culture conditions, e.g. incubation time, temperatures, media composition, combined with mass spectroscopy or 16S ribosomal RNA sequencing as identification tools, was developed to cultivate and identify unknown bacteria [262]. Indeed, designing culture media by macro- and micronutrients supplementation, using e.g. lichen enriched media, for *in vitro* cultivation can provide environmental and nutritional conditions that mimic natural habitats [184].

However, studies integrating the taxonomic, genomic, and functional diversity associated with holobionts being still scarce, microbiologists should be able to minimize the gap between the microbial richness in nature and the number of species in culture [173], for the benefit of both basic and applied microbiology [258] by combining both methods to gain a more complete picture of the microbial diversity [79] and maximize the opportunities to discover new secondary metabolites including the ones belonging to the tremendous ecological niche represented by lichens.

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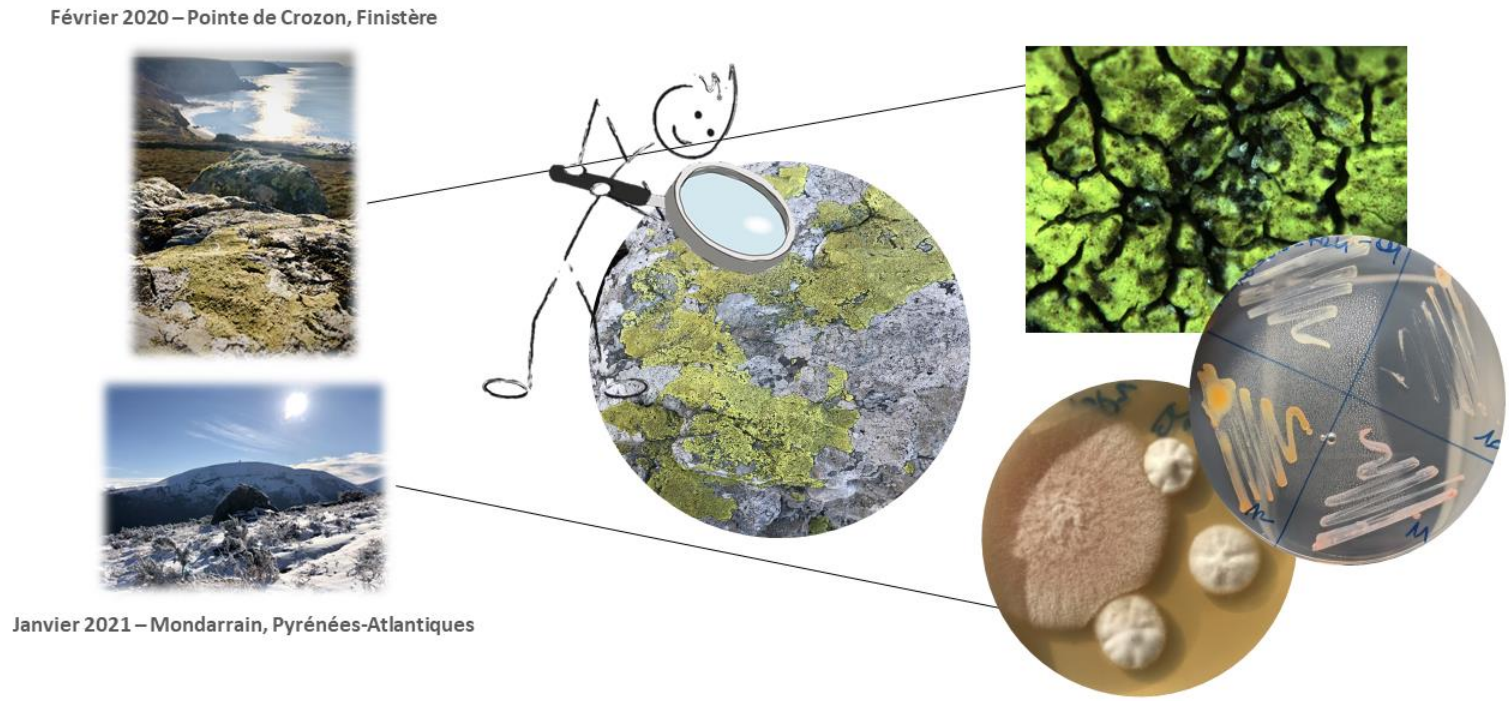
ETUDE DE LA MICROFLORE ASSOCIEE A *RHIZOCARPON GEOGRAPHICUM*



CHAPITRE 2 : ETUDE DE LA MICROFLORE ASSOCIEE A *RHIZOCARPON GEOGRAPHICUM*

I. Introduction et contexte de l'étude

Le chapitre précédent a permis de contextualiser l'intérêt que représentent d'une part, l'isolement microbien à partir des lichens - aussi bien grâce à des méthodes de microbiologie classique pasteurienne, que par celles de haut débit utilisant des approches d'enrichissement – et d'autre part l'étude de la chimiodiversité bactérienne et fongique pour des fins de valorisation biotechnologique. Les lichens constituent des niches écologiques d'une incroyable diversité microbienne (Grube and Berg, 2009) dont seulement une fraction infime a pu être appréhendée. Dans ce contexte, il nous est alors apparu intéressant de réaliser des isolements à partir d'un lichen peu étudié d'un point de vue de sa microflore, le lichen crustacé et très communément répandu *Rhizocarpon geographicum* (présenté dans l'article 1 p.45). Les démarches d'isolements microbiens entreprises dans le cadre de cette étude se fondent sur des stratégies culture-dépendantes ouvrant d'encourageantes perspectives.

En premier lieu, ce travail s'est penché sur un isolement, que l'on peut qualifier de classique, des bactéries et des champignons à partir de deux échantillons de *R. geographicum*. Dans cette étude, nous avons cherché à explorer la communauté microbienne du lichen par des techniques de microbiologie pasteurienne. Ces travaux ont fait l'objet d'une soumission dans le journal *Environmental Microbiology Reports* en collaboration avec Patricia Jargeat (Laboratoire Evolution et Diversité Biologique, Toulouse) et Angèle Lengo Mambu (Laboratoire PEIRENE, Limoges) (Partie II, p 45). Les descriptifs morphologiques des microorganismes isolés sont indiqués dans le tableau S1 p. 1 du Volume 2.

Figure 1 Essai d'isolement par la technologie de l'iChip

En second lieu, nous avons mené un isolement bactérien massif, à partir de 24 échantillons de *R. geographicum* récoltés dans différents environnements bretons et basques, grâce à des méthodes de haut débit, dont l'enrichissement, les traps (Gavrish et al., 2008) et les iChips (Nichols et al., 2010). Cette dernière, n'étant pas adaptée à la morphologie du lichen, n'a pas permis d'aller au-delà de la mise en place des dispositifs (Fig. 1) et ne

sera pas décrite par la suite. En effet, la membrane semi-perméable utilisée et permettant les échanges avec l'environnement extérieur a entrainé l'asséchement total de la gélose empêchant toute croissance bactérienne.

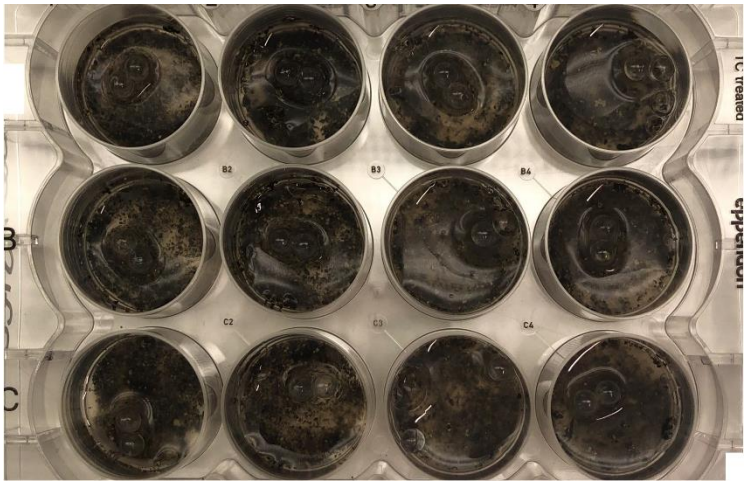


Figure 2 Mise en place de l'isolement par « traps ».

Les essais préliminaires pour l'isolement bactérien à partir de la méthodologie « traps » (Fig. 2) ont nécessité une grande quantité de lichen *R. geographicum* pulvérisé. Considéré comme denrée très précieuse et difficilement récoltable en quantité suffisante pour ces essais, cette méthode d'isolement a également été laissée de côté pour se concentrer uniquement sur la culturomique. Ces travaux ont été réalisés sous la supervision de Claudia Bartoli à l'Institut de Génétique, Environnement et Protection des Plantes (IGEPP), INRAE, de Le Rheu. Les résultats de l'isolement sont finalisés, certains tests biologiques sont en cours et également compilés dans cette version non encore finalisée d'un article devant être soumis à *The ISME Journal* (cf III - p 75).

II. Microbial community associated with the crustose lichen *Rhizocarpon geographicum* L. (DC.) living on oceanic seashore: a large source of diversity revealed by using multiple isolation methods

Microbial community associated with the crustose lichen *Rhizocarpon geographicum* L. (DC.) living on oceanic seashore: a large source of diversity revealed by using multiple isolation methods

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Keywords

Holobiont, lichen-associated microbiota, isolation methods, barcoding

Abstract

Recently, the study of the interactions within a microcosm between hosts and their associated microbial communities drew an unprecedented interest arising the holobiont concept. Lichens, a symbiotic association between a fungus and an alga, redefined as complex ecosystems considering the tremendous array of associated microorganisms satisfy this concept. Culture dependent and independent methods are additional methods in order to explore this particular and still poorly known microbial ecological niches. The present study focuses on the diversity of the microbiota associated with the seashore located lichen *Rhizocarpon geographicum*, recovered by different culture-dependent methods. Samples harvested from two sites allowed the isolation and molecular identification of 68 fungal isolates distributed in 43 phylogenetic groups, 15 bacterial isolates distributed in 5 taxonomic groups and 3 microalgae belonging to 2 species. While Firmicutes was the dominant phylum (15 isolates, 62.5%), Proteobacteria phylum represented 20.8% of total bacterial isolates and Actinobacteria 16.7%. Most of the

identified fungal isolates (91.2%) belonged to the Ascomycota phylum. Among Ascomycota, Dothideomycetes were particularly abundant (44.8%) followed by Sordariomycetes (32.8%). Basidiomycota (7.8%), the second phylum of fungal isolates was represented by Agaricomycetes, Cystobasidiomycetes and Tremellomycetes. The closest site to the cliff, appeared to be richer than the other one, with more undetermined taxa from the thallus as well as from the supernatant. Moreover, for 12 fungal isolates belonging to 10 different taxa, the genus was not described in GenBank. These fungal species have never been sequenced or even described and therefore non-studied. All these findings highlight the novel and high diversity of the microflora associated with *R. geographicum*. While many species disappear every day, this work suggests that coastal and wild environments have still an unrevealed variety to offer and that lichens constitutes a great reservoir of microbial diversity which can be recovered by multiplying the culture-dependent techniques.

Introduction

Coastal environments and cliffs, whether wave-pounded or inland, are binding ecosystems but also very fragile. They occupy one of the most dynamic interfaces on Earth, at the boundary between land and sea and finally correspond with some of the most diverse and productive habitats (McLean, Tsyban, et al., 2001). Considered as climatic refugia, they arouse growing scientific interest. Their ecology and biogeography have been investigated across the world (Kuntz and Larson, 2006; Strumia et al., 2020). As a brittle environment, cliffs and seashores are not only naturally unstable and subject to rapid changes (exposure to waves, local marine currents and wind action), they are also affected by global climate change (rise of temperature, changes in precipitation regimes, sea level, wave exposure, and salt spray) modifying the physical, biological and biogeochemical characteristics of the oceans and coasts and their ecological structure and functions. Due to such factors, several cliff plant species and their underexplored communities are under the threat of extinction, requiring political action for their conservation. The Integrated Coastal Zone Management and the European Biodiversity Strategy are examples of spatial planning strategies such as the protection by Council Directive 92/43/EEC (European Economic Community) (Strumia et al., 2020; Pena et al., 2021).

Coastal plant communities grow under specific environmental conditions explained by the interaction of land and sea, and lichens are an essential component of such communities. According to the classic definition, lichens are symbiotic organisms, highly adapted to extreme

habitats (Sancho et al., 2007), formed with a fungal partner, the mycobiont, and a photoautotrophic partner, the photobiont.

With its attractive green-yellow color, *Rhizocarpon geographicum* (L.) DC. (Rhizocarpaceae, Ascomycota) (McCarthy and Elix, 2014), formed by the fungi *Rhizocarpon* (Ascomycota, Lecanoromycetes, Rhizocarpaceae) and the microalga *Trebouxia* sp. (Chlorophyta, Trebouxiaphyceae), is one of the most widely distributed of crustose lichens and frequently one of the first colonizers of newly exposed rock surfaces, first terrestrial substrates available for living organisms on Earth (Ruibal et al., 2009). This species grows exceptionally slowly, on a broad range of substrates, occasionally found in submontane regions, but more commonly in the high mountains (Armstrong, 2011), and are thereby difficult subjects for laboratory conditions (Armstrong and Smith, 1996). As other rock-inhabiting lichens, it is often exposed to extreme abiotic conditions with broad fluctuations of temperature and humidity providing poor comfort of life and sources of nutriments (Muggia and Grube, 2018), conferring them unique abilities to develop protective mechanisms (Fernandes et al., 2015). As crustose lichens found in high altitude habitat, the samples studied in this work, collected at La Pointe de Crozon, Brittany (France), is strongly affected by hostile environment conditions. As for lichens of the arctomontane group, which is associated with the Arctic and high mountain regions, their presence is probably explained by severe environmental conditions in coastal habitats (Rodnikova, 2012).

Few years ago, it has been admitted that a third partner, the microbial consortia of bacteria and fungi, was part of the evolutionary, long-term successful and intimate lichen lifestyle (Grimm et al., 2021; Grube et al., 2009; Spribille et al., 2016). Nowadays, lichens are considered as holobionts (Simon et al., 2019), an exciting reservoir and unexplored hotspot of more or less specific and persistent members of complex microbial networks (Cao et al., 2018, Cardinale et al., 2006). However, their role in adapting lichens to unfriendly environments and moreover to coastal environments is still not clear (Delmail et al., 2013). If these microbial networks are dependent on these particular habitats, they might be involved in defense and chemical communication pathways as a source of original molecules (Bjelland et al., 2011; Boustie et al., 2011; Suzuki et al., 2016). The diversity and contribution of this third partner, the fungal and bacterial consortia partner, have recently been studied mostly by culture-independent techniques (Cardinale et al., 2006; Muggia and Grube, 2018) due to difficulties in their isolation and uncultivability. They have been thus described as the "microbial dark matter" (Rinke et al., 2013). However, these techniques can lead to biased estimates of microbial community richness and composition. In order to offset molecular analysis' lacks of information and hardship of

high recovering percentage of cultivable strains in axenic cultures, numerous media and different techniques of isolation can be applied (Lagarde et al., 2018; Muggia et al., 2017).
Most of the studies reported the influence of the culture media on the composition and diversity of the isolated microorganisms (Li and Wang, 2017; Medina et al., 2017; Muggia et al., 2017) and that this composition is also affected by the employed method of lichen's surface sterilization (Masumoto and Degawa, 2019). In this study, we aimed to investigate the microbial community of two samples of the lichen *R. geographicum* collected in the westernmost and hostile point of France, facing the Atlantic Ocean, in order to explore the French local biodiversity by depicting the common lichen-associated microbiota but also to improve our microbial diversity knowledge by reporting non-described and non-studied microbial strains.

Results

Isolation and identification of isolated microbiota

The morphological observation of the fungal and bacterial isolates from *R. geographicum* led to the isolation of 68 fungi, 24 bacteria and 6 microalgae.
Among the 68 fungal isolates, 62 were identified on molecular basis, distributed in 43 phylogenetic groups (Fig. 1) and 6 were morphologically related (Tab. 1; Fig. 1). Thirty seven isolates were identified at the species level and 19 at the genus level. Twelve isolates belonging to 9 different phylogenetic groups, could not be identified either at the species or the genus level.
Concerning bacteria, among the 24 isolates, 12 were identified at the species level and 2 isolates at the genus level after comparison with the GenBank database (Table 2). Only 1 isolate was identified at family level and 9 isolates were morphologically related. Out of the 6 microalgal isolates, 3 were identified at the species level and 3 were morphologically related (Table 3).

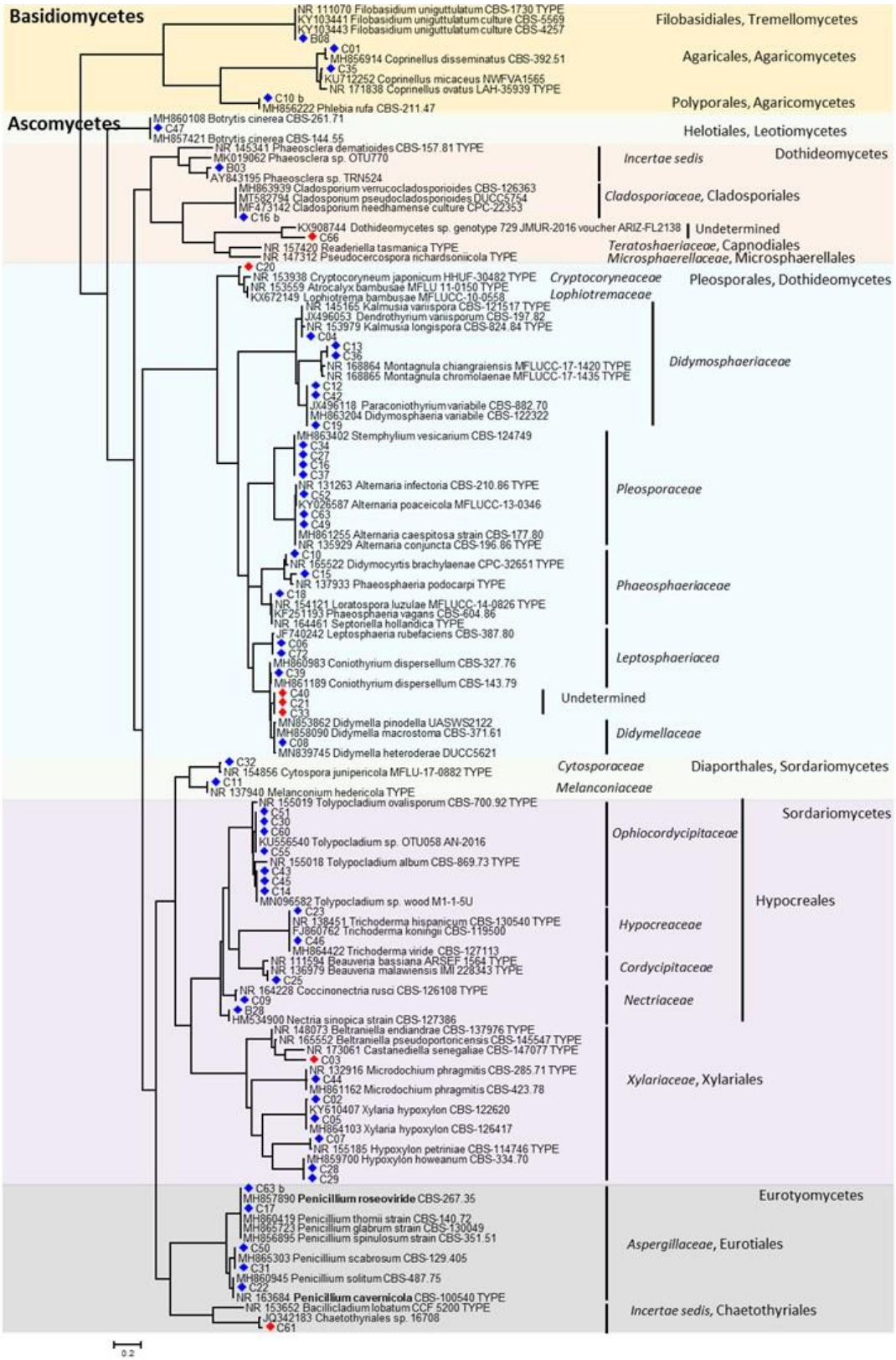

Fig. 1 Maximum-likelihood phylogenetic tree based on samples ITS sequences and closest ITS reference sequences from GenBank. The tree was obtained by applying the PhyML method in the Geneious® plateform. Bootstrap values > 50% are indicated above branches. Sequences generated in this study are indicated with diamonds. Red diamonds correspond to non-identified isolates.

Table 1

Molecular and morphological identification of fungi isolated from *R. geographicum*.

				Classification				
Isolate	BLAST and Phylogenetic identification	Location	Deposit method	Phylum	Class	Order	Family	Accession number
B01	Undet Cystobasidiomycetes *	Site 1	Supernatant	Basidiomycota	Cystobasidiomycetes	*incertae sedis*		OL890684
B03	*Phaeosclera sp.*	Site 2	Supernatant	Ascomycota	Dothideomycetes	*incertae sedis*		OL891597
B05a	*Aureobasidium* sp. *	Site 2	Thallus	Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	OL890685
B08a	*Filobasidium uniguttulatum*	Site 2	Supernatant	Basidiomycota	Tremellomycetes	Filobasidiales	Filobasidiaceae	OL891601
B24a	*Tremella sp. **	Site 1	Thallus	Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	OL890686
B27	Undet Myriangiales *	Site 2	Supernatant	Ascomycota	Dothideomycetes	Myriangiales	N. D.	OL890687
B28	*Thyronectria sinopica*	Site 2	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	OL891600
C01	*Coprinellus disseminatus*	Site 2	Thallus	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OL891602
C02	*Xylaria hypoxylon*†	Site 2	Thallus	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	OL891603
C03	Undet. Xylariales	Site 2	Supernatant	Ascomycota	Sordariomycetes	Xylariales	N. D.	OL891604
C04	*Dendrothyrium variisporum*	Site 1	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	OL891605
C05	*Xylaria hypoxylon*	Site 1	Thallus	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	
C06	*Leptosphaeria rubefaciens*	Site 1	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	OL891606
C07	*Hypoxylon petriniae*	Site 1	Thallus	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	OL891607
C08	*Didymella sp.*	Site 1	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	OL891608
C09	*Coccinonectria rusci*	Site 2	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	OL891609
C10	*Didymocyrtis brachylaenae*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OL891610
C10b	*Phlebia rufa*	Site 2	Thallus	Basidiomycota	Agaricomycetes	Polyporales	Meruliaceae	OL891611
C11	*Melanconium hedericola*	Site 2	Thallus	Ascomycota	Sordariomycetes	Diaporthales	Melanconidaceae	OL891612
C12	*Didymosphaeria variabile*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	OL891613
C13	Undet. Didymosphaeriaceae	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	OL891614
C14	*Tolypocladium sp. (2)*	Site 1	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	OL891615
C15	*Phaeosphaeria sp.*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OL891616
C16	*Stemphylium vesicarium*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	OL891617
C16b	*Cladosporium sp.*	Site 2	Thallus	Ascomycota	Dothideomycetes	Cladosporiales	Cladosporiaceae	OL891618

C17	*Penicillium roseoviride*	Site 2	Thallus	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	OL891619
C18	Undet. Phaeosphaeriaceae	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	OL891620
C19	*Didymosphaeria variabile*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	
C20	Undet. Pleosporales (1)	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	N. D.	OL891621
C21	Undet. Pleosporales (2)	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	N. D.	OL891622
C22	*Penicillium cavernicola*	Site 2	Thallus	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	OL891623
C23	*Trichoderma sp. (1)*	Site 1	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	OL891624
C24	*Beauveria malawiensis*†	Site 2	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	
C25	*Beauveria malawiensis*	Site 2	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	OL891625
C26	*Stemphylium vesicarium*†	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	
C27	*Stemphylium vesicarium*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	
C28	*Hypoxylon howeanum*	Site 1	Thallus	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	OL891626
C29	*Hypoxylon howeanum*	Site 2	Thallus	Ascomycota	Sordariomycetes	Xylariales	Xylariaceae	
C30	*Tolypocladium sp. (1)*	Site 1	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	OL891627
C31	*Penicillium scabrosum*	Site 1	Thallus	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	OL891628
C32	*Cytospora sp.*	Site 1	Supernatant	Ascomycota	Sordariomycetes	Hypocreales	Cytosporaceae	OL891629
C33	Undet. Pleosporales (2)	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	N. D.	
C34	*Stemphylium vesicarium*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	
C35	*Coprinellus micaceus*	Site 2	Thallus	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	OL891630
C36	Undet. Didymosphaeriaceae	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	
C37	*Stemphylium vesicarium*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	
C38	*Stemphylium vesicarium*†	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	
C39	*Coniothyrium dispersellum*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	OL891631
C40	Undet. Pleosporales (2)	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	N. D.	
C41	*Dendrothyrium variisporum*†	Site 1	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	
C42	*Didymosphaeria variabile*	Site 1	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	
C43	*Tolypocladium sp. (2)*	Site 1	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	
C44	*Microdochium phragmitis*	Site 1	Thallus	Ascomycota	Sordariomycetes	Xylariales	Microdochiaceae	OL891632
C45	*Tolypocladium sp. (2)*	Site 1	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	
C46	*Trichoderma sp. (1)*	Site 2	Supernatant	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	
C47	*Botrytis cinerea*	Site 2	Thallus	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	OL891633

C48	*Botrytis cinerea* †	Site 2	Thallus	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	
C49	*Alternaria sp. (2)*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	OL891634
C50	*Penicillium scabrosum*	Site 1	Thallus	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	
C51	*Tolypocladium sp (1).*	Site 1	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	
C52	*Alternaria sp. (1)*	Site 1	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	
C55	*Tolypocladium sp. (1)*	Site 1	Thallus	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	
C60	*Tolypocladium sp. (1)*	Site 1	Supernatant	Ascomycota	Sordariomycetes	Hypocreales	Ophiocordycipitaceae	
C61	Undet. Chaetothyriales	Site 1	Supernatant	Ascomycota	Eurotiomycetes	Chaetothyriales	N. D.	OL891635
C63	*Alternaria sp. (2)*	Site 2	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	
C63b	*Penicillium roseoviride*	Site 2	Thallus	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	
C66	Undet. *Dothideomycetes*	Site 2	Supernatant	Ascomycota	Dothideomycetes	N. D.	N. D.	OL891636
C72	*Leptosphaeria rubefaciens*	Site 1	Thallus	Ascomycota	Dothideomycetes	Pleosporales	Leptosphaeriaceae	

* 18S sequence, † Morphological identification, N. D.: not determined

Table 2

Bacteria isolated from *R. geographicum.*

					Classification				
Isolates	BLAST and Phylogenetic identification	Location	Deposit method	BLAST result	Phylum	Class	Order	Family	Accession number
B02	*Lichenibacterium* sp.	Site 1	Supernatant	98% *Lichenibacterium ramalinae*	Proteobacteria	α-proteobacteria	Hyphomicrobiales	Lichenibacteriaceae	
B04	Undet	Site 2	Supernatant	95.4 % *Arthrobacter* spp	Actinobacteria	Actinomycetia	Micrococcales	Micrococcaceae	OL891637
B05	*Paenibacillus etheri*	Site 2	Thallus	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B07	*Paenibacillus etheri*	Site 2	Supernatant	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B08	*Paenibacillus etheri*	Site 2	Supernatant	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B09	*Paenibacillus etheri*	Site 1	Thallus	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B10	*Paenibacillus etheri*	Site 1	Supernatant	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	OL891638
B11	*Paenibacillus etheri*	Site 1	Supernatant	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B12	*Paenibacillus etheri*	Site 2	Supernatant	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B13	*Paenibacillus etheri*	Site 2	Thallus	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B14	*Paenibacillus etheri*†	Site 2	Supernatant		Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B15	*Paenibacillus etheri*†	Site 2	Supernatant		Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B16	*Paenibacillus etheri*†	Site 1	Supernatant		Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B17	*Paenibacillus etheri*	Site 2	Supernatant	99.8 % *Paenibacillus etheri*	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B23	*Lichenibacterium* sp.†	Site 1	Supernatant		Proteobacteria	α-proteobacteria	Hyphomicrobiales	Lichenibacteriaceae	
B24	*Lichenibacterium* sp.	Site 1	Thallus	97% *Lichenibacterium ramalinae*	Proteobacteria	α-proteobacteria	Hyphomicrobiales	Lichenibacteriaceae	OL891639
B24b	*Lichenibacterium* sp.†	Site 1	Thallus		Proteobacteria	α-proteobacteria	Hyphomicrobiales	Lichenibacteriaceae	
B33	*Microbacterium paraoxydans*	Site 1	Supernatant	99.7 % *Microbacterium paraoxydans*	Actinobacteria	Actinomycetia	Micrococcales	Microbacteriaceae	OL891640
B33b	*Microbacterium paraoxydans*	Site 1	Supernatant	99.7% *Microbacterium paraoxydans*	Actinobacteria	Actinomycetia	Micrococcales	Microbacteriaceae	
B34	*Paenibacillus etheri*†	Site 1	Thallus		Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B35	*Microbacterium paraoxydans*†	Site 1	Supernatant		Actinobacteria	Actinomycetia	Micrococcales	Microbacteriaceae	
B38	*Paenibacillus etheri*†	Site 2	Supernatant		Firmicutes	Bacilli	Bacillales	Paenibacillaceae	
B39	*Caballeronia mineralivorans*	Site 2	Thallus	99.3 % *Caballeronia mineralivorans*	Proteobacteria	β-proteobacteria	Burkholderiales	Burkholderiaceae	OL891641
B46	*Paenibacillus etheri*†	Site 1	Thallus		Firmicutes	Bacilli	Bacillales	Paenibacillaceae	

† Morphological identification

Table 3

Microalgae isolated from *R. geographicum.*

					Classification				
Isolates	BLAST and Phylogenetic identification	Location	Deposit method	BLAST result	Phylum	Class	Order	Family	Accession number
B21	*Apatococcus lobatus*	Site 1	Supernatant	99.6% *Apatococcus lobatus*	Chlorophyta Pascher	Trebouxiophyceae	Chlorellales	Chlorellaceae Brunnthaler	OL891598
B22	*Coccomyxa viridis*	Site 1	Supernatant	100% *Coccomyxa viridis*	Chlorophyta	Trebouxiophyceae			OL891599
B26	*Coccomyxa viridis*	Site 1	Supernatant	100% *Coccomyxa viridis*	Chlorophyta	Trebouxiophyceae			
B40	*Coccomyxa viridis*†	Site 1	Supernatant		Chlorophyta	Trebouxiophyceae			
B41	*Apatococcus lobatus*†	Site 1	Supernatant		Chlorophyta Pascher	Trebouxiophyceae	Chlorellales	Chlorellaceae Brunnthaler	
B44	*Coccomyxa viridis*†	Site 1	Supernatant		Chlorophyta	Trebouxiophyceae			

† Morphological identification

Diversity of isolated fungal communities

Ascomycota is the dominant phylum with 62 isolates, depecting 91.2% of the total fungal isolates (Fig. 2). They are distributed in 4 classes, 10 identified and 2 unidentified orders and 21 families. The most abundant class was Dothideomycetes (32 isolates, 47%) followed by Sordariomycetes (22 isolates, 32.3%) and Eurotiomycetes (6 isolates, 8.8%). Leotiomycetes are represented by 2 isolates. At the order level, Pleosporales (27 isolates, 39.7%) are dominant, followed by Hypocreales (14 isolates, 20.5%) and Xylariales (7 isolates, 10.3%). At the family level, Pleosporaceae (9 isolates, 13.2%) is the most abundant followed by Ophiocordycipitaceae and Didymosphaeriaceae (7 isolates, 10.3% each). Finally, at the species level *Stemphylium vesicarium* (5 isolates, 7.3%) is more present.

Basidiomycota phylum is poorly represented with only 6 isolates corresponding to 8.8% of the total isolates but 5 different taxa were identified. With 2 isolates each, Agaricomycetes and Tremellomycetes classes are the most abundant class (33.3%). Only 1 undetermined isolate represented the Cystobasidiomycetes class.

Comparing the 2 sampling sites, 25 strains were isolated and identified from site 1 versus 43 isolated and identified from site 2 (Table 1). The two sites share 2 taxa: *Didymosphaeria variabile* and *Hypoxylon howeanum*. Among the 25 isolates from site 1, 4 were isolated from the supernatant representing 4 taxa including 2 undetermined; 21 were isolated from the thallus representing 14 taxa. *Tolypocladium* sp. (1) is common to the thallus and supernatant deposit methods. Out of the 43 isolates from site 2, 7 were isolated from the supernatant representing 3 taxa including 3 undetermined; 37 were isolated from the thallus representing 19 taxa including 4 undetermined (Fig. 2). From washing water, only 11 isolates among the 68 isolates were obtained. However, 5 of them were characterized as undetermined taxa. Moreover, one yeast, *Filobasidium uniguttulatum* was isolated.

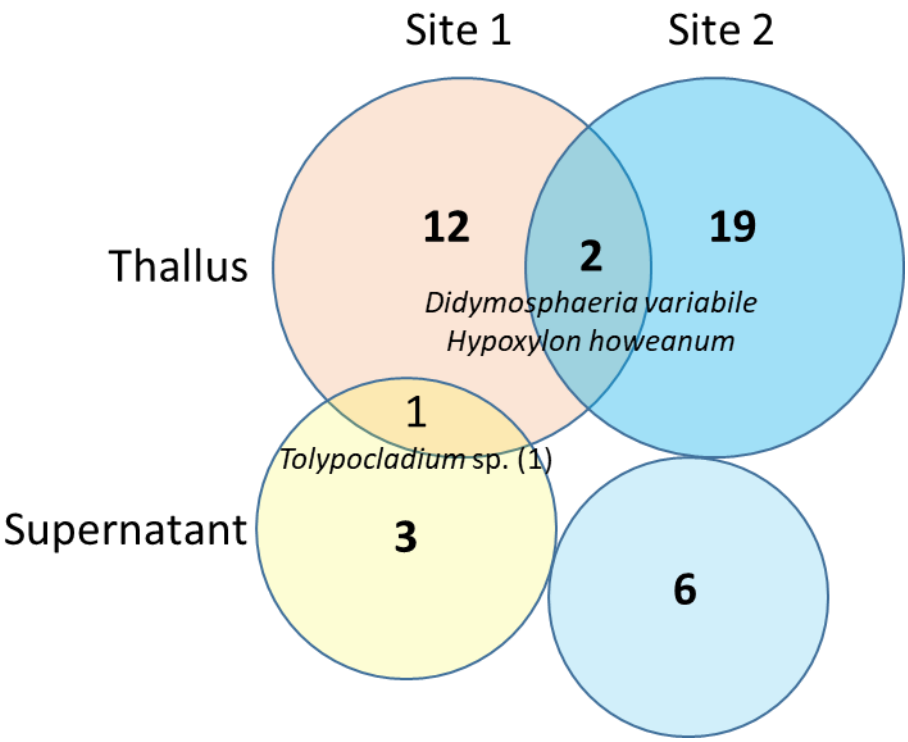

Fig. 2 Venn diagram representing the number of shared and exclusive fungi isolated from *R. geographicum* comparing the different collect locations and methods of isolation.

Diversity of isolated bacterial communities

This work permitted the isolation of 24 bacterial strains belonging to 5 different taxa. Three taxa were recovered from site 1. *Lichenibacterium* sp and *Microbacterium paraoxydans* were only found on the first site while the second site allowed the isolation of one undetermined taxon. *Paenibacillus etheri* was found on both locations.

While Firmicutes is the dominant phylum (15 isolates, 62.5%), Proteobacteria phylum represents 20.8% of total bacterial isolates and Actinobacteria 16.7%. At the class level, Bacilli (15 isolates, 62.5%) is dominant, followed by Actinomycetia and Alphaproteobacteria (respectively 4 isolates, 16.7%). At the order level, Bacillales (15 isolates, 62.5%) is in the majority, followed by Micrococcales and Hyphomicrobiales (respectively 4 isolates, 16.7%). At the family level, the most abundant is Paeanibacilleae (15 isolates, 62.5%), followed *ex aequo* by Microbacteriaceae and Lichenibacteriaceae (respectively 4 isolates, 16.67%). At the genus level the most abundant bacteria are *Paenibacillus* (15 isolates, 62.5%), followed by *Lichenibacterium* (4 isolates each, 16.7%).

Isolation of microalgae

The long incubation period, 6 months, permitted the isolation of 6 microalgae. All of them were isolated from the supernatant and from the site 1. They all belong to the Trebouxiophyceae family and two species were identified including *Apatococcus lobatus* and *Coccomyxa viridis.*

Selectivity of agar media used

Originally, the media were chosen for the isolation of bacteria. As no antifungal compound was used, fungi also grew. The lichen mycobiote diversity was higher with the TY medium (25.6%), closely followed by the GEM (23.3%) and the YS and MEP media (16.3%) (Fig. 3). With taxa diversity of 14%, the PDA and MB media allowed the growth of 6 different taxa each. The lowest diversity recovery has been observed for PYM and LB media with only one taxon (2.3%). On LB medium 6 isolates were purified but 5 of them could not be further cultivated under axenic conditions. Twelve unidentified fungal strains belonging to 9 different phylogenetic groups were mostly recovered on PDA (25%), YS, GYM and MEP (16.7%) media.

Concerning bacteria, YS, MYP, PYM and LB media permitted to recover 2 different taxa (40%). GYM and MEP media did not allow any bacterial growth contrary to previous isolation (not published).

The 6 microalgal strains were able to grow as well on PDA, TY, GYM as on ISP2 media (Fig. 3).

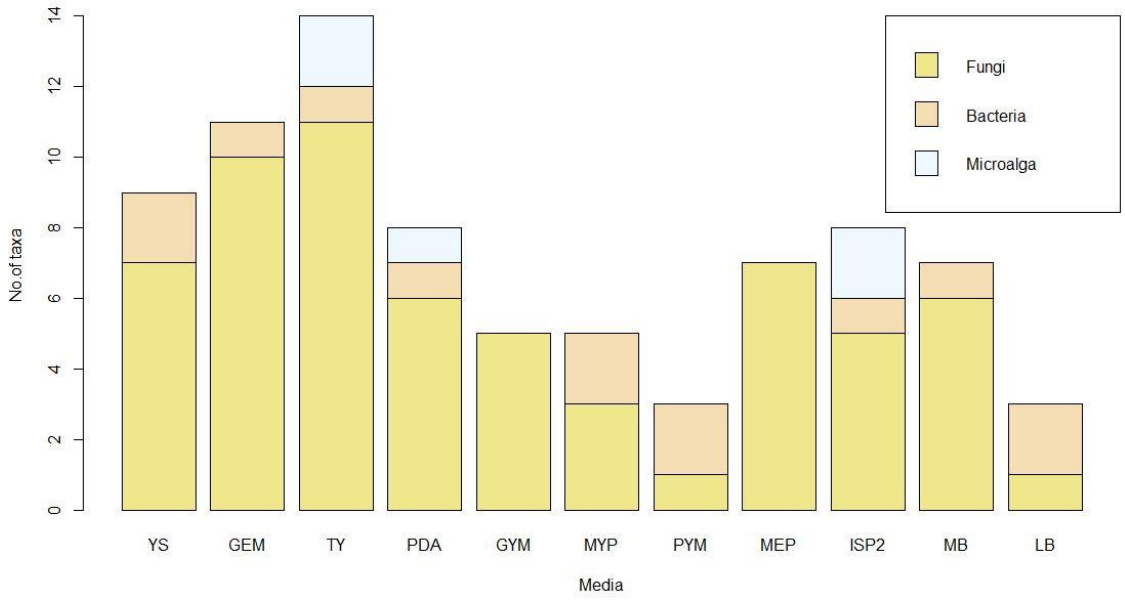

Fig. 3 Comparison of the number of taxa identified according to the media used.

Comparison of different deposit methods

The deposit method of lichen material onto Petri dishes had an effect on microbial growth (Fig. 4). Indeed, for fungi, the suspension spreading led to isolation of 10 different taxa including 5 undetermined. Two of the 10 taxa were shared with the thalli pieces deposit. A *contrario*, the lichen bacteriobiont presented a greater diversity when the suspension was spread. Microalgae were only expanded when the suspension of lichen material was spread plated.

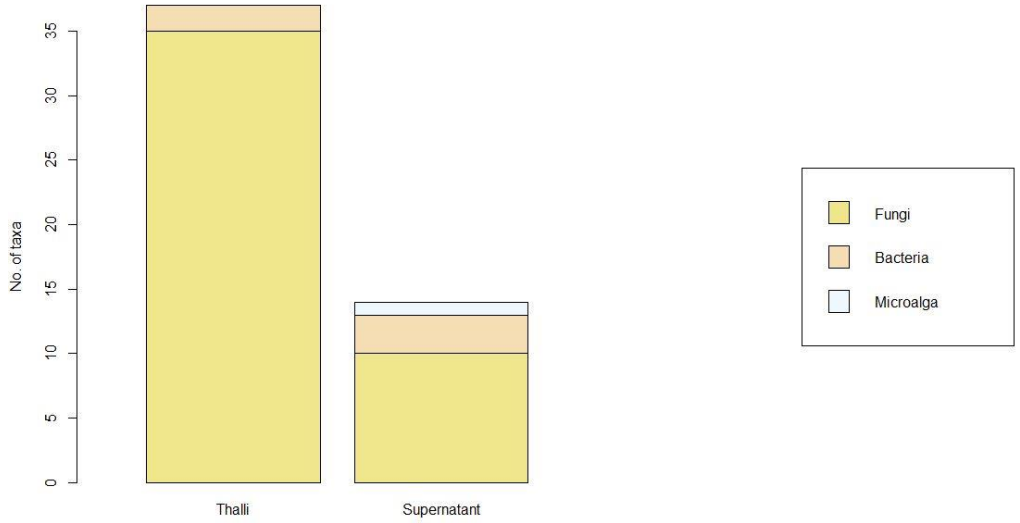

Fig. 4 Comparison of the number of fungal, bacterial and microalgal strains identified according to the deposit method used.

Comparison of locations

Over the 68 fungal isolates, 28 (41.2%) were recovered from the site 1 and 40 (58.8%) from site 2. Site 1 presented 16 different taxa including 2 undetermined while site 2 presented 30 taxa. Sites 1 and 2 had 6 shared taxa. Although, some taxa are not shared as *Tolypocladium* sp. which are only found on site 1. Moreover, *Stemphylium vesicarium*, and undetermined Pleosplorales are only recovered from samples of the second site. Concerning the bacterial isolates, *Paenibacillus etheri* was common to the both sites. The undetermined bacteria and *Caballeronia mineralivorans* were isolated from site 2. *Microbacterium paraoxydans* and *Lichenibacterium* sp. were only found from the site 1. Finally, all the microalgae were isolated from the site 1.

Discussion

The diversity of lichen-associated microbiota of *R. geographicum* was, to our knowledge for the first time, investigated by a culture-dependent approach. The *R. geographicum* lichen

samples were chosen because the rocky sea coasts represent some of the most extreme habitats for living organisms. Two different spatially proximate sites were chosen: site 1, more inland than the site 2 located on the edge of the cliff.

This work allowed the isolation and molecular identification of 62 fungal isolates, 15 bacterial isolates and 3 microalgae. Site 2, the closest to the cliff, appeared to be richer than the site 1 with more undetermined taxa from the thallus as well as from the supernatant. Indeed, for 12 fungal isolates belonging to 9 different taxa, the genus is not represented in GenBank. These genera have never been sequenced or even described and therefore non-studied. While many species disappear every day, this work suggests that coastal and wild environments have still an unrevealed variety to offer and that lichens constitutes a great reservoir of microbial diversity which can be recovered by multiplying the culture-dependent techniques.

Surprisingly, among the Basidiomycota, 3 Agaricomycetes, which were never previously described as endolichenic, were found from thallus and exclusively from site 2. One of them, *Coprinellus disseminates* described as a wood decomposer (Singh et al., 2009), does not have much to decay on such a surface. Another taxa *C. micaceus*, described as being able to biosorb 100% of lead (Albert et al., 2020), could be explained by a pollution fact: Brest Bay is known for its very high level of Pb due to bombing attacks during the World War II and by the intensive agriculture (Chiffoleau, 2017). Endolichenic fungi are often host-generalists with regard to the lichens in which they occur, they are more closely related to endophytic symbionts than to saprotrophic fungi, suggesting that their associations with lichen thalli are not purely incidental (Chagnon et al., 2016). As a matter of fact, *C. micaceus* could thus play a role of a "protector" as it could have been previously described for some bacterial strains (Cernava et al., 2017).

Most of fungal isolates (91.2%) identified belong to the Ascomycota phylum correlating to previous results (Lagarde et al. 2018). Among Ascomycota, Dothideomycetes were particularly abundant (44.8%) followed by Sordariomycetes (32.8%). It is known that the growth form of the lichen hosts influences the diversity of the associated fungi. Hence, taxa belonging to Dothideomycetes are mainly isolated from crustose thalli on rocks (Muggia and Grube, 2018). Very few fungal genera found herein have already been reported from lichens. We can cite endolichenic fungi belonging to *Aureobasidium*, *Cladosporium*, *Penicillium*, *Trichoderma*, *Xylaria* (Lagarde et al. 2018). At the species level, it is interesting to note that, only one species described in our study, *Botrytis cinerea*, a known pathogenic plant fungus, was already isolated from an epiphytic lichen *Ramalina fastigiata* (Lagarde et al. 2018). These findings highlight the novel and high diversity of the microflora associated to *R. geographicum*.

Regarding the bacterial diversity, while one study reported, using fingerprinting method (DGGE) and clone libraries, the presence of Acidobacteria, α and β-proteobacteria from *R. geographicum* (Bjelland et al., 2011). Firmicutes was in our study the dominant bacteria phylum (15 isolates, 62.5%) only represented by the genus *Paenibacillus* followed by α-Proteobacteria represented by *Lichenibacterium* sp. then by Actinobacteria. These results put on the light the importance to use different and complementary methods in order to better describe a microbial community. *Lichenibacterium* species (*L. ramalinae* and *L. minor*), the second most abundant bacterial species, were reported as β-carotene producing bacteria and were already isolated from subantartic lichens. Antartic lichens housed also *C. mineralivorans*, an atmospheric nitrogen fixer (Noh et al., 2021). Strains of *Paenibacillus* has been described as being especially common constituents of the lichen associated microbiota fraction (Grube and Berg, 2009). In addition, trying to explain the low rate of bacterial isolates and the high percentage of *Paenibacillus* strains identified, some studies previously reported that Firmicutes from lichens were widely considered as producers of antibiotics and enzyme inhibitors (Swamy and Gayathri, 2021) and some of them were mycorrhizal helper bacteria (Poole et al., 2001). At the species level, *P. etheri*, the most abundant bacterial species found in this study, was isolated from hydrocarbons polluted soil and has been described as a methyl *tert*-butyl ether degrader (Guisado et al., 2016). Interestingly this species was already isolated from a *R. geographicum* sample collected four years ago on the Brittany Coasts (data not published), converging with the isolation of *C. micaceus*. Moreover, *Arthrobacter* sp and *Microbacterium paraoxydans* have been described as being able of bioremediation (Manzoor et al., 2021; Sayyed et al., 2019) and for the last species as arsenic and lead degrader (Kaushik et al., 2012). The location of lichens harvested, la Pointe de Crozon, being also polluted by the Amoco Cadiz and Erika oil spills in 1974 and 1999 respectively, we can ask ourselves if these bacteria have some kind of ability to improve and help the lichen to live in such an unfriendly environment. This observation could also be supported by the identification of a *Cladosporium* strain from the site 2 which has been described as hydrocarbons tolerant and capable of bioremediation (Birolli et al., 2018; Velez et al., 2020), another "protector" species as described above.

Most of the culture-dependent studies concentrated on filamentous ascomycetes neglecting lichen-inhabiting basidiomycetes or yeasts in general and thus have only rarely been isolated (Duarte et al., 2016; Santiago et al., 2015; Zhang et al., 2016). It was shown that metabarcoding using ITS1 and ITS2 permitted the detection of Basidiomycetes but no Cystobasidiomycetes (Banchi et al., 2018; Fernández-Mendoza et al., 2017). In both of these studies, the lichens surfaces were not sterilized prior to perform metabarcoding. Hence, as nothing went off the

samples, it might explain the infrequency of basidiomycetes isolation in lichen-associated microbiota works. In our study, prior to the isolation steps, the lichen material was not commonly sterilized but just washed (Yang et al., 2021). One Cystobasidiomycete strain (site 1) and *Filobasidium uniguttulatum* (site 2) were then identified and isolated from the supernatant spreading deposit method. This supports the hypothesis that Cystobasidiomycetes are epilichenic rather than endolichenic (Černajová and Škaloud, 2019). In 2016, it was suggested that these yeasts may play a role in lichens' phenotype and hypothesized that the yeasts may represent yet another obligatory constituent of the lichen symbiosis (Spribille et al., 2016), hypothesis criticized (Oberwinkler, 2017). More recently, a positive correlation was made between the abundance of basidiomycete secondary fungal symbionts in the lichen *Bryoria tortuosa* with the visible production of the secondary metabolite vulpinic acid in the shared extracellular matrix between the core ascomycete symbiont and the yeasts (Tagirdzhanova et al., 2021). With added partners, the complexity of species interactions increases (Mark et al., 2020). As the basidiomycete yeasts diversity is still poorly known and opinion diverse, it might be hard to conclude on either one or the other hypothesis but their diversity can be expected to be tremendous.

In addition of the fungal and the bacteria diversity within the lichen *R. geographicum*, two microalgal species were identified. One of the genera *Coccomyxa* can be lichenicolous algae or lichenized photosynthetic partners in lichens (Malavasi et al., 2016) as described in several studies (Cao et al., 2018; Gustavs et al., 2015). It is interesting to note that a strain of *Coccomyxa viridis* was also isolated from the *R. geographicum* sample collected 4 years ago. Finally, this study highlighted the presence of another algal strain corresponded to *Apatococcus lobatus*, striking ecological differences with the lichen photobiont (Chrismas et al., 2021; Gustavs et al., 2016). This evidence must be related to a recent study which described the variation of photobiont diversity depending on the growth stage of the thalli, the geographic location and the habitat. An important point which was also revealed is the risk of overestimation of photobiont diversity from small thalli (Molins et al., 2021). While it has always been possible to assess relationships among the fungal partners through microscopy of their complex structures, the same cannot be said for the algal partner. The tedious culture of lichen photobionts has been the only possibility to distinguish species of microalgae and cyanobacteria isolated from lichen symbioses. The enhancement of molecular methods and development of specific primers for algal gene loci has improved the knowledge of the diversity and variability of photosynthetic partners (Grube and Spribille, 2012). The questions which can be asked are

if they are merely epibionts or only distributed in low abundance within the lichens or only spotted in certain parts of the thallus (Casano et al., 2011; Grube and Muggia, 2010; Guzow-Krzemińska, 2006). A study hypothesized, by analyzing both washed and unwashed lichen samples (Muggia et al., 2013), that the epithalline algae communities host numerous algal species, and if not separately considered, might lead to an overestimation of photobiont diversity in lichens in general and a direct improper function in the lichen symbiosis. But as emphasized on the other hand, it may confer advantages in the lichen's ability to counteract environmental changes or to occupy extreme environments mixing strategies to fine-tune their association (Eva M. et al., 2013; Piercey-Normore, 2006; Sun and Friedmann, 2005). Indeed, a higher variety of symbiotic associations could be helpful when changing environments and therefore might be the rule in lichens living on a wide variety of substrates and in diverse habitats like the very hostile environment of the Britain Atlantic Coast. They could finally correspond to a habitat-adapted symbiosis (Casano et al., 2011; Rodriguez et al., 2008) and different partnerships can be tested at low risk for the entire thallus structure (Muggia et al., 2013).

Even if the Sanger direct sequencing approach gave clues about fungus-algal association, distribution patterns and diversity in lichens, it also can lead to oversimplify diversity (Voytsekhovich and Beck, 2016), thus, underestimating all the complexity of the symbiotic association and underdetecting less abundant cooccurring photobiont partner.

We finally can mention that the deposit method used impact the microbial isolation. Indeed, while fungi were mostly isolated from thalli, bacterial isolates and microalgae were better recovered from supernatant. Comparing the fungal recovery rates of the suspension plate spreading deposit method and the thalli deposit method, the latter one permitted to isolate and identify 87.5% of the total lichen mycobiote. This could be explained by the fact that all lichen thalli host a community of cryptic fungi and that many lichenicolous fungi are endothallic, i.e. form their mycelium inside the thallus (Černajová and Škaloud, 2019). Moreover, even if the size of the lichen thalli deposited could have appeared being very small, the diversity of the fungi recovered was great. Indeed, as previously described, the isolate density and diversity is inversely related to the size of the thallus (Yang et al., 2021).

Experimental procedures

Lichen sample collection

The two thallus samples of *R. geographicum* were carefully collected in February 2020, using sterile gloves and washed instruments, in France at La Pointe de Crozon from two different locations: site 1 (48°13'59''N and 4°33'60''W) and site 2 (48°14'11''N and 4°33'59''W) located in a very particular spot: on a seaside cliff and directly exposed to the ocean sprays. Lichen samples were identified by Joel Esnault from the French Association of Lichenology. After sampling, lichens on rock fragments were transported in sterile Petri dishes stored in individual plastic bags and processed within the 6 hours.

Isolation and culture conditions

As there is no standardized methodology for the isolation of lichen associated microbiota, a protocol for lichen washing was used, based on Parrot et al. (2015) and Petrini, (1991). Two techniques of isolation and eleven different media picked on DMSZ website (https://www.dsmz.de/collection/catalogue/microorganisms/culture-technology/list-of-media-for-microorganisms) were applied: Glucose Yeast Extract Medium (GEM) containing dextrose (20 g L-1); yeast extract (10 g L-1), $CaCO_3$ (20 g L-1), agar (15 g L-1); Malt Extract Peptone Agar (MEP): malt extract (30 g L-1), soya peptone (3 g L-1), agar (15 g L-1); Potato Dextrose Agar (PDA): potato extract (4 g L-1), dextrose (20 g L-1), agar (15 g L-1); Luria Bertani Agar (LB): tryptone (10 g L-1), yeast extract (5 g L-1), sodium chloride (0.5 g L-1), agar (15 g L-1); Tryptone Yeast Extract Medium modified Agar (TY): tryptone (10 g L-1), yeast extract (5 g L-1), sodium chloride (5 g L-1), agar (15 g L-1); Peptone Yeast Extract Medium with MgSO4 Agar (PYM): peptone (10 g L-1, yeast extract (1 g L-1), $MgSO_4$ x 7 H_2O (2 g L-1), $(NH_4)_2SO_4$ (2 g L-1), agar (15 g L-1); Yeast Starch Agar (YS): yeast extract (2 g L-1), soluble starch (10 g L-1), agar (15 g L-1); Mannitol Yeast Extract Peptone (MYP): D-mannitol (25 g L-1), yeast extract (5 g L-1), peptone (3 g L-1), agar (15 g L-1). Marine Agar (MB) bacto peptone (5 g L-1); bacto yeast extract (1 g L-1); agar (15 g L-1), Gym Streptomyces Agar (GYM): dextrose (4 g L-1), yeast extract (4 g L-1), malt extract (10 g L-1), $CaCO_3$ (2 g L-1), agar (15 g L-1); and ISP2 (ISP2): dextrose (4 g L-1), yeast extract (4 g L-1), malt extract (10 g L-1), agar (15 g L-1). Aseptically, the crustaceous lichen *R. geographicum* was scrapped from the rock using a sterile scalpel and lichen sample obtained was split into two sterile 50 mL Falcon® tubes. 20 mL of sterile distilled water was added to the first tube and a 1 min vortexing was applied. After decantation, the supernatant was removed and the washing carried out two more times. The third washing water was kept and used for the isolation. 200 µL of this supernatant were spread

plated on eleven different media and incubated at room temperature until growth of fungi and bacteria. The second part of thalli was washed as described above then transferred into an empty Petri plate to dry in the laminar flow hood. Four little segments (1 x 1 mm) of thalli, were then deposed on the eleven different media previously described, in Petri dishes, and incubated at room temperature until growth of fungi and bacteria. A total of 88 thallus segments were incubated. For each plate, the isolates and colonies were daily examined for 4 days to 6 months. The ones showing distinct phenotypes were transferred into new Petri dishes containing the respective medium until pure culture. All pure isolates were stored at - 80°C in cryotubes containing 20% sterile glycerol.

Molecular identifications

For the molecular fungal identification, two protocols were used depending on the laboratories' habits. The protocol of DNA extraction and ITS PCR amplification used at EDB lab (Toulouse, France) was previously described (Lagarde et al., 2018). For an identification at the species level of the Penicillium isolates, beta-tubuline and calmoduline regions were PCR-amplified using the primers Bt2a/Bt2b (Glass and Donaldson, 1995) and CMD5/CMD6 (Hong et al., 2005), in the same conditions as above, except for annealing temperature (58°C instead of 55°C). PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany) using ITS 5, Bt2a and CMD5 primers, respectively.

For the protocol used at the Bio2Mar platform (Banyuls sur Mer, France), total DNA was extracted from mycelia directly picked from the Petri dishes then transferred onto FTA® paper, using the Whatman FTA Protocol BD05. The disc was then placed in PCR amplification tube (1.5 mL microcentrifuge tube) and 200µL of FTA Purification Reagent was added. The amplification tube was incubated for 5 minutes at room temperature with moderate manual mixing. The Purification Reagent was then removed and discarded with a pipette. The three later steps were repeated once for a total of 2 washes. 200 µL of TE-1 Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) was added and the mix was incubated for 5 minutes at room temperature. The disc was removed and 2 more TE-1 Buffer washes were performed prior the analysis. The ITS rDNA region was PCR-amplified using the primer set ITS1/ITS4 (White et al., 1991) and the 18S rDNA gene was PCR-amplified using the oligonucleotide primers 25F (5'-ACCTGGTTGATCCTGCCAG-3') and 1515R (5'-TGATCCTTCYGCAGGTTCAC-3'). Molecular bacterial and microalgal identification was carried out according to Fagervold et al. (2013) and Hadi et al. (2016).

Sequence data are available in GenBank under accession numbers OLO891597 and OL891600 to OL891636 (fungal ITS), OL890684 to OL890687 (18S), OL891637 to OL891641 (bacterial 16S) and OL891598 to OL89599 (algal ITS) (Tables 1 to 3). Only one sequence per taxa was deposited.

Sequence similarities to sequences available in the NCBI GenBank database were analyzed using the Basic Local Alignment Search Tool (BLASTn program http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). Fungal ITS reference sequences were selected to carry out a phylogenetic analysis. All sequences were aligned with MAFFT v6.814b (Katoh et al., 2002) using Geneious®6.1.8. The PhyML method (Guindon and Gascuel, 2003) was used via the Geneious® plateform to generate maximum-likelihood phylogenetic tree with the following setting: GTR substitution model, 100 bootstraps, estimated transition/transversion ratio, estimated proportion of invariable sites, estimated gamma distribution, branch length, and optimized substitution rate. Phylogenetic tree was visualized and edited with MEGAX (Kumar et al., 2018).

The following criteria were used to determine the taxa from the GenBank database and the phylogenetic tree: for sequence identities > 99 %, the species were accepted; for sequence identities of 97–99 %, only the genus was accepted. When sequences matched at 100% with several species, only the genus was accepted.

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III. Culturomics of *Rhizocarpon geographicum* lichen discloses a highly diversified microbiota carrying antibiotic-resistance, antibiosis, persistant organic pollutants tolerance

Culturomics of *Rhizocarpon geographicum* lichen discloses a highly diversified microbiota carrying antibiotic-resistance, antibiosis, persistant organic pollutants tolerance

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Abstract (200 words)

Introduction

The term symbiosis was first introduced by Albert Bernhard Frank in 1876 to describe the mutualistic association between a fungal partner and a photobiont leading to lichens symbiotic organisms [1]. Lichens are widely distributed organisms with an incredible adaptability to highly contrasted ecosystems. They are pioneer species distributed in perturbated and extreme habitats and they display an incredible tolerance to dryness, UV-exposition, pollutants etc [2]. Despite the extraordinary ecological features harbored by lichens, studies on their ecology and the evolution are still in their infancy and mostly addressed on the fungal partner. Nevertheless, a recent work from Keller et al., 2022 [3] focuses on the impact of algae genomic diversity to explain the evolutionary trajectories of a wide range of terrestrial and aquatic lichens. A third partner, the lichen-associated-microbiota, not strictly associated with the symbiotic interaction, has been described as a key element of the lichen evolutionary history [2, 4–6]. Recently, lichens have been reconsidered as holobionts [1, 7, 8], a concept introduced to describe the host and its endocellular and extracellular microbiota [9]. Looking at lichens as complex holobionts evolving with a cortege of associated microbes, offers new perspectives on how to explore the lichen-associated biodiversity and the associated ecological functions [10].

Since the beginning of the 20th century, lichen associated-bacteria were described to harbor key functions for the maintenance of the lichen symbiotic system [5, 11–14]. This observation is in accordance with the consideration of lichens as complex holobionts instead of simple binary symbionts. In order to dissect the molecular mechanisms behind the microbe-lichen interactions, the culturable as yet the unculturable microbiota need to be thoroughly explored. Studies on the lichen microbiome has been mostly carried out by microbial molecular fingerprints [5, 15, 16], molecular cloning approaches [17] or 16S rRNA gene Illumina sequencing [18]. High-throughput methodologies analysis are undoubtedly powerful to draw a complex picture of the microbial communities associated to lichens. On the other hand, they suffer from important limitations when applied to functional studies concerning the microbiome. For example, depending on the sequencing depth, microbial species may only be detected above a certain threshold, thus low-abundance species harboring central roles in the holobiont, cannot be effectively studied [19]. More generally, functional studied on lichen-associated microbes can only be efforted by deeply isolating the microbial partner. Because many host-associated microbes are uncultivable outside their habitat of origin, improving

cultivability of a wider range of bacterial community members, is a prerequisite for a better understanding of metacommunities interactions and their functions in complex biotic systems [10, 20, 21]. Culturomics is a high-throughput culture approach multiplying culture methods and with mass spectroscopy or 16S ribosomal RNA sequencing in order to detect higher microbial diversity. This innovative strategy, mostly explored in the human-gut microbiome field, expanded our knowledge concerning the human gut bacterial repertoire [22]. Nevertheless, culturomics has been poorly utilized in the non-medical field and even less on lichens.

Here we adopted a novel culturomic approach on *Rhizocarpon geographicum*, a crustose lichen pioneer of exposed rock surfaces and constituting the first terrestrial substrate available for living organisms on Earth [23, 24]. As rock-inhabitants, lichens are often exposed to extreme abiotic conditions with broad fluctuations of temperature and humidity providing poor comfort of life and sources of nutriments [25]. These extreme conditions confer to lichens the unique ability to develop protective mechanisms [26, 27]. We sampled *R. geographicum* in French zones strongly affected by hostile environmental conditions (wind, sea sprays, snow) and used 9 culturing methods to collect a broad bacterial diversity. We amplified a portion of 16S rRNA gene and reconstruct putative functional networks representing the *R. geographicum* microbiome. We pointed out the presence of a set of bacterial species resistant to a wide range of antibiotics and displaying an antimicrobial activity against human and plant pathogens. In addition, a set of bacterial strains were able to tolerate and grow in presence of high level of hydrocarbons.

Material and methods

Collection of Rhizocarpon geographicum *populations*

R. geographicum samples were collected in January 2021, under specific municipality authorizations, in 6 sites situated in France (Fig.1a, b) on : i) the Atlantic coastal area (Ille-et-Vilaine, Finistère and Côtes d'Armor - Brittany) ii) the English Channel coastal area in the northern limit of the Mont-Saint-Michel in Carolles (La Manche, Normandy), iii) the inland area in Baulon and Plounéour-Menez (Ille-et-Vilaine et Finistère) and iv) low mountains of Itxassou (Pyrénées Atlantiques, Nouvelle Aquitaine). Sampling localities were selected based on the habitat diversity (Fig. 1b). For each location (called *R. geographicum* population), lichen samples were collected randomly on four rocks (Supplementary Table S2 p. 20, Volume 2) and

rock fragments were transported in sterile Petri dishes stored in individual plastic bags and processed within the 6 hours post collection.

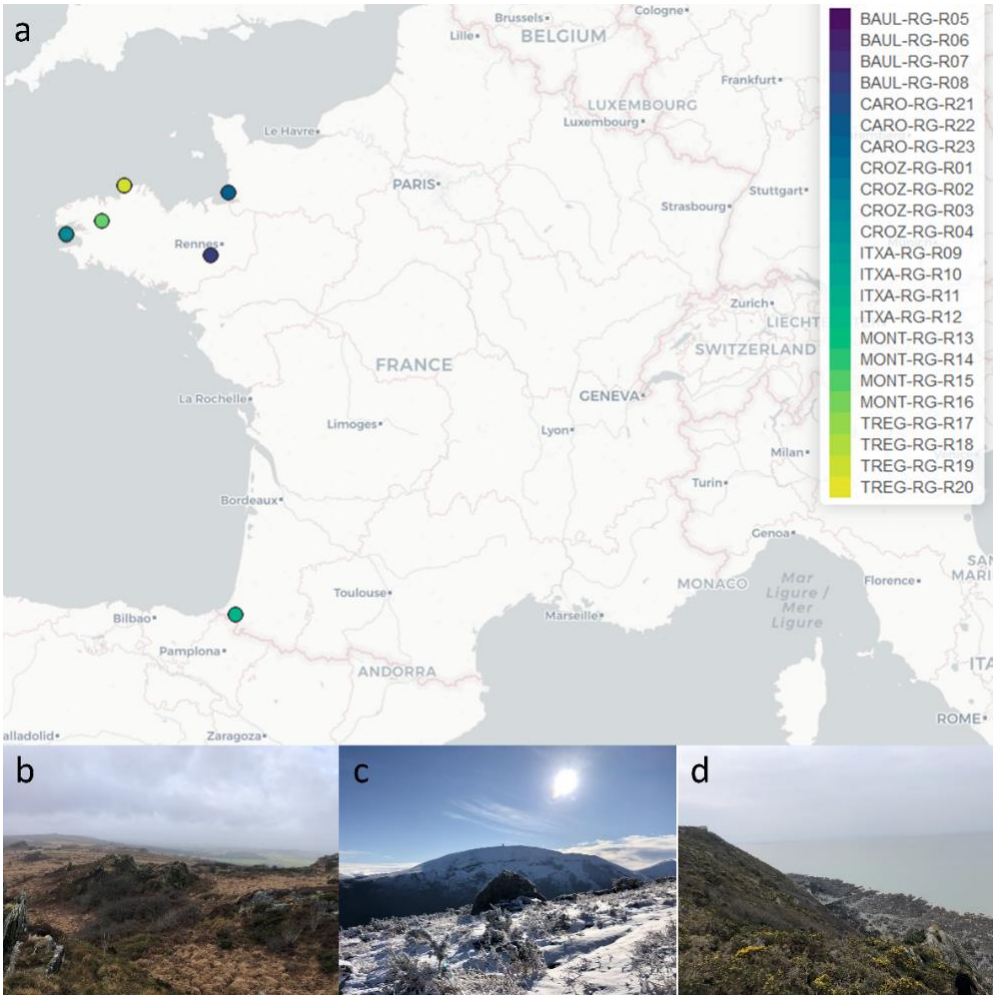

Figure 1. ***Rhizocarpon geographicum*** **populations.** (**a)** locations where populations were sampled across France (north and south). Colored dots represent the *R. geographicum* populations that were collected in the 6 sites, the color gradient within the same localities indicates the different harvest locations. Four populations per site were sampled. (**b)** representing MONT populations, **(c)** ITXA populations, and (**d)** CARO populations diversity of the terrestrial and maritime habitats harboring *R. geographicum*.

Media used for culturomics

Three types of matrices were prepared as organic nutrient source and were supplemented into the media. For the first type of matrix, we developed a lichen filtrate; for this 50 mg of *R. geographicum* were scrapped and mixed with 100 mL of seawater collected from the site of sampling. The suspension was centrifuged for 10 min at 5000 rpm at 4°C. The supernatant was

recovered and sterilized by filtering with 0.22 µm pore-size Millipore® membranes. The second type of organic matrix was performed on a macroalgal filtrate. For this, 155 g of *Ascophyllum nodosum* and 95 g of *Laminaria digitata* collected at la Pointe de Crozon (Britany, France) were rinsed with seawater. These algae co-habit in the ecosystem with *R. geographicum* and are part of the lichen natural habitat. Macroalgae were roughly cut before being homogenized with a clean kitchen blender then bag-mixed with a Stomacher. From the concentrated solutions, 400 mL were recovered and filled with 600 mL of seawater collected at la Pointe de Crozon, previously sterilized by filtration. Once crushed, the algal pastes were passed through a stamen. Filtrates were then centrifuged for 10 min at 5000 rpm at ambient temperature. Filtrates were then filtered with 0.22 µm pore-size Millipore® membranes to guarantee sterility. The third organic matrix consisted in a microalgal filtrate. The *Coccomyxa viridis*, a microalgal associated with *R. geographicum*, collected on the Atlantic coastal area in Crozon, was previously isolated on the International Streptomyces Project 2 medium (ISP2, dextrose 4 g L^{-1} [Sigma-Aldrich, St. Louis, MO, USA], yeast extract 4 g L^{-1} [Sigma-Aldrich, St. Louis, MO, USA], malt extract 10 g L^{-1} [Sigma-Aldrich, St. Louis, MO, USA], agar 15 g L^{-1} [Sigma-Aldrich, St. Louis, MO, USA]). The total mass present in one Petri dish of *C. viridis*, was added into 50 mL of NaCl 0.85 % then transferred into 500 mL of ISP2 liquid medium. Culture was maintained under agitation (120 rpm) for 2 weeks. After this growing time, 500 mL of sea water was added into the *C. viridis* suspension and sterilized by filtering as described above.

The three lichen, macroalgal and microalgal-based matrices were then used to develop culturing media and isolation methods. Medium 17B was used for bacterial enrichment and a total of 9 media were used for bacterial isolation (Supplementary Methods, liste S1 p. 16, Volume 2). Three media (18F, 19F and 20F) were supplemented by 100 mg L^{-1} of chloramphenicol, 300 mg L^{-1} of streptomycin and 100 mg L^{-1} of penicillin to isolate antibiotic resistant strains. Detailed protocols for media preparation are listed in the Supplementary Methods (Liste S1 p.16, Volume 2).

Bacterial isolation, characterization and taxonomic affiliation

The 24 crustose lichen samples were aseptically scrapped using a sterile scalpel and placed into sterile 15 mL Falcon® tubes containing 5 mL of sterilized distilled water. The fresh mass of each lichen used for isolation is listed in Supplementary Table S3 p.21, Volume 2. To isolate the larger bacterial diversity, we adopted two methodologies. Firstly, dilutions (from 10^{-1} to 10^{-3}) were directly plated on the 8 media (media supplemented with 100 mg L^{-1} of cycloheximide:

01B, 08B, 14B, 15B, 16B, media supplemented with 100 mg L^{-1} of chloramphenicol, 300 mg L^{-1} of streptomycin and 100 mg L^{-1} penicillin 18F, 19F and 20F) described in the Supplementary Material (Liste S1 p. 16, Volume 2). The samples were incubated under dark conditions at 15 and 20°C until colonies appeared for 6 weeks. Secondly, the 24 lichen samples were enriched into 500 mL Erlenmeyer containing 50 mL of microalgae filtrate and 500 µL of lichen filtrate. The four samples for each *R. geographicum* population were pooled and 100 µL of each lichen suspension were added to the enrichment broth. Erlenmeyer flasks were sealed with a cotton and aluminum cap then maintained under agitation (100 rpm) at 15°C for 4 weeks. Once a week, the cap was aseptically removed for 15 minutes to aerate the cultures. After 4 weeks, dilutions from the enrichment solutions were plated in triplicate on the 9 media (01B, 08B, 14B, 15B, 16B, 17B 18F, 19F and 20F) (Supplementary Material, Liste S1). Plates were incubated under dark conditions at 20°C for 6 weeks.

For both experimental procedures (direct plating and enrichment), colonies showing distinct morphologies were purified on the medium of origin and stored at -20°C into 30% of glycerol. Pure colonies were placed into 96-well plates containing a DNA stabilizing buffer composed by 10 mM TRIS, pH 8.0, 0.1M EDT, pH 8.0 and 0.5% SDS. Plates were stored at -20°C prior DNA extraction that was performed with the protocol described in Vingataramin and Frost (2015) [28], with minor modifications. Briefly, 300 µl of EtNa DNA extraction reagent were added to preheated DNA plates containing the stabilizing buffer. The mix was heated at 90°C for 10 min and centrifuged at 4000 rpm for 30 min. The supernatant was removed and the pellet resuspended in 100 µl of DNA suspension solution.

Bacterial identification was performed by amplifying a region of the 16S as described in the Supplementary Material (Protocole S1, p. 18, Volume 2).

Characterization of antibiotic resistant strains

Twenty-four bacterial strains (Supplementary Table S4, p. 22, Volume 2) able to grow on media containing antibiotics (Supplementary Methods, Liste S1), were tested for their antibiotic resistance ability against 12 antibiotics (Supplementary Table S5, p. 23, Volume 2). For this, the 24 strains were incubated in 10 mL of Tryptic Soy Broth (TSB) (Sigma, Reference T8907-1KG) from 2 to 4 days at 20°C under agitation (120 rpm). Strains were then tested at both exponential (*exp*) and stationary (*sta*) phase. After incubation, 10 µl microliter of each strain at *exp* or *sta* phase were inoculated into 96-well sterilized plates containing 90 µl TSB and the

appropriate antibiotic concentration (Supplementary Table S5). TSB in absence of the antibiotic was used as control. Three independent experiments (temporal blocks) were performed and at each experiment three replicates were included (N=9 replicates per strain). Plates were incubated for 72h at 20°C under agitation (120 rpm) then the optical densities (OD) were read at $\lambda = 620$ nm with a microplate reader Multiskan™ FC, Thermo Scientific™. The 6 values for each *strain* × *antibiotic* obtained from the 3 independent experiments were used to estimate the antibiotic effect on the bacterial growth on both *exp* and *sta* conditions. Also, *exp* and *sta* conditions were nested into the analysis to estimate their effect on strain growth variability. For this, we first estimate an antibiotic coefficient by dividing the OD of each strain grew in presence of the antibiotic on the OD of the strain grew in absence of the antibiotic. Coefficient values were integrated into a generalized-linear-mixed model that was run by using the glmer function implemented in lsmeans and lme4 R packages (Bates et al., 2015; Lenth, 2016). Replicates and the temporal blocks (i.e. the 3 independent experiments) were integrated in the model as random effects. *P*-values were corrected for FDR and barplots were built using the ggplot2 and reshape2 packages [29, 30] on the lsmeans.

Nine strains showing a high antibiotic resistance range were whole-genome sequenced (Supplementary Table S4, p. 22, Volume 2) and analyzed as described in the Supplementary Methods (p. 19, Volume 2). Two strains that were not identified by 16S sequencing (CARO-RG-8B-R23-01 and CARO-RG-8B-R24-01) were also whole-genome sequenced to investigate their taxonomical affiliation.

Preliminary antimicrobial activity assay

In order to evaluate the potential to produce antimicrobial molecules of the 24 strains showing an antibiotic resistance phenotype, strains were screened for their capacity to inhibit the human bacterial pathogens *Staphylococcus aureus* (CIP 53.156) (gram positive) and *Escherichia coli* (CIP 54.8) (gram negative). The two bacterial pathogens were grown for 24 h onto Luria Broth solid medium (LB, peptone 2.5 g L^{-1}, NaCl 2.5 g L^{-1}, yeast extract 1.5 g L^{-1}, 15 g L^{-1} agar). Each bacterial pathogen at OD 600 nm = 0.110 were then swab-spread into three LB Petri dishes respectively. Three drops of 10 µL of each of the 24 bacterial strains isolated from lichen thalli were deposited on sterile 5 mm diameter paper disc placed on the Petri dishes containing *S. aureus* and/or *E. coli*. A 10-µg gentamicin disc was added as positive control. The negative control consisted into a 10 µl drop of LB broth culture. Inoculated Petri dishes were first incubated at 37°C for 24 h then at room temperature for 7 days. The inhibition zone was estimated by measuring diameters of inhibition halo [31].

Chemical profiling of an antimicrobial strain

The MONT-RG-20F-R14-06 fungal strain and MONT-RG-20F-R14-05 bacterial strain were cultured on plate of trypticase soy agar (TSA, Biokar Diagnostics, France) at 25 °C for 3 days. Then, the strains were inoculated in independent flasks each containing 5 L of trypticase soy broth (TSB). A third flask was inoculated with the same culturing method with both fungal and bacterial inoculums for coculture. Flask cultures were incubated at 25°C on an orbital rotary shaker New Brunswick Innova 42® at 120 rpm. After 10 days of culture, the pellets and supernatants were separated by centrifugation at 3500 rpm during 15 minutes at 4°C and the supernatants were discarded for further liquid/liquid extraction. For this, the 2 L of different supernatants were extracted twice with 650 ml of ethylacetate (EtOAc). EtOAc organic phases were collected and dried on anhydrous sodium sulfate then were evaporated under vacuum yielding a fungal monoculture supernatant extract (29.6 mg), a bacterial monoculture supernatant extract (109.2 mg) and a fungal/bacterial coculture extract (27.8 mg). The coculture experiment was repeated twice in a smaller medium volume.

The extracted supernatants from the three microbial inoculation conditions were analyzed with an HPLC system – Diode Array Detector (LC-DAD) (Shimadzu, Marne La Vallée, France). A Hypersil GOLD™ C18 column (5 µm, 250 × 4.6 mm, ThermoFisher Scientific™) was used for HPLC, and a gradient system was applied: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The following gradient was applied at a flow rate of 0.8 mL/min in the HPLC system: initial: 100% (A); from 0 to 5 min: 100% (A); from 5 to 35 min: 100% (A)/0% (B) to 0% (A)/100% (B); from 35 to 45 min: 100% B; from 45 to 50 min: 100% (A)/0% (B) to 0% (A)/100% (B); from 50 to 55 min: 100% (A). Twenty microliters of 5 $mg.ml^{-1}$ concentration samples were injected.

Tolerance to persistent organic pollutants

A sub-set N=455, showing a good growth rate when re-cultured on the TSA medium after storage, were tested for their ability to tolerate and growth in presence of Persistent Organic Pollutants (POP). For this, the 455 strains (Supplementary Table S6, p. 24, Volume 2) where grown for 3-days of TSA then inoculated into TSB medium supplemented with perofluorooctanoic acid (PFOA) for a final concentration at 1.65 $g.L^{-1}$. Controls consisted on strains inoculated on TSB only. POP screening was performed in triplicates on TSB in 96-well containing a volume of 200 µL of TSB supplemented with PFOA. Each well was inoculated

with one pure bacterial colony of each of the 455 strains. The inoculated 96-well plates were incubated for 7 days at room temperature then the OD were read at $\lambda = 620$ nm with microplate reader Multiskan™ FC, Thermo Scientific™. A POP tolerance coefficient was obtained by dividing the OD of each strain grew in presence of the POP on the OD of the strain grew on the TSB control. A threshold of POP tolerance coefficient >1 was considered as in index of bacterial growth on the POP.

Results

Culturomics revealed a Rhizocarpon geographicum *highly diversified microbiota*

R. geographicum was sampled in 6 highly diversified maritime and terrestrial habitats located in France (Fig. 1). These habitats were selected to maximize niche diversity but also to include French littorals with a history of oil spill [32]. For this, Crozon and Trégastel located in Brittany, and Carolles located in Normandy were chosen to potentially collect *R. geographicum* associated microbes harboring tolerance to hydrocarbons and pollutants. To capture the highest bacterial diversity, we applied a culturomics approach by using 9 isolation media (Supplementary Methods, Liste S1, p. 16, Volume 2) and two culturing methods: direct plating and enrichment with microalgae prior plating. In order to increase the recovery of the bacterial communities, we mimicked the nutritional and environmental conditions of *R. geographicum.* Seven of the selected media consisted in algae-based and/or lichen based media and they were developed in our study with the attempt to reproduce the lichen habitat and isolate recalcitrant bacterial species. Three media were supplemented with antibiotics to isolate bacterial strains displaying resistance to a wide range of antibiotics. In fact, Grube and collaborators have previously detected in the genome of lichen-associated bacteria the presence of genes coding for multidrug resistance efflux pumps and for antibiotic resistance indicating that lichens could be also a reservoir of mechanisms of bacterial antibiotic [11, 33]. Moreover, the use of antibiotics was chosen because there are molecular evidences that persistent organic pollutants may induce the antibiotic resistance genes in contaminated soils [34].

We isolated a total N° = 1913 bacterial isolates from the *R. geographicum* samples. Among the 1913 strains only 1, 063 were amplified and characterized with the 16S marker gene. Based on the 16S sequence, analysis with the CD-HIT software [35] identified 364 bacterial clusters

showing 100% of sequence homology with at least one strain (Supplementary Data Set 1 and Data Set 2, fichier joint Excel). The remaining 699 unique bacterial clusters (i.e. unique bacterial strains) where taxonomically affiliated (Supplementary Data Set 2, fichier joint Excel). Bacterial abundance (i.e. number of bacterial isolated per each medium divided by the total number of bacteria) showed a heterogenous success to isolate bacteria across the isolation media used (Fig. 2). Bar plot clearly shows that 01B and 08B media recovered the maximum of the bacterial diversity. A lower number of bacterial strains were recovered from the algae or lichen-based media (from 14B to 17B) and few antibiotic resistance strains (N = 126) where isolated on the 18F, 19F and 20F media (Fig. 2).

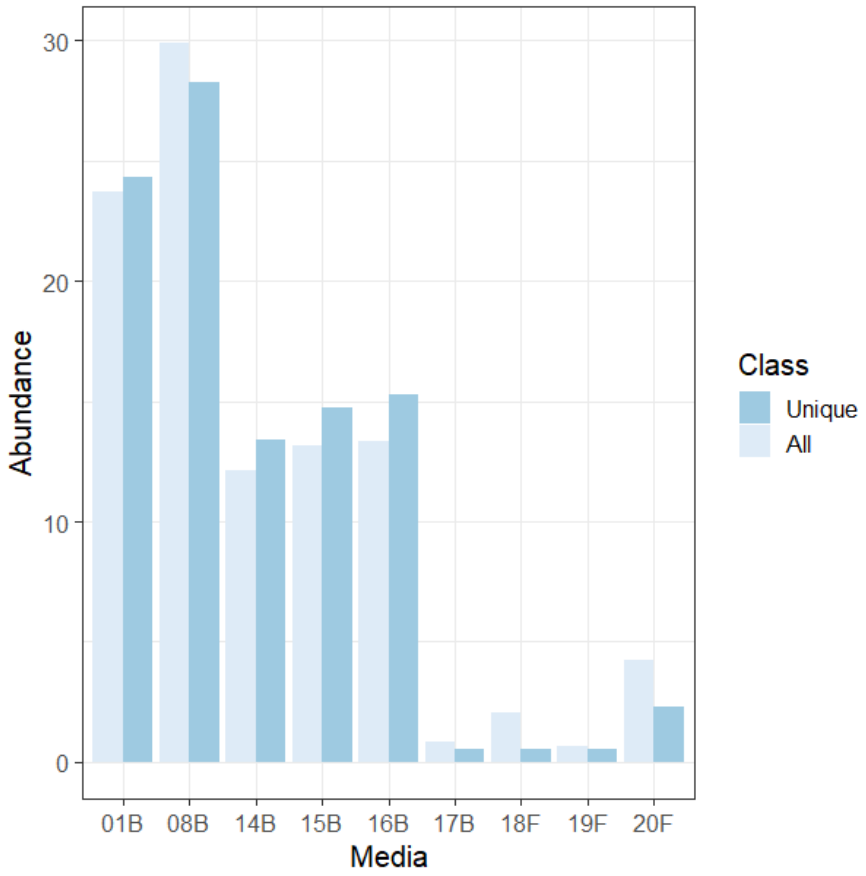

Figure 2. Number of bacteria strains isolated from each medium. Bar plot is based on the abundance of each bacterial strain (expressed in %) calculated by dividing the number of strains recovered on each medium by the number of the total strains.

Similar results were obtained when analyzing the abundance of distinct bacterial isolated species on each medium (Fig. 2). On the other hand, media designed to reproduce the lichen environment (14B, 15B, 16B, 17B, 18F, 19F and 20F, Supplementary Methods, Liste S1)

allowed to isolate 17 bacterial species that were not recovered on the Nutrient Agar and TSA medium and that where specific to the isolating medium (Table 1).

Table 1. Bacterial species isolated specifically from lichen and/or algal-based media. Full media description is provided in Supplementary Methods.

Bacterial species	Isolation medium	Medium description
Aquaspirillum arcticum	15B	Lichen algal-based medium
Geobacillus sp.	16B	Lichen-algal based minimal medium
Jeongeupia chitinilytica	15B	Lichen algal-based medium
Klenkia taihuensis	14B	Macroalgae-based medium
Kocuria polaris	14B	Macroalgae-based medium
Microterricola gilva	16B	Lichen-algal based minimal medium
Paracoccus chinensis	19F, 20F	Lichen-algal antibiotic based
Pseudomonas gessardii	20F	Lichen-algal antibiotic based
Pseudomonas helmanticensis	16B, 20F	Lichen-algal based medium & Lichen-algal antibiotic based
Roseomonas mucosa	15B	Lichen algal-based medium
Sinomonas sp.	15B	Lichen algal-based medium
Skermanella sp.	14B	Macroalgae-based medium
Tardiphaga sp.	14B	Macroalgae-based medium
uncultured Erythrobacter sp.	16B	Lichen-algal based minimal medium
uncultured Gordonia sp.	16B	Lichen-algal based minimal medium
uncultured Nakamurella sp.	8B, 16B	TSA and Lichen-algal based minimal medium
Zafaria cholistanensis	14B	Macroalgae-based medium

Most of the species isolated from media reproducing the lichen environment are known to inhabit water reservoirs and polluted environments. For instance, *Aquaspirillum arcticum, Erythrobacter sp, Klenkia taihuensis, Paracoccus chinensis, Microterricola gilva,* and *Kocuria polaris* (Table 1), were previously isolated from marine environments [36–39], sediments [40, 41], seaweed [42] or cyanobacteria [43]. It is to note that various *Erythrobacte*r, *Paracoccus Kocuria*, *Pseudomonas* or *Gordonia* species were already isolated from marine or maritime Brittany lichens and *P. helmanticensis* from an inland Austrian lichen [39]. *Geobacillus* sp. and *Gordonia* sp. are described to grow on thermal area and polluted environments contaminated with cyclic hydrocarbons [44–50]. *Geobacillus* sp. is also described to produce volatile antibiotics [51]. Our results suggest that media reproducing the habitat of origin help in isolating rare species that can play important roles on the ecological network of the habitat.

Based on 16S taxonomy affiliation, the most abundant bacterial class isolated by culturomics belong to α-Proteobacteria followed by Actinomycetia, β-Proteobacteria and γ-Proteobacteria (Fig. 3a). Among the three most representative bacterial class, the α-Proteobacteria are mostly constituted by the Hyphomicrobiales and Sphingomonadales families (Fig. 3b). The Actinomycetia are mostly represented by the Micrococcales and the Frankiales orders (Fig. 3c). The β-proteobacteria are almost exclusively represented by the Burkholderiales order. These results are coherent with those obtained previously by a metagenomic study on different crustose lichens, including *R. geographicum* [16].

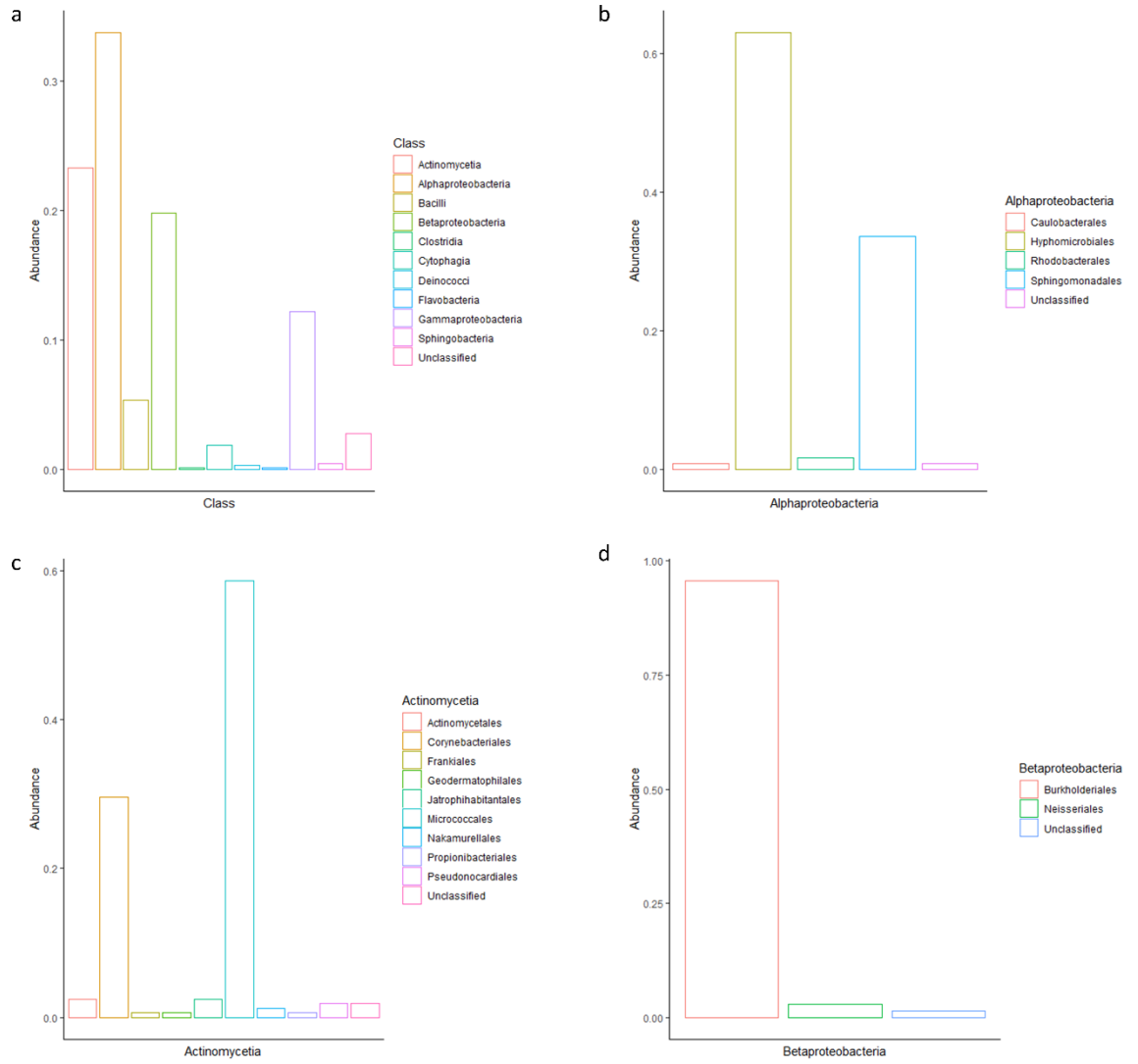

Figure 3. Abundance of bacterial class and families isolated from *Rhizocarpon geographicum*. (a) Proportions of the different bacterial class (N° of each bacterial class/N° of total isolated strains) (b) Proportions of the bacterial families included in the α-proteobacteria class, (c) Proportions of the bacterial families included in the Actinomycetia class and (d) Proportions of the bacterial families included in the β-proteobacteria class.

Three strains CARO-RG-8B-R24-01, CARO-RG-8B-R23-01 and MONT-RG-14B-R14-06 did not match with any 16S sequences in GenBank. We then extracted the total DNA of these isolates for whole-genome sequencing. For MONT-RG-14B-R14-06, DNA did not pass the quality test and was not sequenced. Statistics on the genome quality for the two genomes is reported in Supplementary Table S7, p. 42, Volume 2. Phylogeny on the whole genome performed by the Type Strain Genome Server (TYGS) https://tygs.dsmz.de/ showed that

CARO-RG-8B-R23-01 strain belongs to the *Microbacterium lacticum* species, on the other hand CARO-RG-8B-R24-01 belongs to a potential new species of the *Sphingomonas* genus (Supplementary Fig. S2, p. 45, Volume 2)

Culturomics allows to isolate taxonomically diversified antibiotic resistance bacterial species inducing inhibition to human pathogens.

We identified 126 bacterial strains from media containing antibiotics. Among the 126 only 87 were selected because of the quality of their 16S sequences (Supplementary Data Set 3, fichier joint Excel). Based on the phylogenetic diversity obtained on a fragment of the 16S sequence (Supplementary Fig. S3, p. 46, Volume 2) we selected 24 strains (Supplementary Table S4, p. 22, Volume 2) for testing their antibiotic resistance degree against 12 antibiotics used as therapeutics. Linear-mixed model performed on the antibiotic resistance coefficient showed a high effect of the Strain *factor* for the response to the antibiotics (Supplementary Table S8, p. 43, Volume 2), by confirming the heterogenicity of the strain resistance (Fig. 4). The *exp* and *sta* determining the Phase *factor* (whether strains have been inoculated at the exponential or stationary phase) was significant on 5 of the 12 antibiotics tested (gentamicin, kanamycin, vancomycin, colistin and cefalexin, Supplementary Table S8). On the other hand, a nested effect Strain × Phase *factor* was found for all antibiotics except penicillin (Supplementary Table S8), by confirming that the bacterial phase can have an impact for the same strain on different antibiotic classes (Fig. 4). Globally, as showed in Fig. 4, resistance to antibiotics was stronger when strains were inoculated at the stationary phase. Few strains were resistant to gentamicin, kanamycin and streptomycin, however for the vancomycin strains showed higher resistance when inoculated at the stationary phase (Fig. 4).

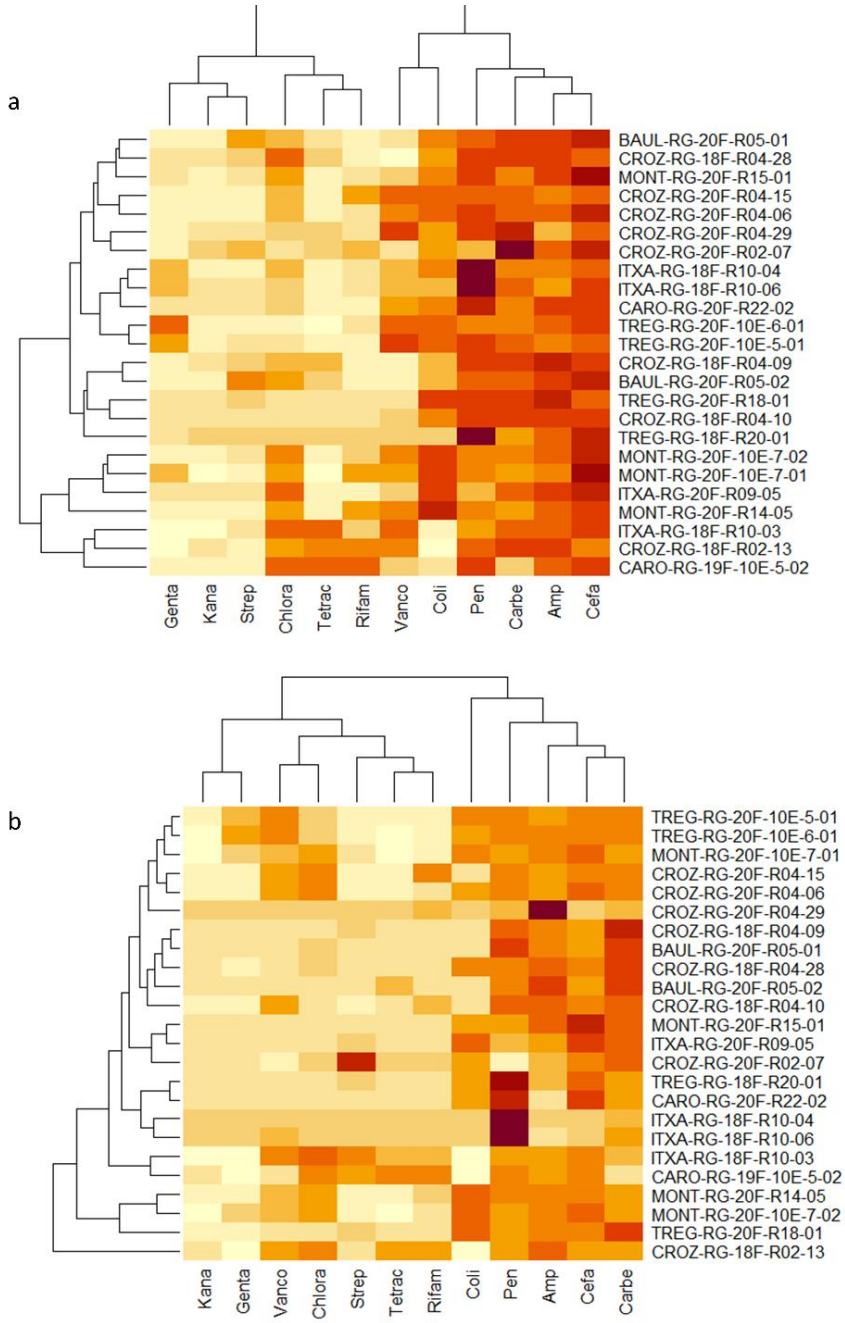

Figure 4. Heatmaps on the antibiotic resistance coefficient. Heatmaps were performed on the lsmeans obtained by running a liner-mixed model on the antibiotic resistance coefficient and by controlling for the block and replication effect. (a) heatmap on the antibiotic coefficients obtained when strains where inoculated at the stationary phase; (b) heatmap on the antibiotic coefficients obtained when strains where inoculated at the exponential phase. Name of the tested strains are reported in the *y*-axis antibiotics are reported in the *x*-axis. Abbreviations: Genta = gentamicin, Kana = kanamycin, Vanco = vancomycin, Chlora = chloramphenicol, Tetrac = tetracycline, Rifam = rifampicin, Strep = streptomycin, Coli = colistin, Pen =penicillin G, Carbe = carbenicillin, Amp = ampicillin, Cefa = cefalexin.

As highly antibiotic resistance bacteria can also produce antimicrobial molecules [52, 53], we then selected 9 strains, BAUL-RG-20F-R05-02, CROZ-RG-20F-R02-07, CROZ-RG-20F-R04-06, CROZ-RG-20F-R04-15, MONT-RG-20F-R14-05, MONT-RG-20F-10-5-01, TREG-RG-20F-R18-01, TREG-RG-20F-10-5-01 and TREG-RG-20F-10-6-01, showing a high rate of resistance to test them as growth inhibitors of two human GRAM + and GRAM - pathogens *Staphylococcus aureus* (CIP 53.156) and *Escherichia coli* (CIP 54.8), respectively. Antimicrobial assay performed on LB agar medium showed that two strains, CROZ-RG-20F-R04-15 and MONT-RG-20F-R14-05 were able to inhibit *S. aureus* (CIP 53.156) with inhibition zone diameters of 15 and 20 mm respectively when compared with the gentamicin control showing an inhibition zone of 23 mm. All strains did not show any bioactivity against *E. coli* (CIP 54.8) (Fig. 5). Inhibition zones were detected in 3 independent experiments.

Figure 5. Antibiotic resistance bacterial species inducing inhibition to human pathogens. (a) positive control; (b) MONT-RG-20F-R14-05. The human pathogen inoculated is *S. aureus* (CIP 53.156)**.**

Antibiosis property is extended to a fungus of the lichen microbial community

Here, streptomycin, chloramphenicol and penicillin were used to supplement isolation media as a screening of microbes carrying a broad spectrum of antibacterial activity and production of novel antimicrobial compounds [54–58]. During the isolation process, we observed that the bacterial isolate MONT-RG-20F-R14-05, showing a high degree of resistance to antibiotics and inhibiting the growth of the human pathogen *S. aureus* (CIP 53.156) was also responsible of a strong antibiosis with the fungal isolate MONT-RG-20F-R14-06. This fungal isolate was recovered isolated from the same thallus ofas the bacterial strain MONT-RG-20F-R14-05 and on the same lichenic habitat and it was affiliated to the species *Neurospora retispora* according with the ITS sequence (Supplementary Data Set 4, fichier joint Excel) (Fig 6). To characterize the chemical dialog of this negative interaction, a liquid co-culture was performed by

demonstrating that in presence of the bacterial strain the fungus was unable to grow (Fig. 6). HPLC analysis carried out on crude extracts obtained from the bacterial and fungal mono-cultures and the bacterial/fungal co-culture, showed that co-cultivation has an impact on the metabolites production. Indeed, two peaks (R_t 22 and 24 min) from the fungal mono-culture chromatogram disappeared in those of co-culture. Moreover, a peak of the bacterial extract chromatogram significantly decreased in co-culture (R_t 37.2 min) (Fig 6).

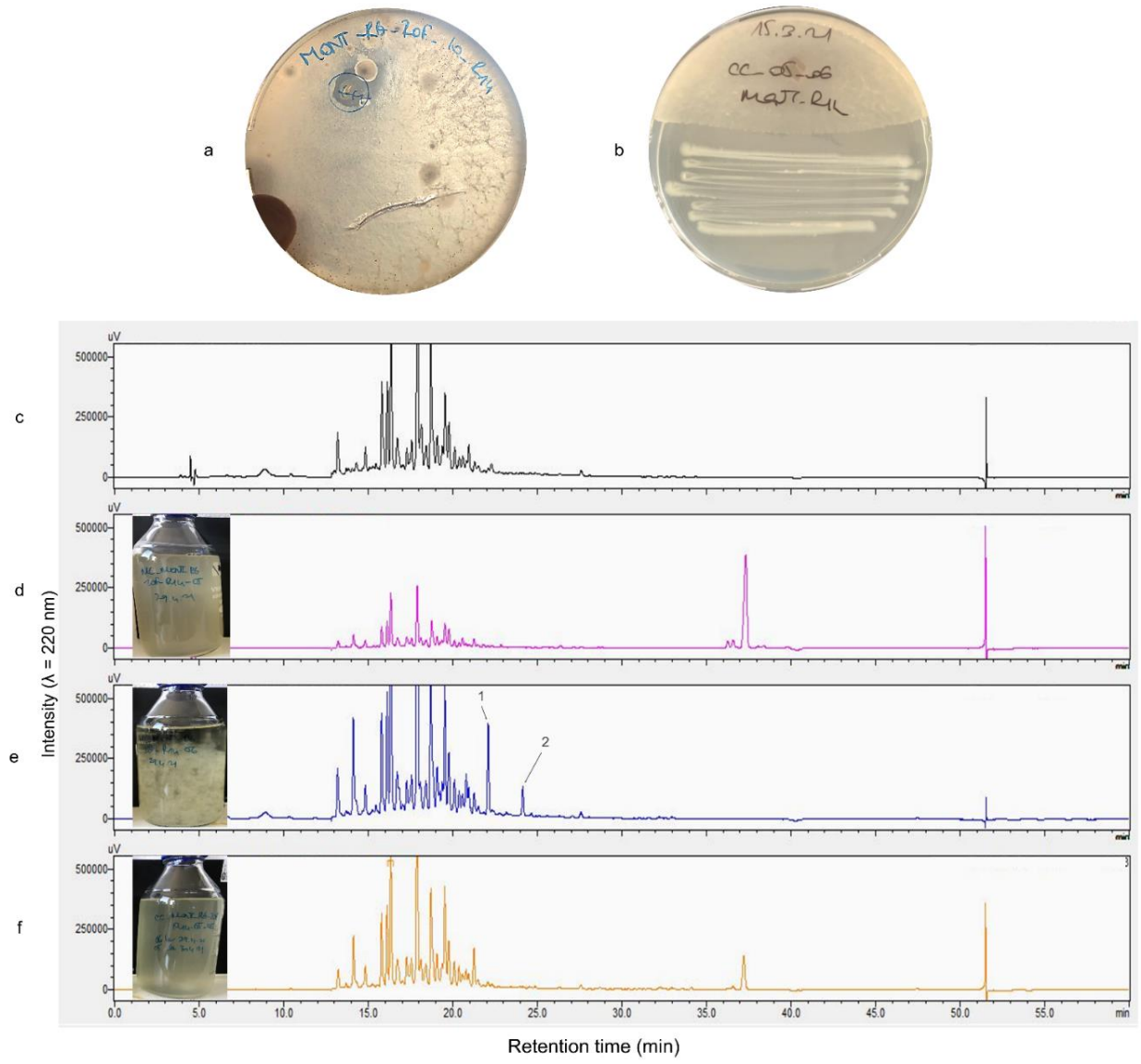

Figure 6. Antibiosis and chromatograms of mono-cultures and co-culture extracts. (a) bacterial/fungal antibiosis on isolation plate; (b) solid co-culture of bacterium and fungus isolated; HPLC chromatogram of (c) liquid culture medium EtOAc crude extract (d) *Pseudomonas gessardii* liquid culture EtOAc crude extract (e) *Neurospora retispora* liquid culture EtOAc crude extract (f) *P. gessardii* and *N. retispora* liquid co-culture EtOAc crude extract. Peaks corresponding to compounds 1-2 are missing in co-culture.

Whole-genome sequencing of antibiotic resistant bacteria inhibiting human pathogens and fungal isolates suggests potential novel species harbouring Antimicrobial Resistance (AMR) proteins

We sequenced and analysed 9 bacterial strains showing a high degree of resistance to the tested antibiotics. Phylogenetic affiliation of the strains performed on the TYGS database [59], suggested that the 9 strains are potential new species. The TYGS database is updated daily, however, type strains closely related to the sequenced strains could be missing. Based on the phylogenetic inference on the whole-genomes (Fig. 7, Fig. 8), the 9 potential new species are distantly related to: i) *Pseudomonas crudilactis* with 47.8% of homology with the CROZ-RG-20F-R04-06, MONT-RG-20F-R14-05 and CROZ-RG-20F-R04-15 strains ii) *Pseudomonas antartica* with 41% of homology with TREG-RG-20F-10-E-5-01, TREG-RG-20F-10-E-6-01 and MONT-RG-20F-10-E-7-02 iii) *Sphingomonas glacialis* with 20% of homology with TREG-RG-20F-R18-01 and BAUL-RG-20F-R05-02 and iv) *Sphingomonas ginsenosidivorax* with 19% of homology with the CROZ-RG-20F-R02-07 strain (Fig. 7 and Fig. 8). The closely related strains were isolated from various different substrates. Indeed, *P. antarctica* and *S. glacialis* are psychrophile bacteria isolated from Antarctica water bodies [60] and Alpine glacier cryoconite [61]. Moreover, *P crudilactis*, firstly isolated from raw milk, was shown to be resistant to several antibiotics [62] alike *S. glacialis* [61]. *P. antarctica* on the other hand was described as having antimicrobial activity [63]. *S. ginsenosidivorax*, isolated from ginseng [64] did not exhibit such activities or resistance but is able to biotransform ginsenosides, possessing antimicrobial activities [65].

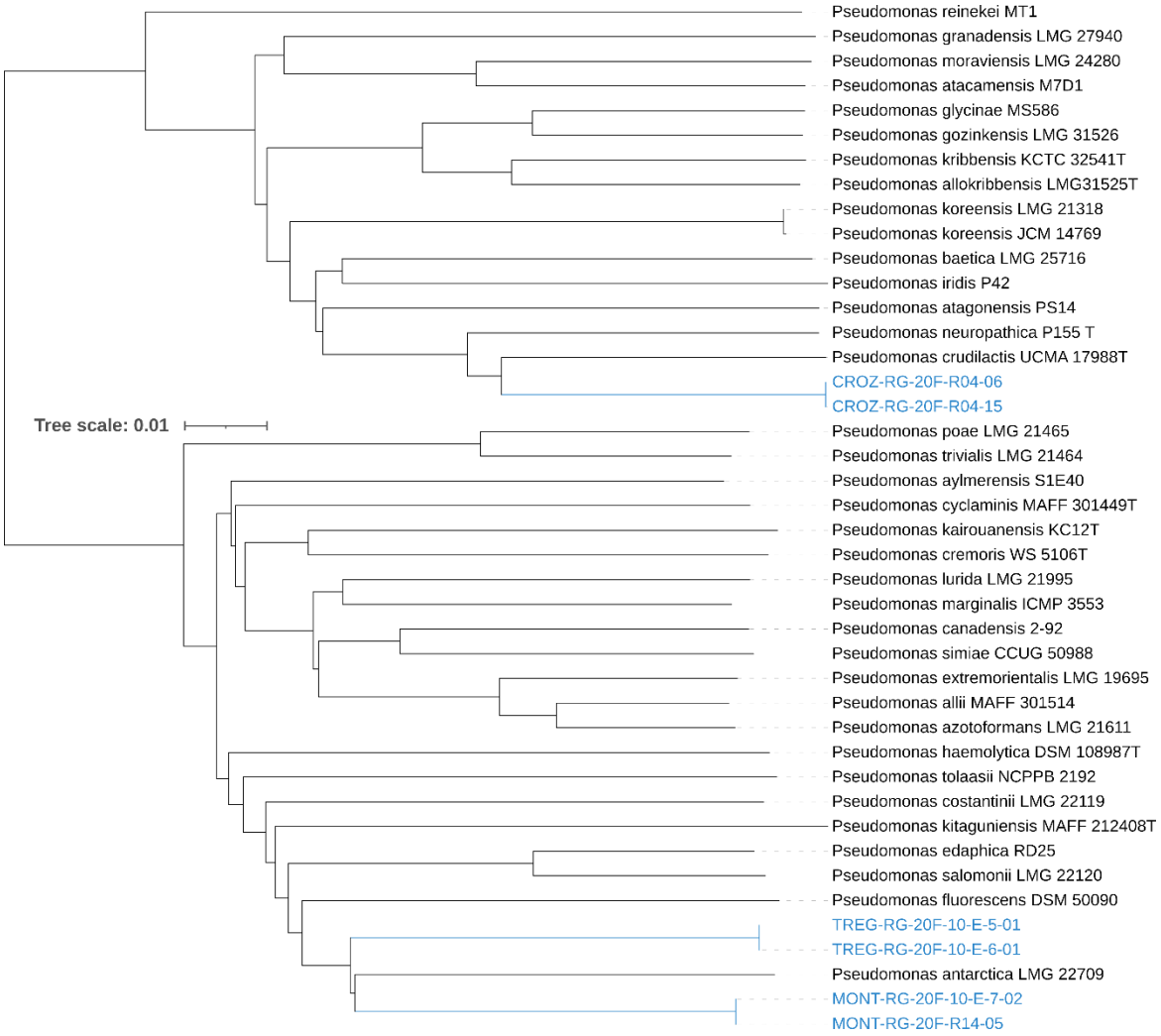

Figure 7. Phylogeny of bacterial strains carrying resistance to antibiotics. Phylogenetic tree was inferred with FastMe 2.1.6.1 [73] on the TYGS platform [59] from GBDP distances calculated from whole-genomes. The tree was rooted ad midpoint. Tree was edited by iTOL [74]. Strains isolated from *R. geographicum* and the related tree branches are indicated in blue.

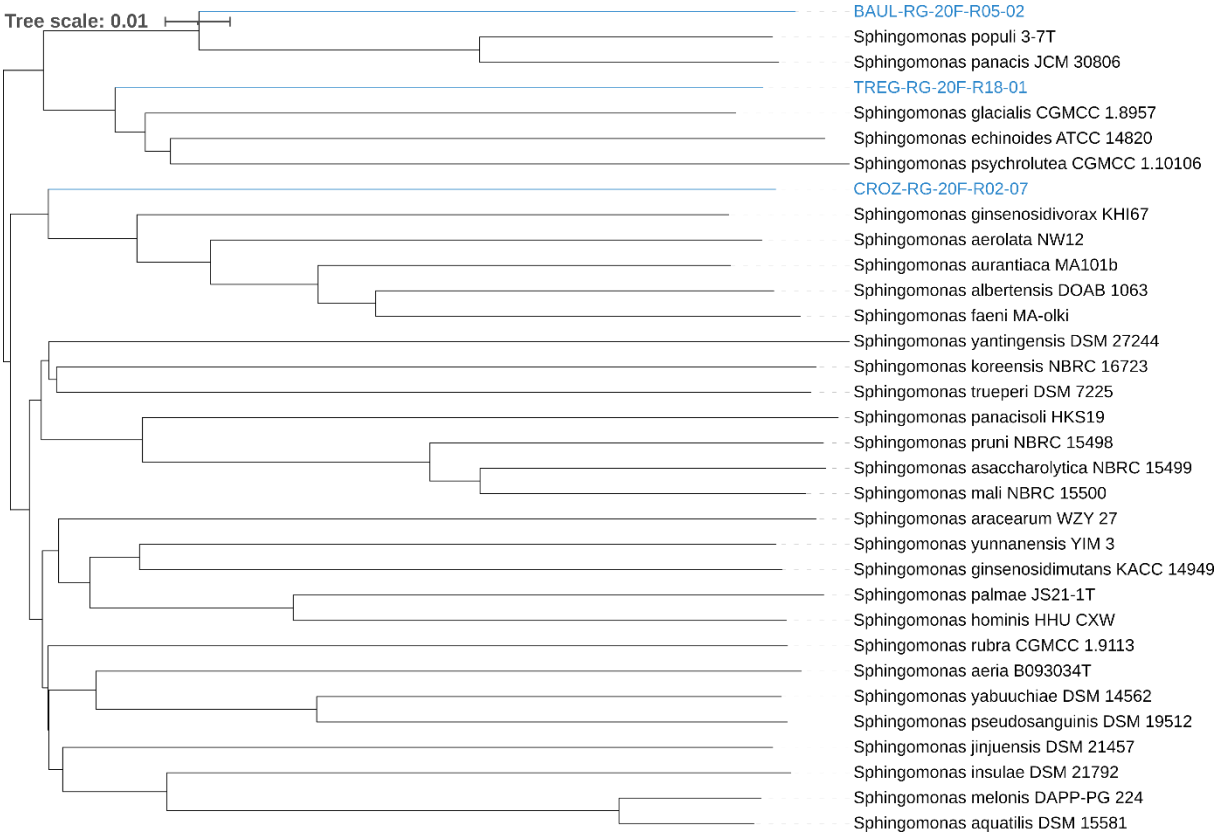

Figure 8. Phylogeny of bacterial strains carrying resistance to antibiotics. Phylogenetic tree was inferred with FastMe 2.1.6.1 [73] on the TYGS platform [59] from GBDP distances calculated from whole-genomes. The tree was rooted ad midpoint. Tree was edited iTOL [74]. Strains isolated from *R. geographicum* and the related tree branches are indicated in blue.

Analysis of the predicted KEGG metabolic pathways performed on the 9 genomes and the two genomes of the strains not affiliated by 16S, showed diversity among the strains (Supplementary Data Set 5, fichier joint excel). The main variabilities reside in carbon degradation, nitrogen, sulfur and hydrogen cycles, vitamins and transporters as well as secretion systems.

To better understand the genetic bases of the antibiotic resistance activity of the 9 strains, we investigated the presence of Antimicrobial Resistance (AMR) proteins by using AMRFinder Plus [66] enabling accurate assessment of AMR gene content. We found that bacterial strains isolated from the Baulon lichen population carried class A β-lactamase and subclass B3 metallo-β-lactamase proteins (Supplementary Data Set 6, fichier joint excel). Strains from the Crozon lichen population displayed a wider range of AMR proteins that were annotated as efflux RND transporter permease subunit EmhB, efflux RND transporter outer membrane

subunit EmhC, class C β-lactamase and copper resistance metal-translocating P1-type ATPase CueA (Supplementary Data Set 6, fichier joint excel). Strains from the Plounéour-Menez population on which the MONT-RG-20F-R14-05 strain inhibiting the *Staphylococcus aureus* (CIP 53.156) human pathogen and fungal isolates were found to carry NAD(+)-rifampicin ADP-ribosyltransferase, class C β-lactamase and fosfomycin resistance glutathione transferase proteins. Finally strains coming from the Trégastel population and showing the highest level of antibiotic resistance were found to harbour AMR proteins annotated as fosfomycin resistance glutathione transferase, class C β-lactamase, arsinothricin resistance N-acetyltransferase ArsN1 family B and APH(3') family aminoglycoside O-phosphotransferase. Interestingly, the AMR profile was defined by the lichen population, suggesting that specific antimicrobial strategies are adopted at local/habitat level.

Lichen bacterial strains harbour tolerance to persistent organic pollutants that is not correlated with the antimicrobial activity

In order to investigate the putative tolerance to persistent organic pollutants (POP) and the possible connection with antibiotic resistance activity, perfluorooctanoic acid, a perfluoroalkyl substance found in aqueous film forming foams, was added under bacterial growing conditions. Results demonstrated that 62.2% of the 455 tested strains were able to tolerate the POP when considering a tolerance coefficient >1. Strains CROZ-RG-16B-R04-02, ITXA-RG-15B-R12-05 and BAUL-RG-1B-R07-09 were the highest tolerant with a coefficient of 43.2, 37.4 and 24.5 respectively. We then calculated the proportion of strains showing tolerance for each lichen population by dividing the N° of tolerant strains on the total N° of strain of a given population (Table 2). We found that POP tolerance was not correlated with the habitat (i.e. lichen populations with a spill oil history), except for the Trégastel population with 73.3% of the tested strains with a tolerance coefficient above 1. These results suggest that POP tolerance can also be induced by habitat selective pressures that are not strictly related with the presence of the POP.

Table 2. Total number and % of strains tolerant to PFAS for each lichen population. Localities indicated in bold correspond to habitats that have been subjected to oil spills in their history

Lichen population	**Locality**	**N° Strains showing a tolerance coefficient > 1**	**Total N° of strains**	**% of POP tolerant strains**
BAUL	Baulon	38	74	51,4
CARO	**Carolles**	54	86	62,8
CROZ	**Crozon**	59	103	57,3
ITXA	Itxassou	49	76	64,5
MONT	Plénéour-Menez	39	55	70,9
TREG	**Trégastel**	44	60	73,3

In our study, POP tolerance was not correlated with antimicrobial activity as described previously [34]. Indeed, the most tolerant bacterial strains, CROZ-RG-16B-R04-02, ITXA-RG-15B-R12-05 and BAUL-RG-1B-R07-09, with coefficient > 20 were not tested for antibiotic resistance because they were not isolated from media supplemented with antibiotics. On the other hand, the most resistant bacterial strains MONT-RG-20F-10-E-7-02, MONT-RG-20F-R14-05, TREG-RG-20F-10-E-5-01 and TREG-RG-20F-10-E-6-01 did not show a strong POP tolerance. Globally, our results corroborate with the evidence that lichen habitats are a source of POP tolerance and antibiotic resistance strains with antimicrobial activities but that those activities are not strictly correlated.

Discussion

This extensive work dealing with the isolation of lichen-associated bacteria provides for the first time data on the abundance and diversity of culturable bacterial consortium that naturally colonize *R. geographicum* collected in French zones strongly affected by hostile environment conditions (wind, sea sprays, snow). Most of the culture-dependent studies only use classic media or adapted media [39]. To our knowledge, only few works used original media enriched with lichen extract [67, 68]. The “Great Plate Count Anomaly” or the “Microbial Dark Matter” [69], the uncultivated microbial consortium, could be cultured and its recovery in laboratory conditions might be possible via a reasonable imitation of conducive natural conditions. In our study, media tailored to mimic the lichen environment (14B, 15B, 16B 17B, 18F, 19F and 20F)

allowed isolation of bacterial species which were not recovered on conventional media suggesting that the nutrient encountered into the specially designed media play a substantial role in isolating rare species. Indeed, among the 699 single clusters amplified and characterized with the 16S marker gene, 19.9% of bacteria were identified as uncultured including an unclassified strain and three strains with no match based on 16S sequences in GenBank. Moreover, the long incubation time allowed the recovery of a strain belonging to the Frankiales order [70] hardly isolated from lichens.

Our data based on KEGG metabolic pathways confirmed the presence of potential functions of some bacteria which protect against toxic compounds, e.g. those contributing to resistances to the toxic arsenic for all the whole genome sequenced bacteria. Besides, resistance mechanisms for antibiotics were also found, like different classes of β-lactamases and efflux pumps. Additionally, the most resistant bacterial strains, present sulfur assimilation pathway. The inhibition of this function in bacterial pathogens permits the enhancement of antibiotic activities [71]. The lichenized fungus producing specialized metabolites with antimicrobial properties, as depsidones, depsides and dibenzofurans [72], the presence of such protective bacterial functions could be explained to confer to the bacterial microbiota the right arsenal to live happily within the lichen holobiont reflecting the symbiosis as a natural reservoir of bacterial resistance mechanisms [33].

As preliminary results, this study also highlighted the tolerance of these lichen-associated bacteria library to high concentrations of POP arguing their potential use for bioremediation, giving a link with the known use of lichens as pollutant biomonitors.

All these evidence finally shed on the light the real biotechnological interest of this library. Moreover, our novel functional-ecological study on the *R. geographicum*-associated bacterial communities stress the need for considering lichens as sources of important ecosystem's functions still poorly deployed.

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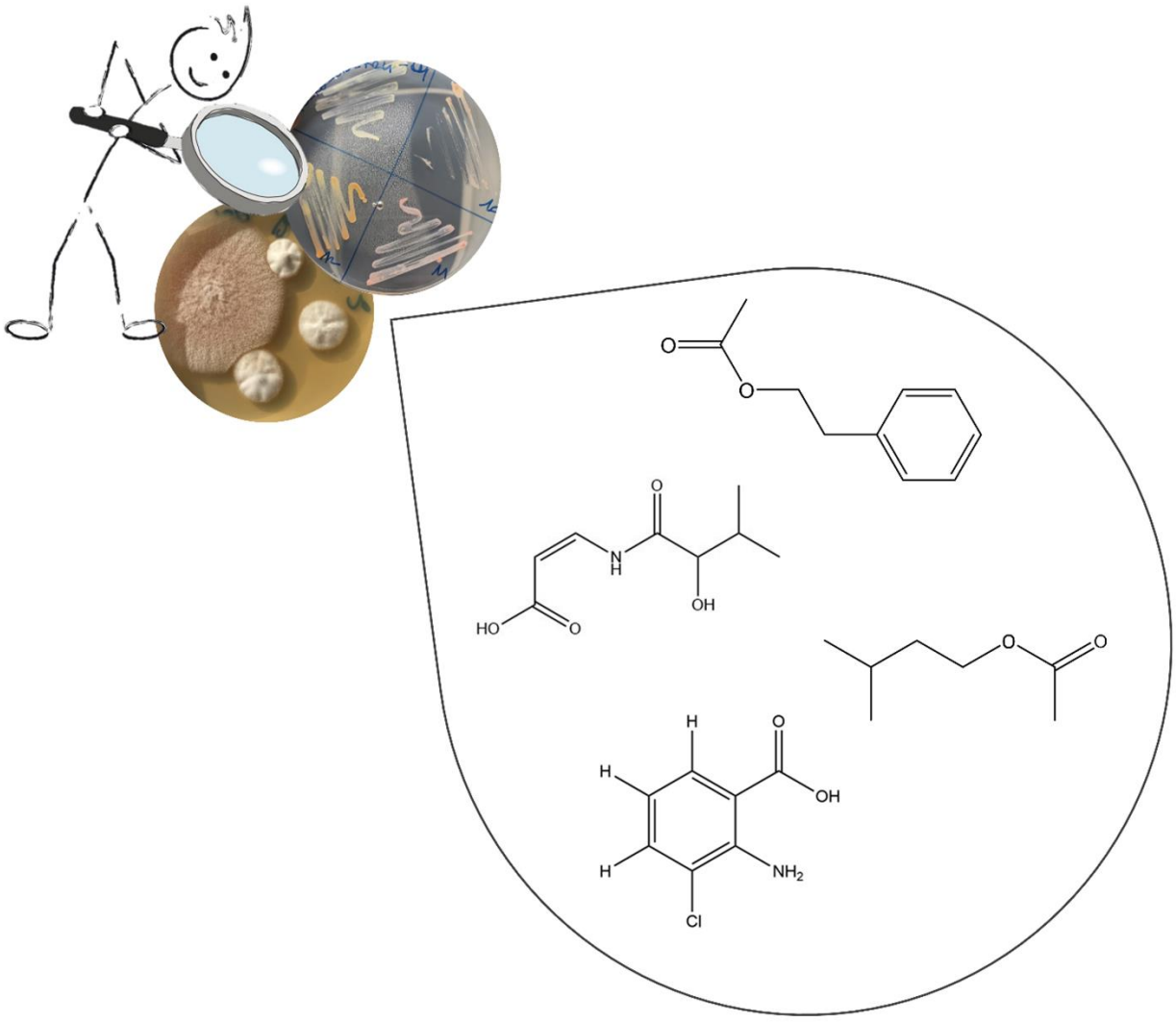
IV. Conclusion

Les lichens sont des écosystèmes autonomes. Beaucoup d'entre eux ont des distributions géographiques extrêmement étendues et tolèrent des habitats hostiles, défavorables à la survie des partenaires dans leur individualité. La diversité du troisième coéquipier au sein de la symbiose est considérable, mais nombre de ces micro-organismes reste incultivable dans les conditions de culture classiques de laboratoire donnant ainsi une image incomplète de la diversité microbienne. En multipliant les milieux et les méthodes de culture, nous avons voulu capturer une plus grande variété du microbiote associé à *R. geographicum*.

Compte tenu, non seulement, de l'abondance, de la diversité et de l'originalité des isolats identifiés, mais aussi des fonctions abritées par certains gènes bactériens, la question soulevée par de tels résultats est de savoir quels rôles écologiques ces micro-organismes peuvent avoir au sein de l'holobionte lichénique et comment ils peuvent être appliqués d'un point de vue biotechnologique. Pour apporter une contribution à cette compréhension, la production métabolique de certains microorganismes isolés précédemment, va être étudiée dans la partie suivante.

VALORISATION DE LA SOUCHOTHEQUE



CHAPITRE 3 : VALORISATION DE LA SOUCHOTHEQUE

I. Introduction et contexte de l'étude

A travers l'étude de la diversité microbienne cultivable du lichen crustacé *R. geographicum* qui a été présenté dans le 2ème chapitre de ces travaux de thèse, un second axe de travail entrepris a eu pour objectif de valoriser cette incroyable diversité microbienne par le prisme de leur production métabolique.

Une première partie de cette valorisation a consisté en l'approfondissement de l'étude du métabolome d'une bactérie en particulier, *Paenibacillus etheri*. En effet, cette bactérie avait déjà été isolée précédemment, mais identifiée comme étant *Paenibacillus odorifer* en 2016, lors des travaux de thèse de Thi Bach Le NGUYEN (Nguyen, 2018). En 2019, un nouveau séquençage sur la base de l'ARN 16S a permis de la ré-identifier comme étant *Paenibacillus etheri*, puis quelques mois plus tard, le nouvel isolement réalisé et décrit dans la 2ème partie du Chapitre II a permis de retrouver cette dernière. Lors de cette nouvelle campagne d'échantillonnage lors de l'hiver 2020, la surface du lichen a été observée au microscope et la présence d'un nématode bactériophage a été constatée. Cette observation associée à une étude bibliographique des molécules isolées au laboratoire (données non publiées) a mené à l'initiation d'une nouvelle collaboration avec Sylvain Fournet, Josselin Montarry et Catherine Porte au sein de l'équipe IGEPP de l'INRAE de Le Rheu. Au cours de ces travaux, l'activité nématicide de la suspension bactérienne de *P. etheri*, de son surnageant de culture ainsi que de son volatolome ont été testés contre les nématodes à kystes *Heterodera schachtii* et *Globodera pallida*. Ce travail sera présenté sous forme de publication intitulée « Volatile organic compounds from a lichen-associated bacterium, *Paenibacillus etheri*, interact with plant parasitic cyst nematodes » qui sera prochainement soumise dans *Journal of Agricultural and Food Chemistry* (cf II p. 104).

La seconde partie de cette valorisation a porté sur la mono- et la co-culture de champignons deux à deux en milieu solide. Ces champignons ont également été isolés comme décrit dans la 2ème partie du Chapitre II et ont été choisis du fait de l'observation d'une zone de compétition entre eux sur les boîtes d'isolement initial avant purification (Fig. 3). Lors de cette observation, dix champignons ont présenté des zones de confrontation. Suite à une étude bibliographique sur les champignons identifiés ainsi

qu'une analyse mycochimique préparatoire, trois couples de champignons on été sélectionnés (Fig. 3).

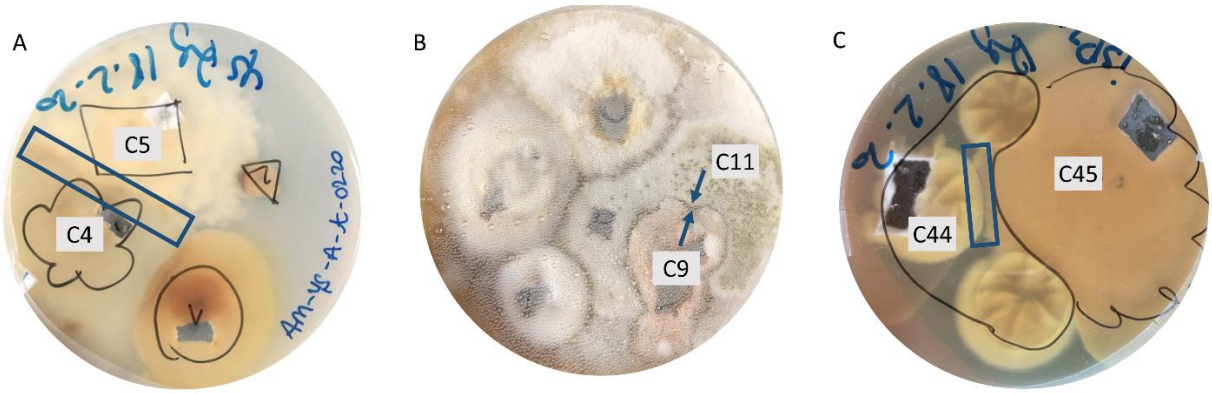


Figure 3 Boîtes de Petri d'isolements issues de la campagne d'hiver 2020. (A) Zone de compétition encadrée en bleu entre Dendrothyrium variisporum C4 et Xylaria hypoxylon C5. (B) Zone de compétition fléchée en bleu entre Coccinonectria rusci C9 et Melanconium hedericola C11. (C) Zone de compétition encadrée en bleu entre Michrodochium phragmitis C44 et Tolypocladium sp. C45.

Outre le fait de présenter une zone d'interaction particulièrement visuelle avec leur voisin, ces six champignons ont été sélectionnés parmi dix du fait du peu, voire de l'absence, d'études relatant leur chimie à l'exception de *Xylaria hypoxylon* (C5) (Hu et al., 2012; Schüffler et al., 2007; Zhou et al., 2021). Ainsi, dans le cadre du stage de Master 2 de Leila Affes, ces souches ont été cultivées en mono- et en co-cultures sur des milieux gélosés de compositions différentes selon des méthodes OSMAC (One Strain Many Compounds) et sur différents supports : boîtes 6 puits, boîtes de Pétri classique d'un diamètre de 9 cm et boîtes de Petri de 9 cm comprenant deux compartiments. Une fois cette étude menée et la comparaison réalisée, le choix du milieu et du support a été fait pour l'entreprise d'une culture à plus grande échelle dans le but d'isoler, caractériser et tester biologiquement des molécules produites par ces champignons. L'étude du couple C4-C5 a notamment permis l'isolement et l'élucidation structurale de 7 nouveaux composés de type sesquiterpènes érémophilanes. L'étude chimique des couples C9-C11 et C44-C45 est en cours. Ces différentes études vont être détaillés dans la partie III p. 120.

II. Volatile organic compounds from a lichen-associated bacterium, *Paenibacillus etheri*, interact with plant parasitic cyst nematodes

Volatile organic compounds from a lichen-associated bacterium, *Paenibacillus etheri*, interact with plant-parasitic cyst nematodes

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ABSTRACT: Healthy food is one of the major challenges to develop in this century. Plant parasitic nematodes cause important damage to many crops worldwide and till now, the use of chemical nematicides are the main means to control their populations. These chemical products must be replaced by more environmental-friendly control methods. Biocontrol methods seem to be one promising option and the number of biopesticides derived from living organisms has increased in the last decades. To develop new plant protection products, we have decided to combine our skills, in natural products chemistry and nematology, and to focus on lichen microecosystem as underexploited ecological niches of microorganisms. We will present herein the potential of lichen-associated bacterial suspensions from *Paenibacillus etheri*, the supernatant of their culture and their metabolites, as nematicides against the beet cyst nematode *Heterodera schachtii* and the potato cyst nematode *Globodera pallida*, notably using a newly developed method to evaluate the effects of volatile organic compounds (VOC). A Solid Phase Micro-Extraction method (SPME) associated to GC-MS analysis of 14 days cultures were performed to analyse these VOC and try to identify as precisely as possible those responsible of the effects on the tested cyst nematodes.

KEYWORDS: *Paenibacillus etheri*; *SPME; mVOCs; GC-MS; Plant-parasitic nematodes; Biological control.*

INTRODUCTION

Changes in agricultural practices, such as mono-culture cropping, intensive tillage and the use of synthetic fertilizers and pesticides have led to a decline in soil structure and an increase in soil borne plant diseases.[1] Plant pests and diseases are major factors leading to the destruction of food crops across the globe and have been raised in history as a public health and society matter apart from economic and environmental issues in agriculture. Around, 20–40 % loss in

global agricultural productivity is owed to insects, diseases, and weeds.[2] However, healthy food is one of the major challenges to develop in this century. To produce good and enough food for each inhabitant living in this planet, the pest's control is crucial and the chemical products must be replaced by more sustainable management practices. Biocontrol agents and products seem to be one promising option and the number of biopesticides derived from microorganisms has increased in the last decades. Biological control comprises natural rivals or disease suppressive bacterial and fungal species, their genes or metabolites, to offset the pathogen population with a consequent improvement in plant health.[3] Plant parasitic nematodes (PPNs) are one of the major constraints to crop production and especially in high-value vegetable and fruit crops. They can cause significant economic yield loss estimated to be more than US$100 billion annually[4]. The cyst nematodes (*Heterodera* and *Globodera* spp.) are among the most damaging plant-parasitic nematodes, based on scientific and economic importance throughout the world [5]. Control of those species is very difficult due to their ability to survive for prolonged periods in the soil as a cyst, in the absence of their host plant. To control their population chemical soil fumigants have been used for more than a century and they remain nowadays the standard practice in many crops. As limitations of chemical soil fumigants are becoming more apparent, there is an urgent need to find new soil fumigation compounds that are safer for the soil ecosystem and the environment.

Microorganisms produce a great variety of secondary metabolites including antibiotics, toxins, pigments, and others. Curiously, small molecular mass metabolites such as volatile organic compounds (VOCs) were for a long time overlooked. Research conducted the last decades demonstrated that bacteria produce a large set of VOCs including hydrocarbons, ketones, alcohols, sulfur- and nitrogen containing compounds, terpenes and others[6,7] with a limited knowledge about their biological and ecological function[8] except for their long- and short distance infochemicals mediating inter- and intra-specific interactions[9,10]. VOCs are a broad group of lipophilic compounds with low molecular weight (100-500 Da), high vapor pressure and low boiling point. Due to their physico-chemical properties, VOCs can easily diffuse through gas- and water-filled pores and can, therefore, have a wide effective range in soil.[11]

Few studies focused on managing PPNs with bacteria, also isolated from a lichen,[12] but *Paenibacillus* strains were described as having a deleterious effect on PPNs[13–15]. *Paenibacillus* is a cosmopolitan and ubiquitously occurring bacterial genus which is predominantly isolated from the rhizosphere. Although it occurs naturally in soil and marine sediments, plant associated habitats like the rhizosphere and roots of crop plants are its preferred environments. The genus *Paenibacillus* has an enormous potential in biotechnology as a source of novel

bioactive compounds and this potential has only partially been exploited.[16,17] For instance, *P. polymyxa* is an agriculturally important microbe already reported as an efficient biocontrol agent and biofertilizer.[18,19] *Paenibacillus* can influence plant growth and health directly by the production of phytohormones, by providing nutrients, by fixing nitrogen and/or by the suppression of deleterious microorganisms through antagonistic functions.[20] Furthermore, *Paenibacillus* species can also be harbored in lichens,[21,22] a symbiotic association between a fungal (mycobiont) and an algal (photobiont) partner and where occurs a rich microflora. We previously isolated *P. etheri* from the lichen *Rhizocarpon geographicum* and it appeared that the strain represented 62.5% of total bacterial isolates.[23] Looking closer, on the lichen surface, we have also observed microscopically a bacteriophage nematode. Indeed, the existence of various invertebrates inhabiting lichens such as arthropods or nematodes is well established [24,25] while within different genera sheltered, *Paenibacillus* was identified as "protectors" in the lichen holobiont.[26,27] Taking into account these insights, we asked ourselves if the abundance of this bacterium was correlated with the presence of such nematode and investigated further this natural symbiotic system for novel biotechnological applications.

The objectives of the present study were to investigate the potential *in vitro* nematicidal activity of a lichen associated bacterium's metabolome, *P. etheri's,* and, considering the importance of microbial VOCs (mVOCs), to assess the mVOCs' activity against two nematode species of agronomical interest: the sugar-beet cyst nematode *Heterodera schachtii* and the potato cyst nematode *Globodera pallida*. The bioassays were conducted, at 6 h and 24 h, with different kinds of *P. etheri's* culture extracts using 12-microwell plates for the evaluation of direct contact or using a process specifically developed here to test mVOCs by gaseous contact.

MATERIALS AND METHODS

Chemicals. Solvents of analytical standard grade were purchased from Merck_Sigma Aldrich (St Quentin Fallavier, France). Malt extract, yeast extract, dextrose and $CaCO_3$ were acquired from Sigma-Aldrich (Saint Quentin Fallavier, France), isoamyl acetate and $NaHCO_3$ from ThermoScientific Acros (Illkirch, France) and 2-phenylethyl ester from Thermo Fisher (Kandel, Germany).

Bacteria and Nematodes. *P. etheri* was isolated from the lichen *R. geographicum*[28] and stored at -80°C. The strain was grown on GYM Streptomyces agar medium containing dextrose (4 g L^{-1}), yeast extract (4 g L^{-1}), malt extract (10 g L^{-1}), $CaCO_3$ (2 g L^{-1}) for 48 h at 25°C. A total plate of *P. etheri* was inoculated in 10 mL of NaCl solution at 0.8% then transferred to 1000 mL of liquid GYM medium ($CaCO_3$ was replaced by $NaHCO_3$ (2 g L^{-1})) in 1 L glass bottle

hermetically sealed with a septum and incubated for 14 days on a rotary shaker (120 rpm) at 25°C. This step was repeated twice.

Nematode populations used in this study were maintained in collection at the IGEPP laboratory and regularly multiplied on sugar-beet cv. Ardan for the AM2 population of *Heterodera schachtii* and on potato cv. Désirée for the Chavornay population of *Globodera pallida*. To assess the nematicidal activity of *P. etheri* and its VOCs, hatching of second-stage juveniles (J2) was performed in water for *H. schachtii* and in potato root exudates for *G. pallida*.

Samples Preparation. After 14 days of culturing, an amount of *P. etheri*'s fermentation broth was then saved in order to separate the supernatant and the pellet: 2 L were centrifuged for 15 min at 4°C and 3500 rpm. The supernatant obtained was split: 100 mL were kept for the further experiments on cyst nematodes, 1 L was feezed for further freeze-drying and the final 900 mL were used for a liquid/liquid partition repeated twice with 450 mL of ethyl acetate. After freeze-drying of 1 L of freeze-dried supernatant 411 mg were obtained. Moreover, after evaporation of organic solvents, an organic crude extract (m = 82.86 mg) was finally obtained.

mVOCs Extraction by Solid Phase MicroExtraction (SPME). To analyze the VOC profiles produced by the bacterium, cultures were prepared as described above. After 14 days of culture, the collection of volatile compounds was performed using a static headspace system. A SPME stable flex fiber 75 mm DVB/C-WR/PDMS (Agilent) was inserted into the headspace of one bottle through the septum and incubated further 30 min at 40°C under agitation (120 rpm). The VOCs from 1 L modified GYM Streptomyces broth were used as controls.

mVOCs Analysis by Gas Chromatography-Mass Spectrometry (GC-MS). A Hewlett Packard 7820AGC/5975MS (Agilent Technologies, USA) equipped with a HP-5MS (30 m x 0.25 mm x 0.25µm) capillary column was used to separate and identify the VOCs. The carrier gas was helium with a flow rate of 1 mL/min in splitless mode. The SPME fiber was then directly inserted into the injector of the gas chromatograph and desorbed at 260°C for 4 minutes. The following oven temperature protocol was used: 40°C for 5 min, 40-250°C at a rate of 5°C/min and held at 250°C for 5 min. The mass spectrometer was operated at 70 eV and 260°C in the electron impact (EI) ionization mode with a full scan from 50 to 250 *m/z*. Identification of VOCs was based on a comparison with mass spectra from a library NIST 17 (National Institute of Standards and Technologies) and confirmed with the injection of commercially available standards.

Nematicidal Activity Assay. Two kind of bioassays were conducted to determine the effects of different kinds of *P. etheri's* culture extracts on the motility of juveniles by direct (broth fermentation, supernatant, EtOAc crude extract or freeze-dried supernatant) or gaseous contact

(mVOCs and chemical standards). By direct contact, each treatment was done on *H. schachtii* and replicated 6 times on a 12-well plate. Culture medium or water were used as controls (6 replicates). Observations were made after 6 and 24 hours of contact. 250 µL of nematodes suspension was transferred to 12-well plates (approximately 30 juveniles/well). ***Broth fermentation and supernatant***. In the 6 treatment wells, 1 mL of broth fermentation or supernatant were added to the 250 µL suspension of nematodes. The controls consisted in adding 1 mL of GYM Streptomyces modified medium. ***Crude extract.*** In the 6 treatment wells, 1 mL of an aqueous solution at 26 mg mL^{-1} were added to the 250 µL suspension of nematodes. The controls consisted in adding 1 mL of water.

After 6 or 24 hours of contact, the number of moving and not moving juveniles was counted, and the 6 wells of each treatment were pooled and rinsed onto a 10 µm sieve. Each pool was then submitted to active passage through an absorbent paper with 45 µm mesh (TORK, France Fournitures, France). After 18 hours of active passage, living juveniles, i.e. those able to pass through the paper, were transferred into counting cells and counted. ***mVOCs.*** Gaseous contact experiments were realized by using 14 days old culture of *P. etheri* performed in 1 L culturing bottles with a 100 mL head space and sealed by a septum. To ensure that culturing bottles were in surpression, 40 mL of air were injected through the septum using a syringe prior to the experiment. Thereafter, culturing bottles were connected with a canula to a 500 µL quartz-cell, sealed with a septum, maintained at the atmospheric pressure by a syringe needle pricked through the septum and containing a suspension of nematodes juveniles. Then, 40 mL of air were injected using a syringe in the culturing bottle head space, bubbling in the nematode suspension contained in the quartz cell. After bubbling, the syringe needle was immediately removed and the potential VOCs effect was observed under a stereo microscope using a transillumination unit. Indeed, the phenotype of each juvenile contained in the suspension was assed as mobile (the larva was still moving) or immobile (the larva showed no motility). The VOCs of culture medium contained in the bottle's headspace were used as control. For each modality, treated or control, the status of juveniles was assessed before (T0) and after (T1) bubbling. For each nematode species, *H. schachtii* and *G. pallida*, experiments were repeated 6 times, and for each replicate the number of juveniles placed into the quartz cell ranged from 28 to 51 for *H. schachtii* and 33 to 58 for *G. pallida*. ***Chemical standards***. Isoamyl acetate and 2-phenylethyl ester were assessed in the same conditions than above for mVOCs.

Statistical Analysis. Statistical analyses were carried out using the appropriate packages of the statistical software R, version 4.1.1 (R Core Team, 2021). For each nematode species, the effect of modality (control-T0, control-T1, treated-T0 and treated-T1) on the frequency of

moving juveniles was tested through a Wald chi-square test on a generalized linear mixed model (function 'glmer') with binomial error and a logit link function. Replicate effect was tested in the modality effect and introduced as a random factor in the model. Normality and homogeneity of variances of residuals were checked with the Shapiro-Wilk and the Levene tests, respectively. As significant effects were detected, pairwise comparisons of mean values were computed using an analysis of contrasts (function 'emmeans', $\alpha = 0.05$).

RESULTS AND DISCUSSION

Samples and mVOCs Analysis. The liquid growth medium was selected with the aim of the best growth of *P. etheri,* based on the monitoring of Optical Density (OD) at 620 nm. This medium was derived from GYM Streptomyces agar medium and $CaCO_3$ (2 g L^{-1}) was replaced by $NaHCO_3$ (2 g L^{-1}). After some optimization, the best parameters for *P. etheri* growth were a culture at 25°C and 120 rpm during 14 days, time necessary to reach the stationary phase. At the end of the culture, various samples were obtained: broth fermentation, supernatant obtained after removal of bacterial cells by centrifugation, crude extract after ethyl acetate extraction of supernatant, freeze dried supernatant and a SPME collection of mVOCs. The approach adopted for mVOCS qualitative analysis was the use of static SPME fiber in order to trap most of the analytes present even at trace level, on a short period of time, to provide a true representation of the studied system. The advantage of SPME fibers is that the volatiles are enriched on the fibers and no solvent is needed. Considering that the goal of volatilomic profiling is to analyze as many metabolites as possible, the use of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibers appeared to be the most suited to increase the number of analytes that can be trapped on the fiber because it can allow the capture of VOCs in a wide range of polarity and molecular weight.[30] Then the SPME/GC-MS analysis of mVOCs produced by *P. etheri* was assessed by using an intermediate non-polar HP-5MS GC capillary column. The culture medium is itself a source of volatiles, particularly as the autoclaving process forms several volatiles. It is therefore essential to run blank analyses on the medium alone to distinguish the volatiles of the medium from those of the bacteria. The comparison of GC-MS total ion chromatograms from broth fermentation and culture medium highlighted the presence of 4 additional peaks, from which two were identified after NIST mass spectral matching as isoamyl acetate (**1**) and 2-phenylethyl ester (**2**). Moreover, the subsequent injection of two commercially available analytical standards confirmed these identifications (Figure 1). Even if the major peak (at Rt 20.5 min) comes from the culture medium, compounds (**1**) and (**2**) appeared relatively more abundant than the other peaks (**3**) and (**4**) identified as unidentified

aliphatic compounds. Note that isoamyl acetate (**1**) has been frequently reported as mVOCs from microorganisms[7] and also described as being produced by another species of *Paenibacillus.*[31] To our knowledge, it is the first report of mVOCS profile of *P. etheri*, a known pollutant degrader.[32]

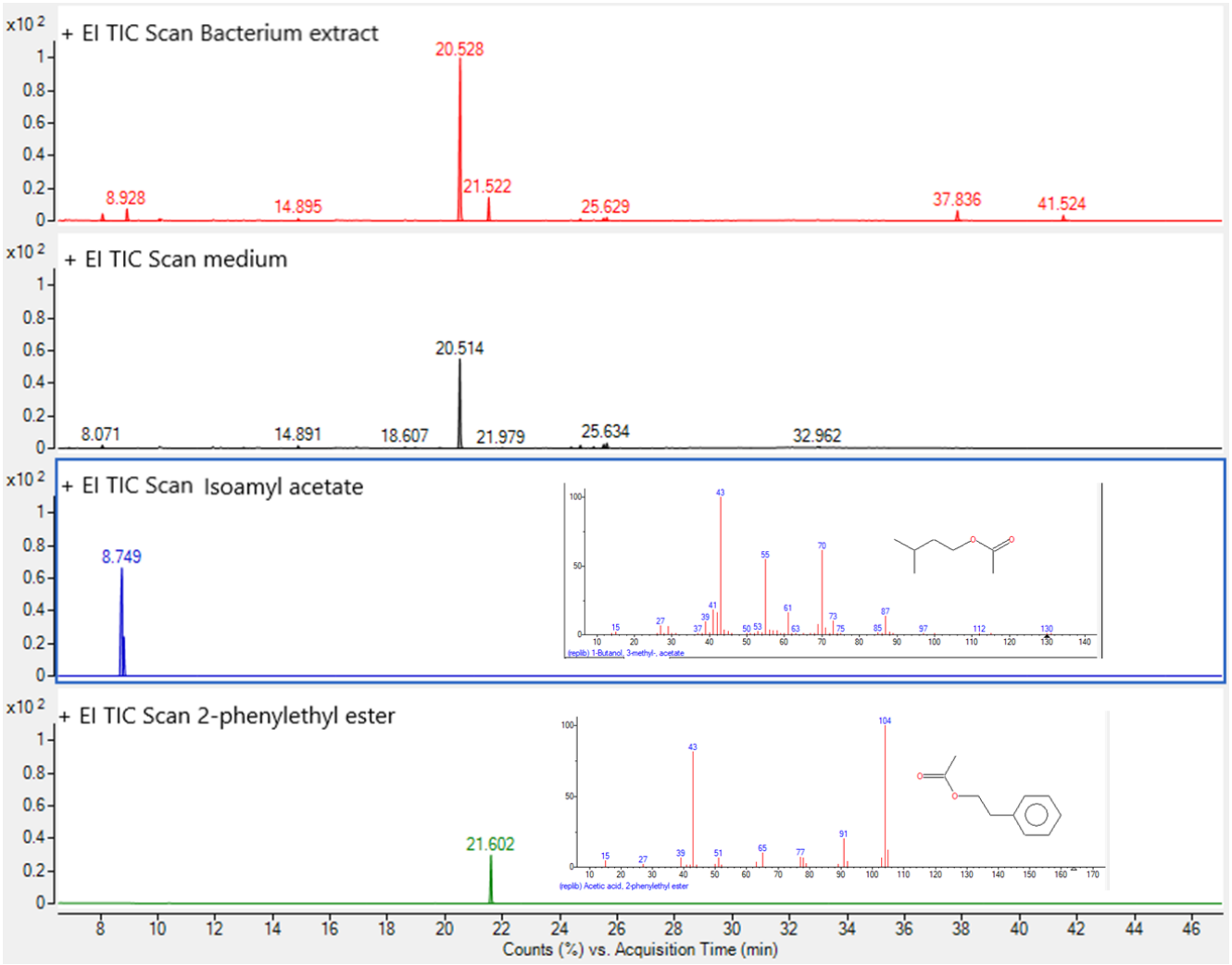

Figure 1 GC-MS total ion chromatograms (TIC) using electron impact (EI) ionization of (A) SPME extraction of P. etheri fermentation broth's head space; B) SPME extraction of GYM Streptomyces medium's head space; (C) isoamyl acetate standard; (D) 2-phenylethyl ester standard. Peaks: (1) isoamyl acetate, (2) 2-phenylethyl ester, (3) and (4) unidentified sesquiterpenes. Peaks 1 and 2 were identified after comparison of their mass spectrum with those of NIST 17 and after injection of the analytical standards.

Nematicidal Bioactivity. While the three controls (water, medium without *P. etheri* and crude extract without *P. etheri*) have no effect on nematodes, broth fermentation and supernatant from *P. etheri* cultures strongly inhibited motility of *H. schachtii* juveniles (from 91% to 100%) after 6 and 24 h of direct contact (Table 1). This effect being reversible, as juveniles were noted alive in the subsequent active passage test, those samples have a clear

nematostatic activity. This nematostatic effect was also exhibited by the crude extract obtained after ethyl acetate extraction of supernatant, but it was associated with a nematicidal activity as the mortality rate was higher (up to 69% at 24h – Table 1). Interestingly, a treatment of freeze-drying on crude extract led to the loss of these activities on nematode juveniles (Table 1).

Table 4 Effect of different P. etheri *extracts on motility of juveniles of* H. schachtii[a]

	After 6h of contact		After 24h of contact		
	% of moving juveniles	% of living juveniles	% of moving juveniles	% of living juveniles	
H_2O (control)	97	87	95	86	No effect
Medium without *P. etheri* (control)	98	89	96	86	No effect
Broth fermentation	2	98	0	87	Nematostatic effect
Supernatant	9	97	2	91	Nematostatic effect
Crude extract of medium without *P. etheri* (control)	96	79	93	86	No effect
Crude extract	14	60	0	31	Nematostatic effect
Freeze-dried crude extract	97	93	95	77	No effect

With no effect observed with the freeze-dried crude extract contrary to the other samples, we hypothesize that the mVOCs may cause the *in vitro* activity against nematodes. Indeed, solvent extraction is the method most commonly used for the isolation of natural products. Nevertheless, when solvent extraction is used, it remains unclear whether a given compound is volatile enough to be released into air and thus absent from the final crude extract. As there is no standard procedure for testing the effects of mVOCs on plant parasitic nematodes, we developed a bioassay to test these VOCs using gas contact by bubbling them on the two cyst nematodes. Motility assays results from mVOCs were reported in Figure 2.

Regarding the *H. schachtii* experiment, the modality effect on the frequency of moving juveniles was highly significant ($\chi^2 = 139.04$, df = 3 and $P < 0.0001$). The proportion of moving juveniles was high before bubbling (0.90 for control-T0 and 0.92 for treated-T0) and mVOCs produced by *P. etheri* reduced significantly the proportion of moving juveniles (0.02 for treated-T1). Bubbling had no effect on the motility of *H. schachtii* juveniles, as control-T1 and control-T0 were not significantly different (Figure 2A).

Regarding the *G. pallida* experiment, the modality effect on the frequency of moving juveniles was also highly significant ($\chi^2 = 207.54$, df = 3 and $P < 0.0001$). The proportion of moving juveniles was high before bubbling (0.91 for control-T0 and 0.93 for treated-T0) and mVOCs produced by *P. etheri* reduced significantly the proportion of moving juveniles (0.05 for treated-T1). The comparison of means showed that *G. pallida* juveniles were slightly impacted by bubbling, as control-T1 (0.69) was significantly lower than control-T0. However,

as treated-T1 was significantly lower than control-T1, there was also a strong mVOCs effect on this nematode species (Figure 2B).

Interestingly, mVOCs produced a significant effect on the two cyst nematodes used in this study and belonging to two different genera of cyst nematodes. This ubiquitous effect could be relied on their hydrophobic and uncharged nature which allow them to easily penetrate cell membranes and subsequently induce perturbations such as increased permeability and leakage of intracellular components.[10]

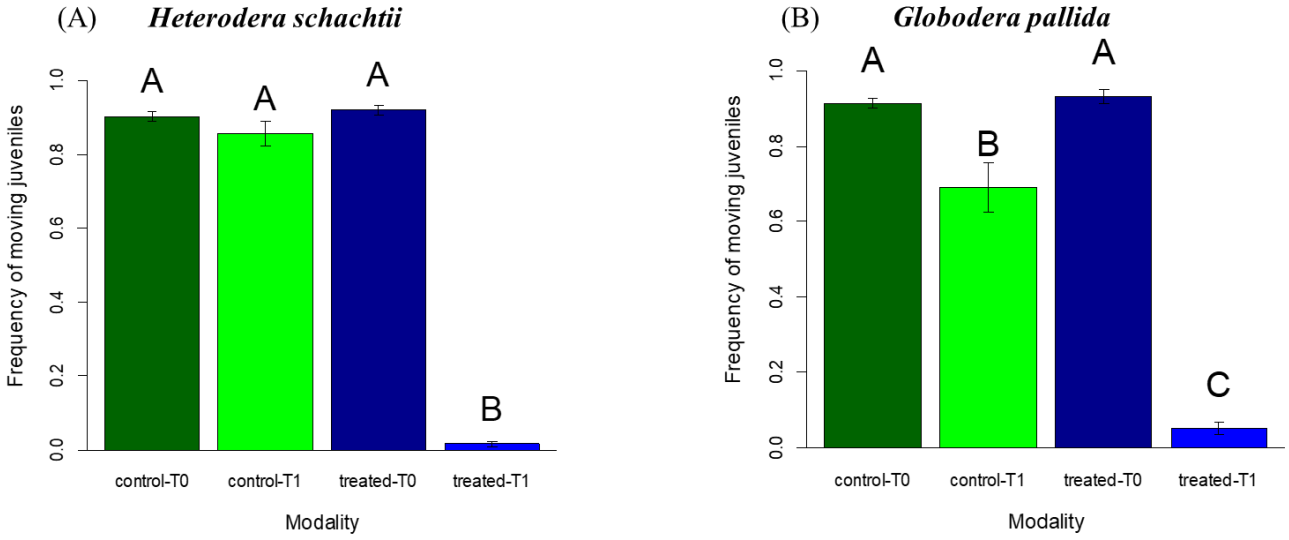

Figure 2 Bioassays of P. etheri *'s mVOCs against* ***(A)*** H. schachtii *and* ***(B)*** G. pallida. *Frequency of moving juveniles (means ± se) for each modality: control-T0 = GYM Streptomyces modified medium before bubbling; control-T1 = GYM Streptomyces modified medium after bubbling; treated-T0 =* P. etheri *broth medium before bubbling; treated-T1 =* P. etheri *broth medium after bubbling. Letters represent significant differences between modalities (pairwise comparisons of means, α = 0.05).*

Isoamyl acetate (**1**) and 2-phenylethyl ester (**2**) were then evaluated, separately or in mixture (v/v; 1:1), in order to eventually correlate their presence to the observed nematicidal effect. As these compounds have very low water solubilities, they were tested in their gaseous forms. To do so, they were incubated à 37°C for 6 days in sealed flasks, and the head-spaces were bubbled in the same way as for the fermentation broth. In preliminary assays, while 2-phenylethyl ester appeared no efficient alone, isoamyl acetate, exhibited nematicidal effect against *H. scharchtii* after 30 min bubbling. Further experiments are in progress in order to confirm these interesting results.

Lichens are known to be populated with bacteria, fungi and proto- and metazoan organisms. In order to cope with this competitive environment, lichen-associated micro-organisms can release a large number of metabolites, which can modify the performance of co-

habitants from the neighborhood.[33,34] In this context, we have evaluated crude extracts and mVOCs derived from the culture of a bacterium associated to a crustaceous lichen which can inhabit nematodes. It is known that mVOCs could effectively act as the first signals or chemical 'weapons' to reach a target organism.[35] We have thus demonstrated the nematicidal effects of *P. etheri* either in the form of crude extract or of mVOCs. The ability to inhibit motility of the two cyst nematodes by one of the constituents of this mixture, isoamyl acetate, is reported for the first time. These evidences open many new doors[36] for the application of either volatile-emitting strains such as *P. etheri* or pure compounds and represent promising alternatives to currently used pesticides.

ABBREVIATIONS USED

GC-MS: gas chromatography coupled to mass spectrometry

mVOCs: microbial volatile organic compounds

SPME: solid phase microextraction

TIC: total ion chromatogram

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Notes

The authors declare no competing financial interest.

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III. Etude de la chimiodiversité issue de champignons lichéniques et de leur interaction

i. Introduction et contexte de l'étude

En raison de leur diversité métabolique, leur capacité de production élevée, leur efficacité de sécrétion et leur capacité à effectuer des modifications post-traductionnelles, les champignons sont largement exploités en tant qu'usines cellulaires efficaces pour la production de métabolites, de substances bioactives et de protéines natives ou hétérologues. L'utilisation commerciale de micro-organismes fongiques est répandue dans plusieurs secteurs industriels, tels que ceux impliqués dans la production de composés organiques simples comme les détergents, les aliments et les boissons ou encore les produits pharmaceutiques (Archer, 2000; Meyer, 2008). Afin de pouvoir mener une investigation complète de la chimiodiversité d'une souche microbienne et notamment fongique, l'intégralité de son métabolome devrait être étudié. En parallèle, de nombreux travaux ont montré que la production microbienne de métabolites spécialisés dépend également des conditions de culture (Bode et al., 2002). Ainsi, dans des conditions standards de laboratoire, des voies de biosynthèse peuvent rester silencieuses. Ces voies biosynthétiques, aussi appelées « cryptiques », peuvent être activées en modulant les paramètres de culture et ainsi permettre l'accès à de nouveaux métabolites spécialisés possédant de potentielles bioactivités (Rämä and Quandt, 2021; Reen et al., 2015). Les métabolites spécialisés qui diffusent dans un milieu de culture liquide ou solide et qui influent sur leur voisin lors de co-cultures ont largement été étudiés tandis que le volatolome, faisant partie intégrante du métabolome d'un organisme, est trop souvent négligé. Pourtant les micro-organismes dialoguent également grâce aux composés organiques volatils (COV). Par exemple, l'interaction entre le phytopathogène fongique *Verticillium longisporum* et son antagoniste bactérien *Paenibacillus polymyxa* induit, la sous ou sur-régulation dépendante du temps d'incubation de certains COVs, et entraîne l'inhibition de la croissance du phytopathogène (Rybakova et al., 2017).

De nombreuses stratégies d'activation des gènes silencieux existent dont certaines sont simples, efficaces et facilement réalisables. Ainsi, pour étudier la diversité chimique des champignons sélectionnés, nous avons opté pour la méthode OSMAC (Bode et al., 2002) et la co-culture (Arora et al., 2020) qui s'apparente à une stratégie OSMAC puisqu'un

même champignon, par le biais de signaux émis par un champignon co-cultivé, produira des métabolites spécialisés nouvellement induits.

L'approche OSMAC (One Strain – Many Compounds), ou « une souche plusieurs composés » en français, permet l'obtention de nouveaux composés en cultivant les micro-organismes dans différentes conditions de laboratoire (Fig. 4). Les paramètres qui peuvent être modulés sont notamment la composition du milieu (Wang et al., 2014), le taux d'aération (Fuchser et al., 1995), le type de support utilisé (Adelin et al., 2011) ou encore l'ajout d'inhibiteurs d'enzymes (Bode et al., 2002). Aussi insignifiant que ce changement puisse paraître, l'utilisation d'eau du robinet à la place d'eau distillée pour la préparation des milieux de culture peut entraîner un changement dans la production chimique des champignons (Paranagama et al., 2007).

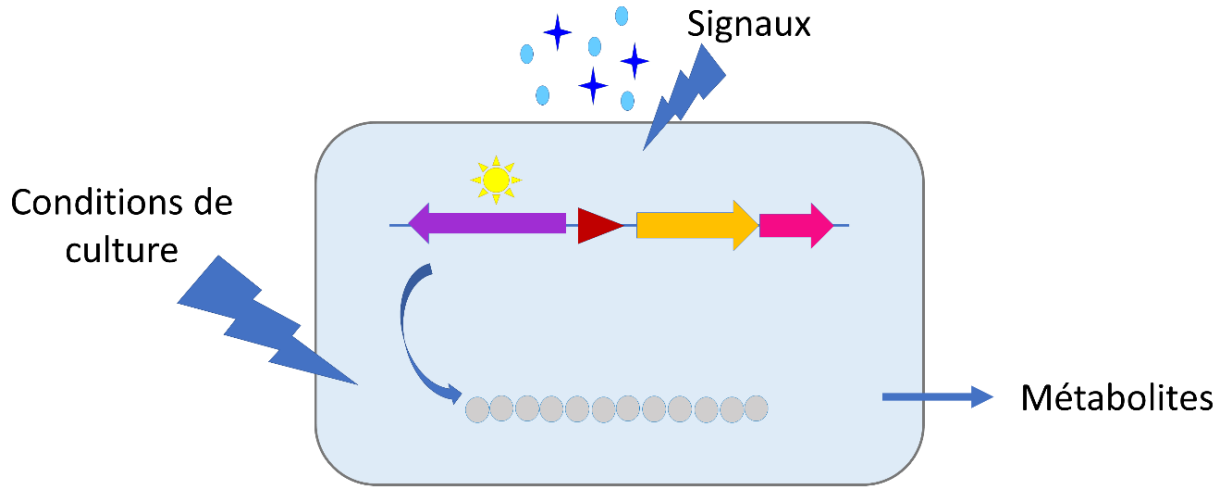


Figure 4 Influence des conditions de culture, des signaux extérieurs sur la biosynthèse de produits naturels (Scherlach and Hertweck, 2009).

La co-culture de micro-organismes implique la culture de deux micro-organismes ou plus dans un même environnement confiné (Bertrand et al., 2014b) dans lequel sont produits des métabolites spécialisés, principalement dans le cadre de la défense ou de la compétition nutritive (De Roy et al., 2014). La co-culture permet de mimer et d'étudier des interactions naturelles entre les populations, l'amélioration des cultures pour certaines populations par rapport aux mono-cultures ou l'établissement d'interactions « artificielles » entre les populations (Fig. 5) (Goers et al., 2014). En effet, ces micro-organismes développent, comme indiqué prélablement, des moyens de communication

ainsi que des moyens de défenses chimiques via des molécules de signalisation et des métabolites spécifiques (Arora et al., 2020). La présence d'un partenaire et/ou d'un adversaire peut engendrer l'activation de certains clusters de gènes donnant l'accès à des voies métaboliques dites cryptiques qui aboutissent à la production de métabolites spécialisés qui n'apparaitraient pas en mono-culture (Benoit et al., 2015). Cette méthodologie de culture est de plus en plus utilisée pour étudier les interactions possibles entre différents micro-organismes et pour découvrir de nouveaux métabolites bioactifs spécifiquement dans les zones de confrontation (Bertrand et al., 2014b). Au cours de la dernière décennie, l'induction de la biosynthèse de métabolites fongiques par co-culture a été largement explorée. Par exemple, la mise en co-culture de deux endophytes fongiques *Cophinforma mamane* et *Fusarium solani* a permis l'induction de la production de cinq composés *de novo* non présents en mono-culture (Barakat et al., 2016). De plus, la co-culture d'un autre système endophyte-endophyte a également permis une diversification dans la production chimique de nouvelles perylenequinones (Bazioli et al., 2020). Ces deux exemples démontrent l'intérêt de l'étude de ces interactions dans le cadre de la recherche de nouveaux composés potentiellement bioactifs.

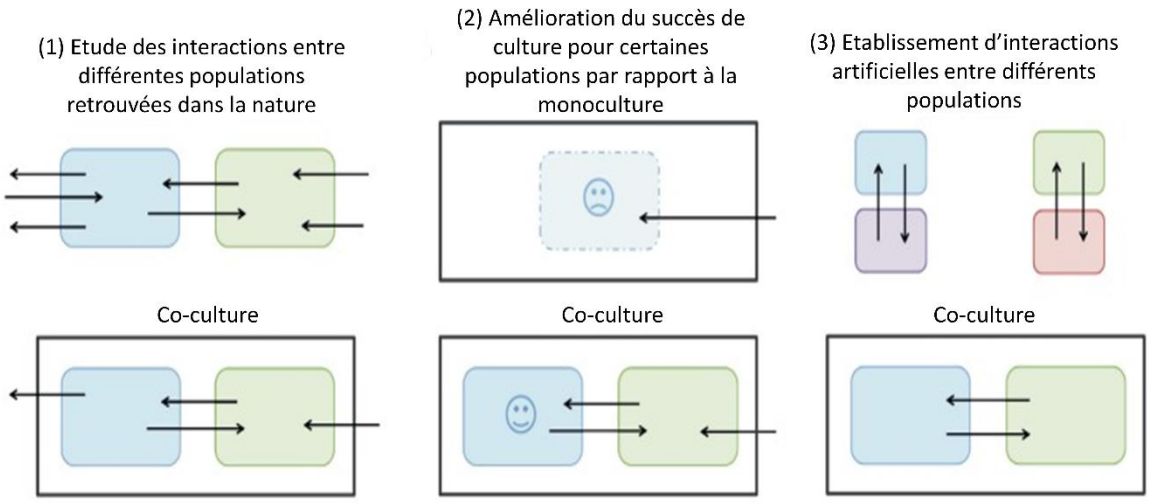


Figure 5 Définition et intérêt de la co-culture. 1) étudier les interactions naturelles entre les populations ; 2) améliorer la croissance des cultures pour certaines populations et 3) établissement d'interactions « artificielles » entre les populations (Goers et al., 2014).

ii. Etude préliminaire : méthodologie et sélection des paramètres d'études mycochimiques

Dans le cadre de nos travaux, la valorisation de la souchothèque fongique a nécessité différentes étapes allant de la sélection de souches d'intérêt à la valorisation des composés isolés. Chacune de ces étapes a représenté un choix stratégique (Fig. 6). La première étape, le choix des souches fongiques étudiées, s'est basée sur une observation et une étude bibliographique des différents champignons impliqués dans des confrontations. Une fois ce choix fait, il a été question de multiplier les conditions de culture selon une approche OSMAC puis d'étudier les profils chimiques obtenus pour chacune des conditions. Après cette étude préliminaire, champignons et conditions de culture ont été sélectionnés pour une mise en culture à plus grande échelle afin d'obtenir suffisamment d'extrait brut pour les étapes de fractionnement et de purification en vue d'une caractérisation chimique des molécules isolées originales et/ou bioactives. Le choix des conditions d'extraction représente également une étape cruciale dans le sens où une extraction optimisée permettra non seulement un meilleur rendement d'extraction (Bringmann et al., 2007) mais également la sélection des composés produits par les champignons plutôt que la sélection des constituants du milieu. Une fois le fractionnement réalisé l'activité antibactérienne, pour mimer une potentielle activité antimicrobienne, de certaines fractions ont été testées de manière à purifier en priorité les fractions bioactives. Enfin, une fois les molécules isolées, leur activité biologique peut être testée pour une potentielle valorisation.

Figure 6 « Workflow » suivi pour l'étude chimique des champignons lichéniques

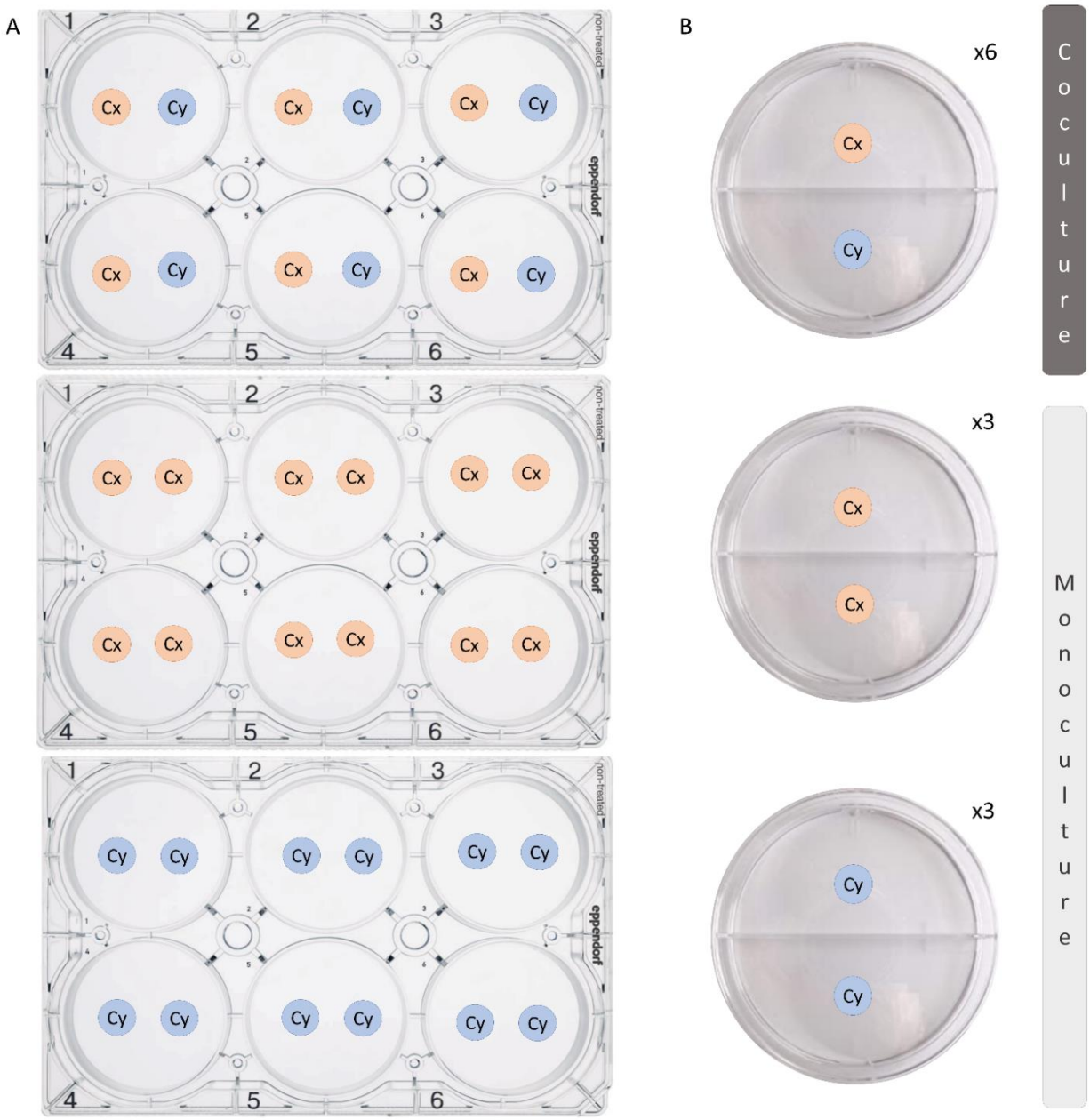
1. Choix des paramètres de culture (champignons, milieux, support)

Au cours des étapes d'isolement que nous avons décrites dans le second chapitre de ce manuscrit (p. 45), cinq interactions par effet d'antibiose entre deux couples de champignons ont pu être observées (Tab. 1). Dans le cadre des essais OSMAC, les champignons impliqués dans une zone d'antibiose ont été déposés sur des milieux de compositions différentes comme indiqués dans la figure 7 parmi les onze milieux décrits préalablement. Ces milieux ont été choisis selon la capacité des couples à y croitre lors de premiers essais réalisés sur des boites de Petri classique.

Tableau 1 Identification des dix champignons interagissant deux à deux par antibiose

Codes	BLAST et identification phylogénétique	N° d'accession
C4[1]	*Dendrothyrium variisporum*	OL891605
C5[1]	*Xylaria hypoxylon*	OL891603
C9[2]	*Coccinonectria rusci*	OL891609
C11[2]	*Melanconium hedericola*	OL891612
C21[3]	Undet. Pleosporales (2)	OL891622
C22[3]	*Penicillium cavernicola*	OL891623
C44[4]	*Microdochium phragmitis*	OL891632
C45[4]	*Tolypocladium sp. (2)*	OL891615
C63a[5]	*Alternaria sp. (2)*	OL891634
C63b[5]	*Penicillium roseoviride*	OL891619

Pour ces essais de modulation du métabolome par la méthode OSMAC, les mono- et les co-cultures ont ensuite été réalisées dans des plaques 6 puits (Fig. 7) (Bertrand et al., 2014a) ainsi que sur des boîtes de Petri de 9 cm de diamètre à 2 compartiments, séparant ainsi par une partie rigide la gélose en deux mais permettant de partager un milieu aérien commun, (Fig. 7) de manière à observer si les composés organiques volatils peuvent également avoir un effet sur le métabolome. Ces champignons ont été cultivés pendant 14 jours à température ambiante et les essais ont été réalisés en 6 réplicas en mono- comme en co-culture pour les cinq couples de champignons et leurs milieux respectifs. Au final, en éliminant les conditions ne permettant pas une croissance correcte des champignons et après une extraction à partir des géloses et des mycéliums fongiques en mono et co-culture et également de la zone de confrontation de manière isolée, 585 extraits ont été préparés et analysés.



Couples	Milieux de mise en culture à petite échelle	
	Plaque 6 puits	*Boîte compartimentée*
C4-C5	YS	MEP et YS
C9-C11	GY, ISP2 et TY	GY, ISP2 et TY
C21-C22	GYM et ISP2	ISP2
C44-C45	ISP2, MYP et YS	ISP2, MYP et YS
C63a-C63b	ISP2 et TY	ISP2 et TY

Extraits analysés par HPLC

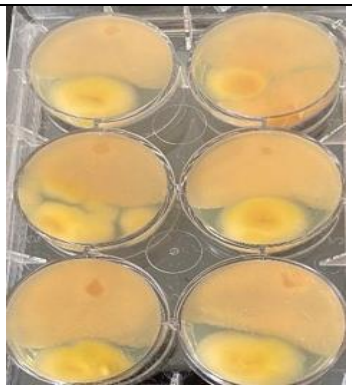
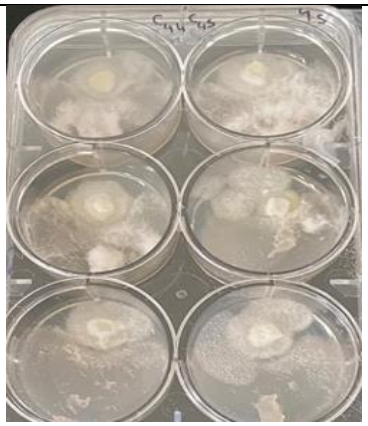
Couples	Milieux de mise en culture à grande échelle
C4-C5	YS
C9-C11	ISP2
C44-C45	YS

Figure 7 Méthodologie mise en place pour l'étude des co-cultures de 5 couples de champignons sur (A) plaque 6 puits (P6P) et (B) boîte de Petri compartimentée (BS) en replicas (x3) ou (x6) et selon les milieux adaptés aux couples de champignons

2. Observation phénotypique et profilage métabolique des extraits par HPLC-DAD

Une fois la mise en culture sur 14 jours réalisée, il a été intéressant d'observer une variation du phénotype des champignons selon les milieux de culture utilisés en particulier pour le couple C44-C45, où la couleur du mycellium variait en fonction des milieux de culture (Tab.2). Néanmoins la zone d'interaction est toujours visible.

Tableau 2 Différences phénotypiques des champignons C44-C45 en fonction du milieu de culture (ISP2, YS et MYP).

Milieu	Recto co-culture C44-45	Verso co-culture C44-45
ISP2		
YS		
MYP		

Pour les cultures en boites de Petri compartimentées, du fait d'une vitesse de croissance différente entre les champignons d'un même couple, nous avons pu constater un passage du mycélium de certains champignons dans le compartiment voisin avant même la fin de la durée d'incubation de 14 jours des cultures (Fig. 8).

Figure 8 Culture de C44 et C45 en boîte compartimentée à t = 10 jours, débordement de C44 sur le compartiment de C45

L'intégralité des extraits bruts ont été injectés en HPLC-DAD à une concentration de 2 mg/mL. Les profils UV à λ 254 nm des six réplicas de chaque mono- et co-culture ont été comparés entre eux et avec un blanc correspondant au milieu respectif et ont montré une bonne reproductibilité comme nous pouvons le voir sur la figure 9 dans laquelle sont reportés trois exemples de comparaison obtenus. L'observation à d'autres longueurs d'onde des extraits a permis d'indiquer que la longueur d'onde à 254 nm était celle pour laquelle nous pouvions observer le plus de pics.

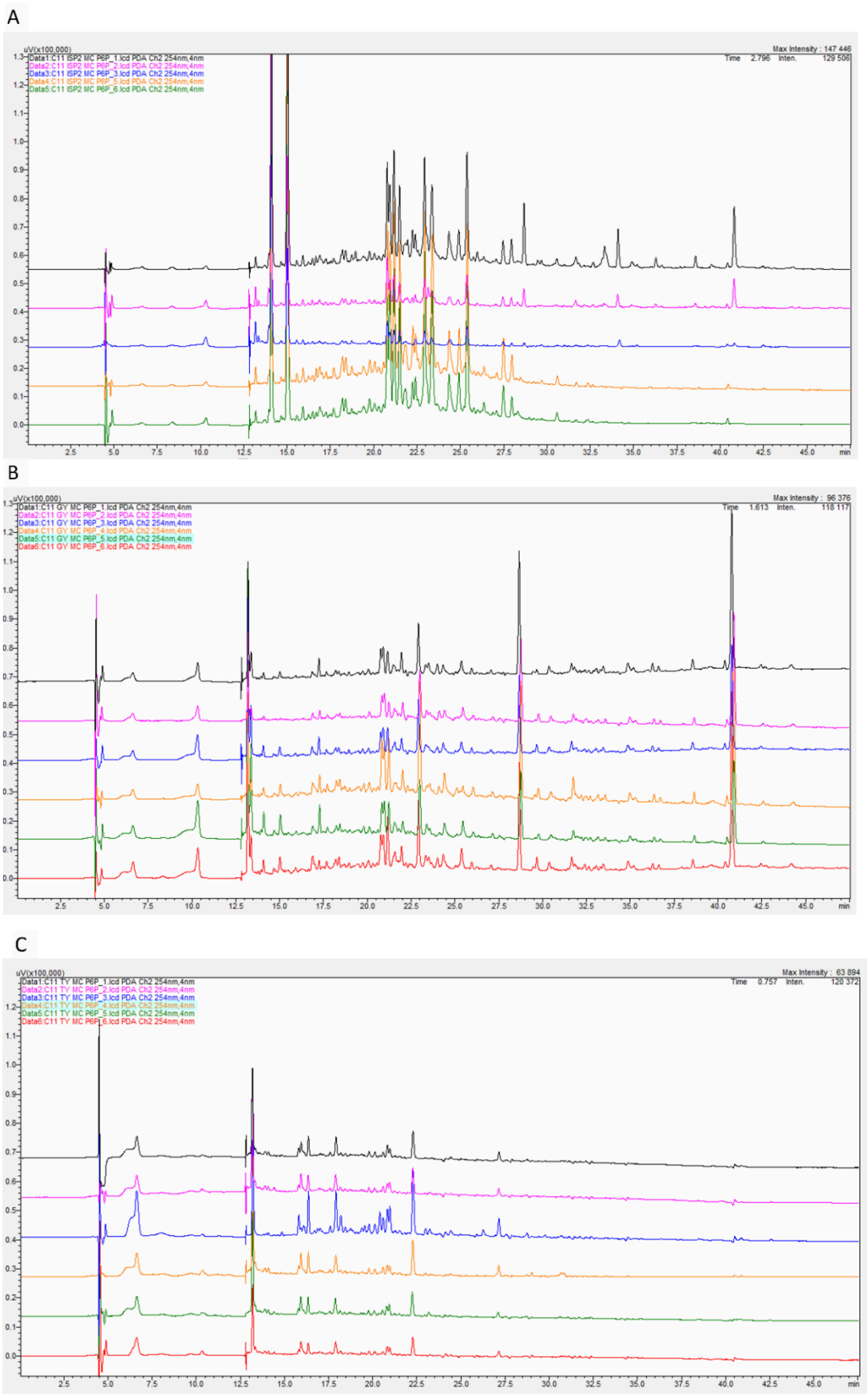


Figure 9 Chromatogrammes obtenus par HPLC-DAD analytique (observation à λ 254 nm) des réplicas de co-culture en plaque 6 puits pour A) C11 sur le milieu ISP2 ; B) C11 sur le milieu GY et C) C11 sur milieu TY.

3. Choix des couples à étudier pour l'isolement de métabolites actifs, de leurs milieux de culture et de leur support

Une première discrimination sur la base des analyses des chromatogrammes HPLC-DAD observés à λ 254 nm a permis d'écarter certains couples du fait d'une production métabolique de base très riche et rendant difficile la lecture des chromatogrammes comme pour le couple C21-C22 sur le milieu GYM (Fig. 10). De même les cultures sur boite à deux compartiments n'ont finalement pas été sélectionnées du fait du peu d'intérêt en termes de production métabolique (pas d'induction de composés supplémentaires) mais également du fait de la croissance plus rapide et plus aisé de certains champignons débordant dans le compartiment voisin (Fig. 8).

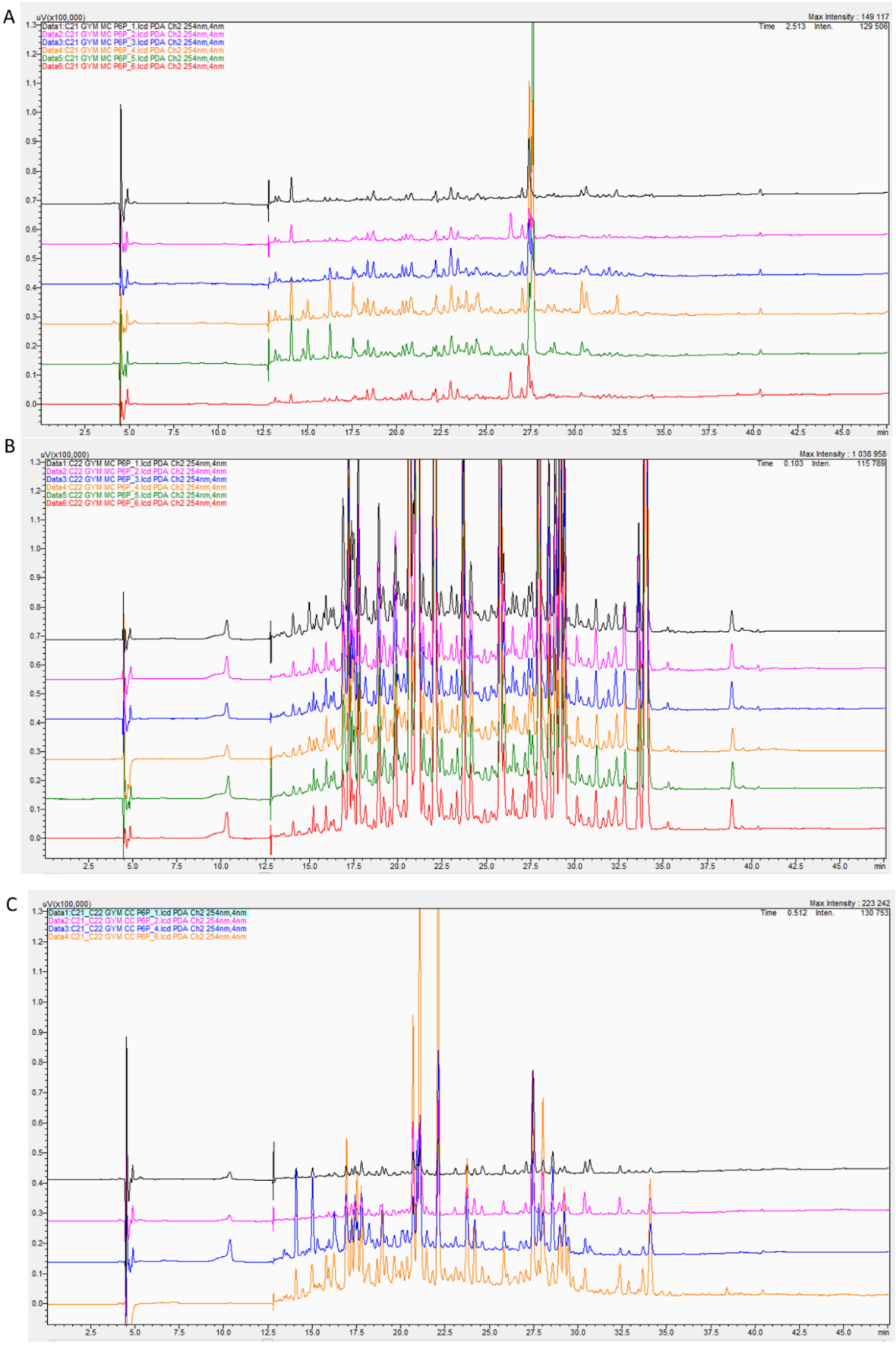


Figure 10 Chromatogrammes des 6 réplicas en boîte 6 puits de : A) monoculture de C21 ; B) monoculture de C22 et C) co-culture de C21-C22.

L'apparition de nouveaux pics sur les chromatogrammes (Fig. 11), après comparaison des données obtenues entre les mono-cultures, co-cultures et zones de confrontation obtenues sur plaques 6 puits, ont permis de sélectionner trois couples de champignons à cultiver sur un milieu donné pour des cultures à plus grande échelle. Les milieux et les couples finalement conservés pour la suite de l'étude sont les milieux ISP2 pour le couple C9-C11 et YS pour les couples C4-C5 et C44-C45 (Fig. 11).

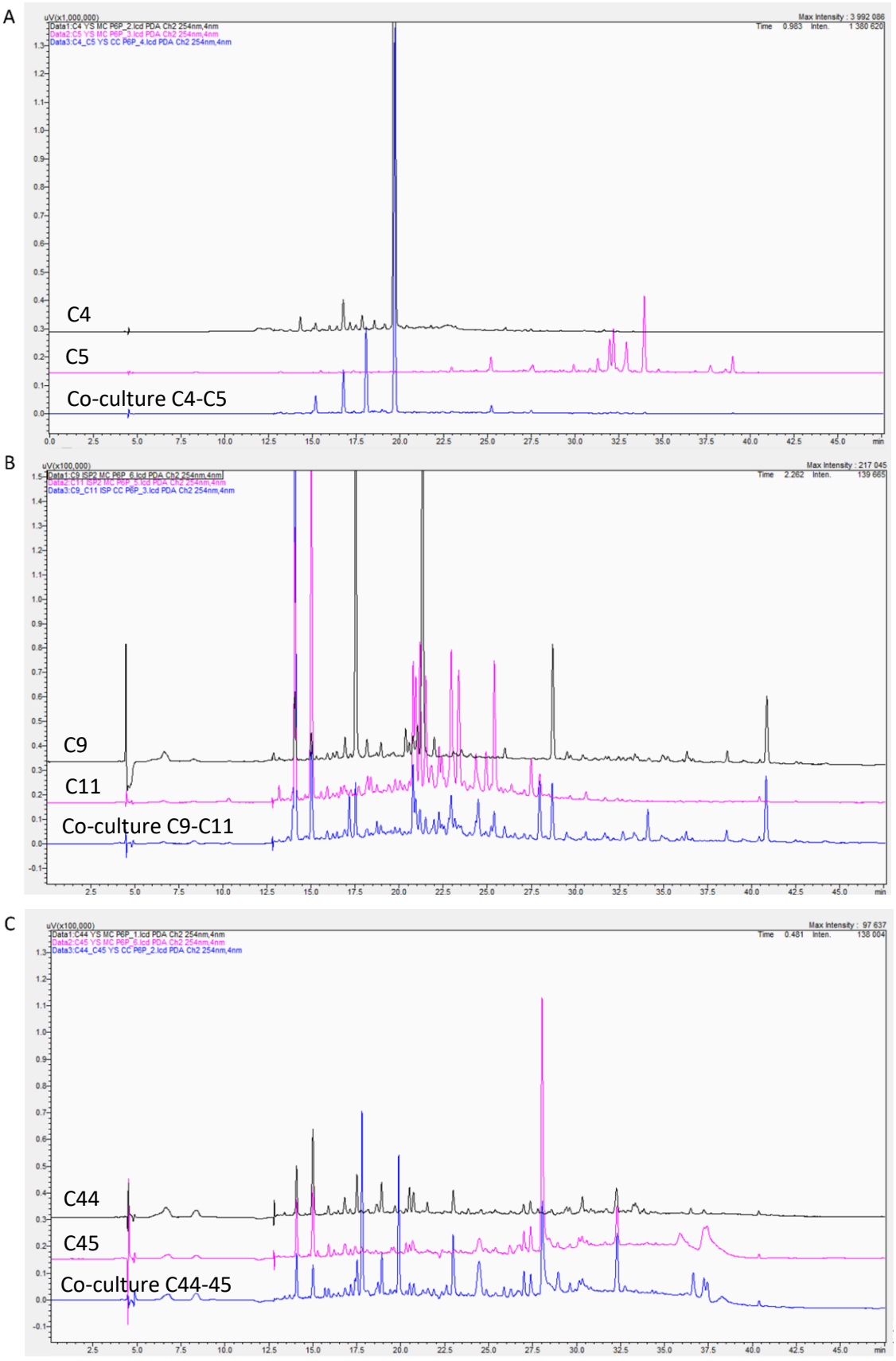


Figure 11 Comparaison des chromatogrammes obtenus par HPLC-DAD analytique (observation à λ 254 nm) entre les mono- et co-cultures en plaque 6 puits pour A) C4-C5 sur le milieu YS ; B) C9-C11 sur le milieu ISP2 et C) C44-C45 sur milieu YS.

iii. Etude approfondie de trois co-cultures fongiques

1. Etude mycochimique du couple *D. variisporum* (C4) et *X. hypoxylon* (C5)

A. Etat de l'art bibliographique

Comme souligné dans une revue (Song et al., 2014), le genre *Xylaria* contient un grand nombre d'espèces productrices de composés présentant des structures chimiques variées dont certains sont bioactifs. Des dérivés de type cytochalasines, dérivés naphtalènes ou pyrones ont ainsi été isolés de *X. hypoxylon*. De plus, des études plus récentes ont révélé que des extraits bruts obtenus à partir de la culture de ce champignon présentaient des activités antimicrobiens contre les bactéries GRAM + (Canli et al., 2016; Özbey et al., 2021).

Concernant les études mycochimiques déjà réalisées sur *D. variisporum*, l'équipe de Stadler a pu isoler douze métabolites : trois dicétopipérazines, cinq dérivés anthraniliques, deux dérivés furanones et deux dérivés massarilactones (Teponno et al., 2017). Parmi ces douze métabolites, deux d'entre eux, des dérivés anthraniliques dont les structures sont indiquées dans la figure 12, ont montré des activités antimicrobiennes à large spectre contre le champignon *Mucor hiemalis* DSM 2656, la levure *Rhodoturula glutinis* DSM 10134, les bactéries *Bacillus subtilis* DSM 10 et *S. aureus* DSM 346 avec des concentrations minimales inhibitrices (CMI) comprises entre 16.33 et 66.67 µg/mL.

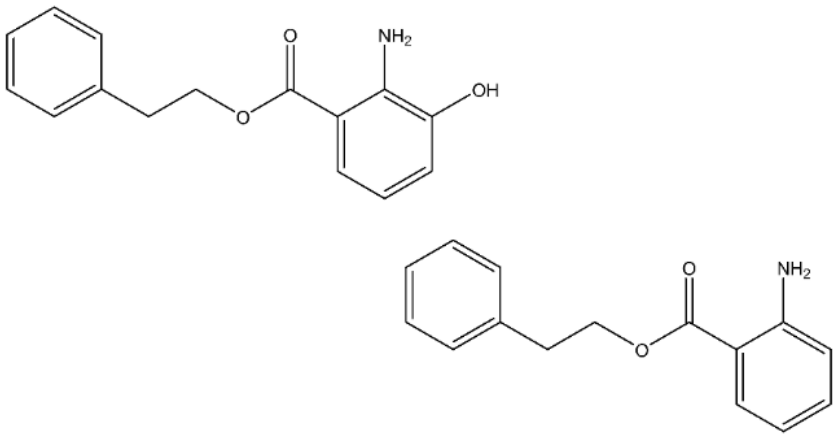


Figure 12 Structures des composés anthraniliques à acitivté antimicrobienne isolés à partir d'une culture de D. variisporum (Teponno et al., 2017)

Les résultats de ces différentes études nous ont confortés dans le choix de ces deux champignons en co-culture pour les essais d'induction de la production ou de surexpression de certains métabolites antimicrobiens.

B. Résultats

Afin de mener l'étude chimique de ces deux champignons en mono et co-culture (Fig. 13), nous avons réalisé une succession de trois séries de culture à plus grande échelle sur, respectivement 40 puis 100 et enfin 350 boîtes. Les résultats sont présentés ci-dessous.

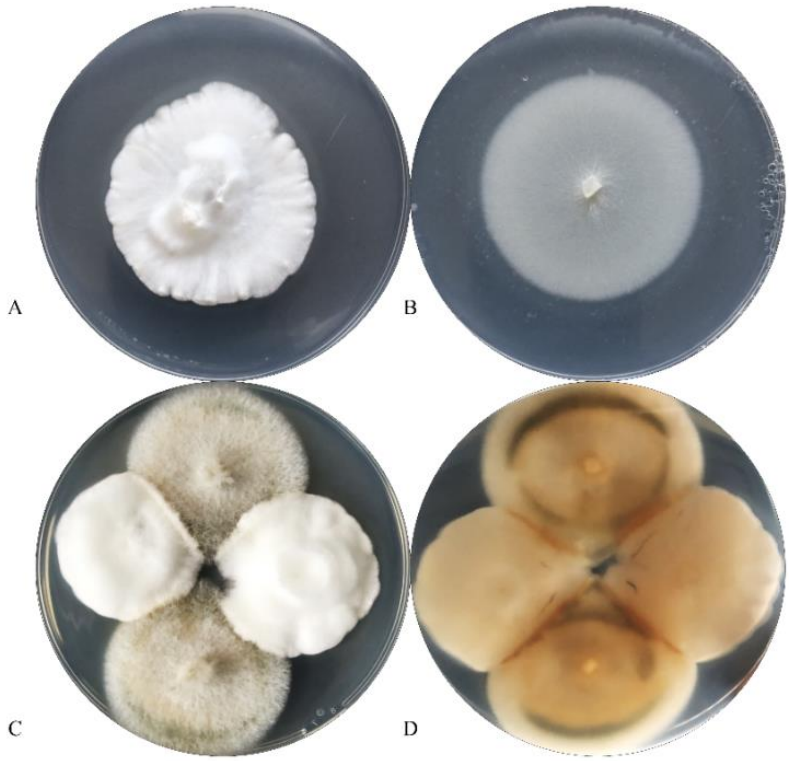


Figure 13 A) X. hypoxylon *(C5) en mono-culture. (B)* D. variisporum *(C4) en mono-culture. (C) vue supérieure de* X. hypoxylon *et* D. variisporum *en co-culture. (D) vue inférieure de* X. hypoxylon *et* D. variisporum *en co-culture*

a. Analyses des extraits obtenus

L'extraction de la première série de culture à plus grande échelle (20 boîtes en mono-culture ; 40 boîtes de co-culture avec extraction spécifque de la zone de confrontation (ZC) comme la deuxième série de culture (100 boîtes de co-culture) ont été réalisées avec le mélange de solvants suivant MeOH:CH_2Cl_2:AcOEt 1:2:3 + 0.1% d'AF . Suite à cette

extraction solide/liquide et après évaporation à sec, l'extrait brut obtenu est resolubilisé puis soumis à une partition liquide/liquide H_2O:CH_2Cl_2 (1:3 v/v) a été réalisée. L'opération d'extraction liquide/liquide a été répétée trois fois est les phases aqueuses et organiques ont été regroupées indépendamment puis évaporées sous vide donnant deux extraits secs, l'un aqueux et l'autre organique. Les quantités d'extraits obtenus sont répertoriées dans le tableau 3 ci-dessous.

Tableau 3 Quantités d'extraits (en mg) obtenues selon les conditions de culture pour C4 -C5.

	Extraits	**Nombre de boîtes**	**Quantité obtenue (mg)**
Phase organique	C4_MC	20	1.9
	C5_MC	20	2.4
	C4-C5_CC	40	44
	C4-C5_ZC	40	4.4
	C4-C5_CC	100	236.7
	C4-C5_CC	350	1500
Phase aqueuse	C4_MC	20	1.5
	C5_MC	20	9.5
	C4-C5_CC	40	63.9
	C4-C5_ZC	40	33
	C4-C5_CC	100	97

L'analyse en HPLC-DAD des extraits préparés à 2 mg/mL dans l'acétone a permis de de confirmer les résultats obtenus en plaque 6 puits (Fig. 14) et de mettre en évidence la présence de composés nouvellement induits en co-culture à 18.2 min et à 23.2 min (Fig. 15).

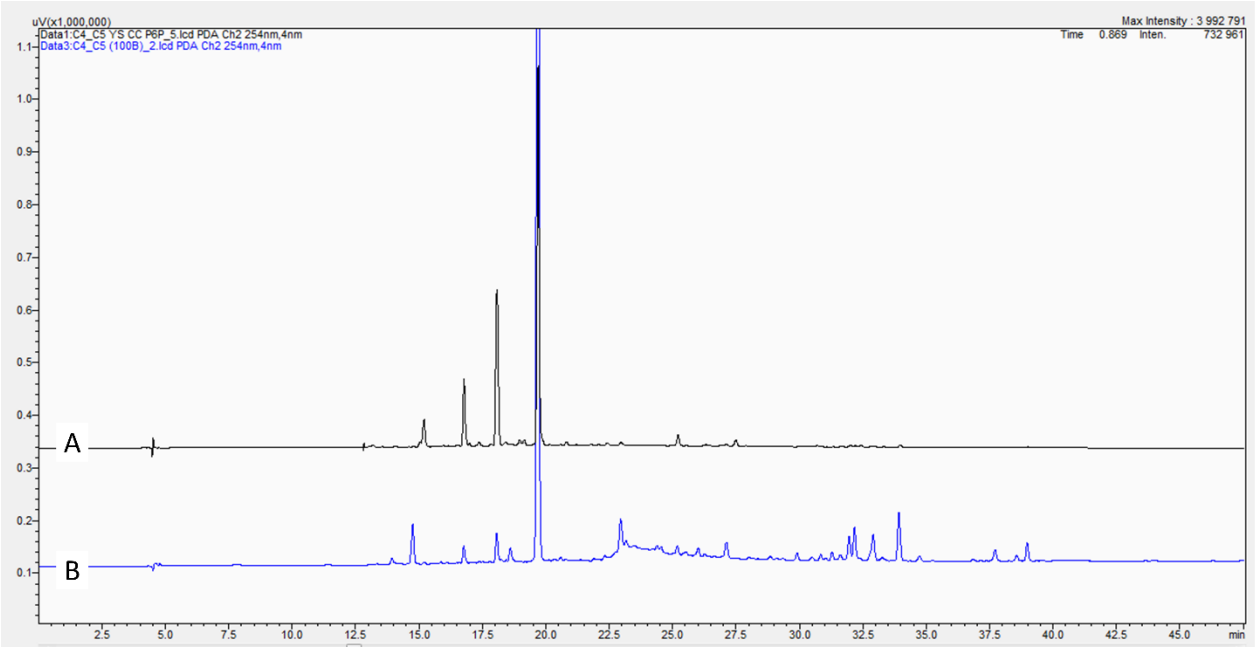


Figure 14 Comparaison des chromatogrammes en fonction du support de culture : A) co-culture C4-C5 en plaque 6 puits et B) co-culture de C4-C5 en boîtes de Petri classique à grande échelle (100 boîtes)

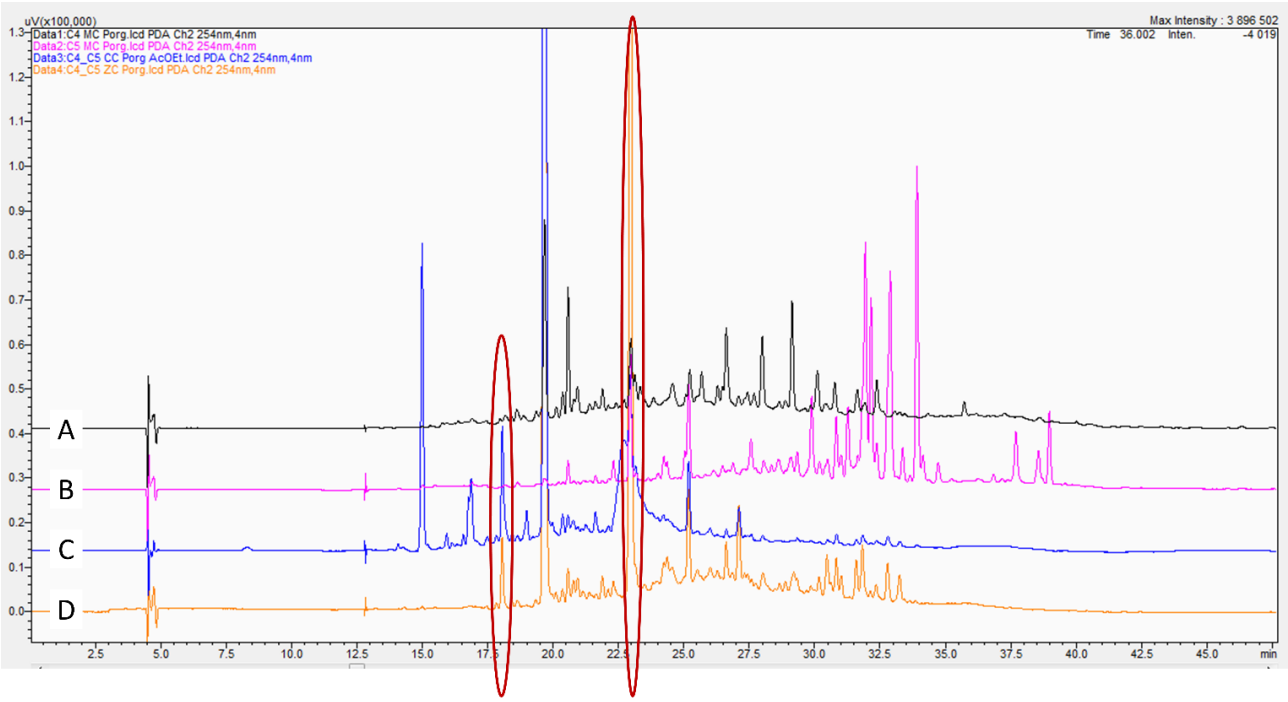


Figure 15 Comparaison des chromatogrammes obtenus par HPLC analytique (observation à λ 254 nm) entre les mono- (20 boîtes) et co-cultures (40 boîtes) A) C4 monoculture ; B) C5 monoculture, C) C4-C5 coculture et D) zone de confrontation de la coculture C4-C5

b. Fractionnement de deux séries de co-culture et isolement de composés purs

Les schémas de fractionnement et de purification des différentes séries de co-culture du couple C4-C5 sont présentés dans la figure suivante (Fig. 16).

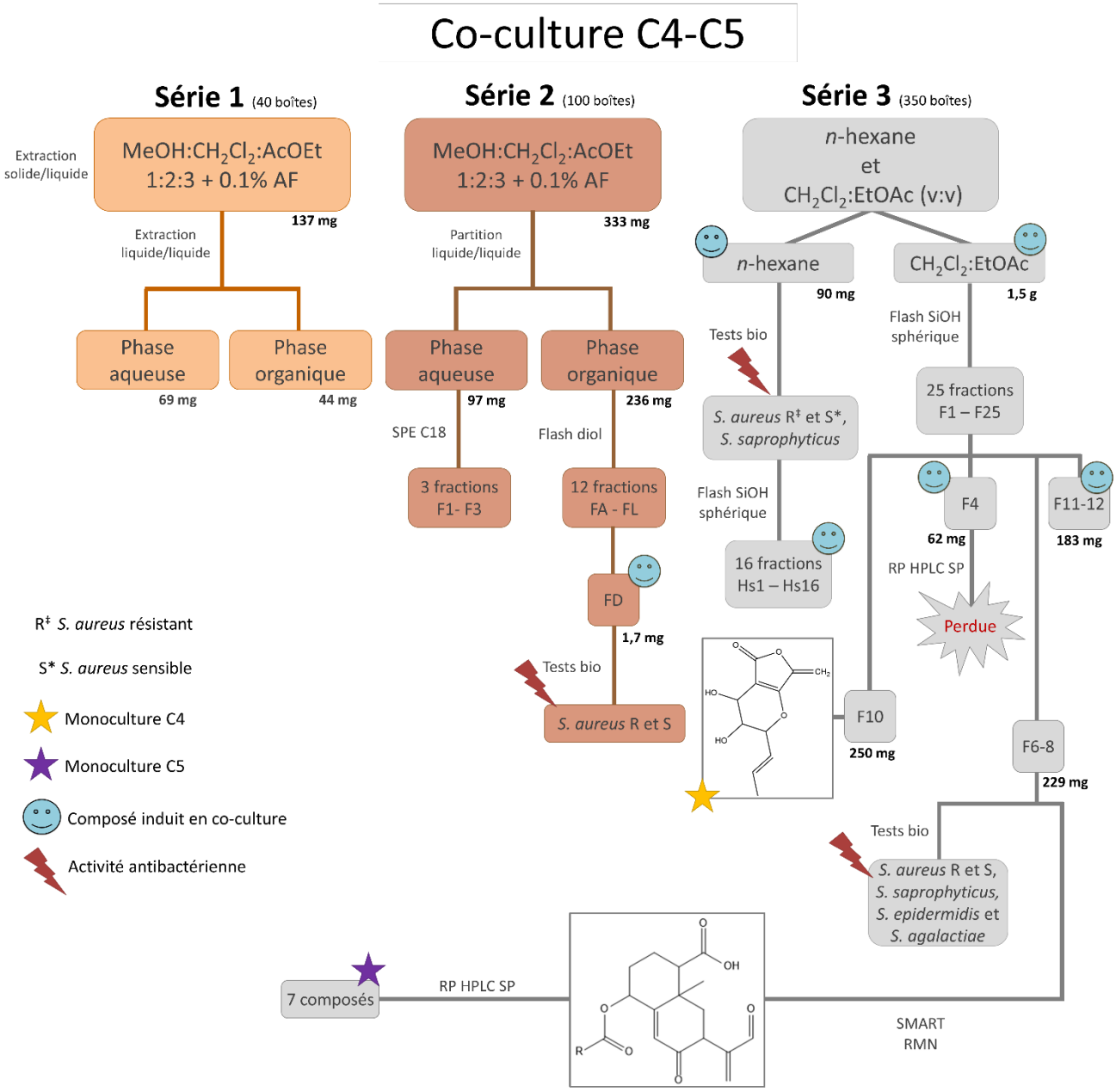


Figure 16 Fractionnement et purification des extraits bruts de la co-culture C4-C5 et évaluation biologique de certaines fractions

Du fait des trop faibles quantités des extraits obtenus à partir de la co-culture sur 40 boites, les étapes de fractionnements et de purification n'ont pas été menées sur cette première série.

- *Fractionnement et isolement de composés spécifiquement induits par la co-culture*

L'extrait brut organique obtenu à partir de 100 boites de co-cultures (m = 236 mg) et après extraction liquide/liquide, a été fractionné par chromatographie de type flash à l'aide d'une cartouche Flash Diol 12 g avec une phase mobile composée de *n*-hexane, CH_2Cl_2, AcOEt et MeOH selon le gradient 1 (cf materiels et méthodes p. 169). 12 fractions (FA-FL) ont été récoltées et certaines d'entre elles ont été en parallèle testées vis-à-vis de bactéries pathogènes humaines (Fig. 16). La fraction contenant le composé d'intérêt à 23.2 min, fraction FD (1.67 mg), en mélange, s'est révélée être active vis-à-vis de deux souches de *S. aureus* sensible et résistante (Tab. 4) et a confirmé l'intérêt de ce composé induit dans la co-culture comme antimicrobien.

Tableau 4 Résultats de la CMI (concentration minimale inhibitrice en µg/mL) de la fraction FD sur deux souches de S. aureus.

	S. aureus **(CIP 53.156)**	***S. aureus*** **resistant (DSM 13661)**
Fraction FD	25,0	50,0
DMSO	-	-
Gentamicine	3,9	>250

- : non actif

Une culture à grande échelle sur 350 boites a ensuite été entreprise dans les mêmes conditions de culture (à savoir sur YS pendant 14 jours) afin d'obtenir suffisamment d'extrait brut pour réaliser et faciliter l'isolement des composés d'intérêt, leur élucidation structurale et les essais biologiques. Pour faciliter le fractionnement, des essais d'extraction avec des solvants moins polaires, *n*-hexane ou mélange CH_2Cl_2:AcOEt (v:v), ont été réalisés. L'extraction solide/liquide a d'abord été faite deux fois avec du *n*-hexane sur la totalité des boîtes puis deux nouvelles fois avec un mélange CH_2Cl_2:AcOEt (v:v). En

éliminant le méthanol dans la procédure d'extraction, le but visé a été de ne pas extraire les composés trop polaires provenant du milieu pour ainsi éviter une étape supplémentaire de partition liquide/liquide (eau/phase organique) (Fig. 16). Les quantités d'extraits obtenus sont répertoriées dans le tableau suivant (Tab. 5).

Tableau 5 Quantités d'extraits (en mg) obtenues pour la troisième série de co-culture C4 -C5.

Extraits organiques	Série	Quantité obtenue (mg)
C4-C5_CC *n*-hexane	3 : 350 boîtes	90
C4-C5_CC AcOEt:CH_2Cl_2 (v:v)	3 : 350 boîtes	1500

L'analyse et la comparaison des chromatogrammes en HPLC-DAD montre le pic d'intérêt à 23.2 min présent dans la fraction hexanique (Fig. 17).

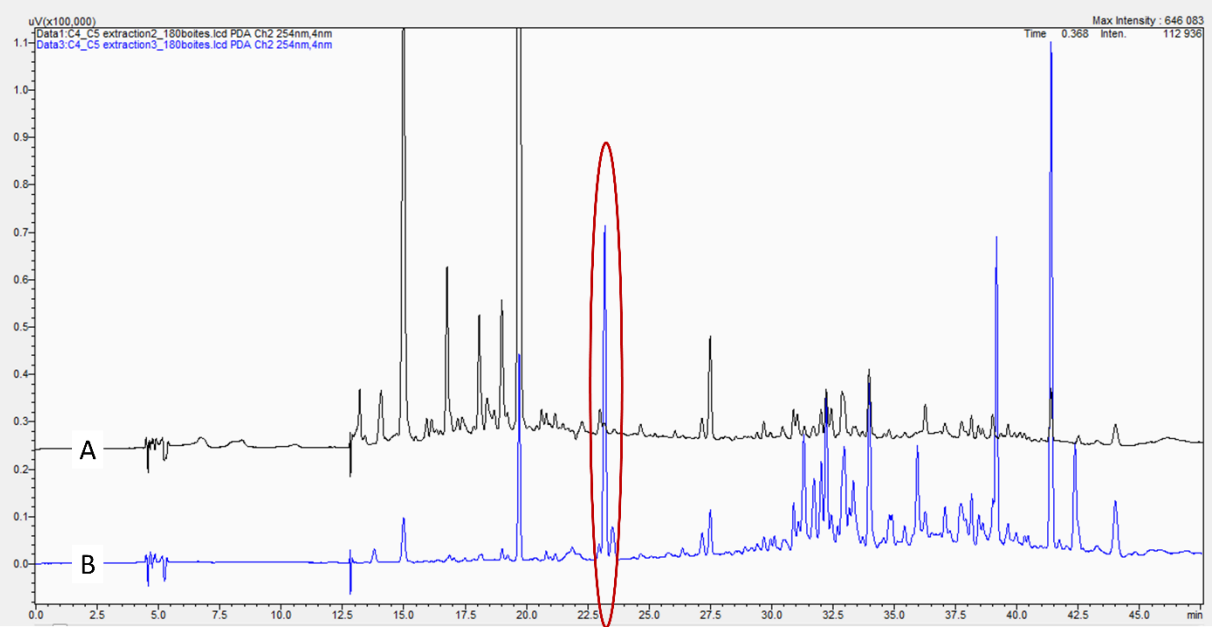


Figure 17 Comparaison des chromatogrammes obtenus par HPLC analytique (observation à λ 254 nm) entre les différents extraits de la série 3 de coculture A) extraction AcOEt:CH2Cl2 (v:v) et B) extraction n-hexane

L'extrait hexanique, moins riche, mais contenant le composé d'intérêt a d'abord été traité par chromatographie de type flash à l'aide d'une cartouche flash de silice sphérique selon le gradient 5 détaillé dans la partie matériels et méthodes (cf p. 170) et a permis d'obtenir 16 fractions (Hs1 – Hs16). Là encore l'activité antibactérienne significative vis-à-vis de *S. aureus* (CMI = 25 µg/mL) et *S. saprophyticus* (CMI = 50 µg/mL) validait l'intérêt d'étudier cette fraction. Malheureusement, le fractionnement n'a pas été probant et la

fraction d'intérêt Hs5 (7.2 mg) étant trop riche nous avons décidé d'isoler ce composé d'intérêt à partir de l'extrait AcOEt:CH_2Cl_2.

1.5 g de l'extrait brut organique de cette troisième série de co-culture a donc été fractionné (Fig. 16) sur une colonne Chromabond Flash RS 25 SiOH avec une phase mobile composée de cyclohexane, AcOEt, MeOH et H_2O selon le gradient 4 pour obtenir 25 fractions de F1 à F25. La fraction F10 (249,6 mg) étant la seule à être pure après fractionnement a été directement analysée en RMN. La fraction F4 (62,2 mg) contenant le composé d'intérêt à 23,2 min (identique à FD et Hs5) (Fig. 18) a été ensuite purifiée par HPLC semi-préparative en phase inverse (cf protocole 1 p.171) pour l'isolement du composé d'intérêt. Une fois l'optimisation pour la purification de la fraction F4 effectuée, le collecteur de fractions présentant un dysfonctionnement n'a pas correctement collecté les fractions nous faisant ainsi perdre l'intégralité de la fonction d'intérêt.

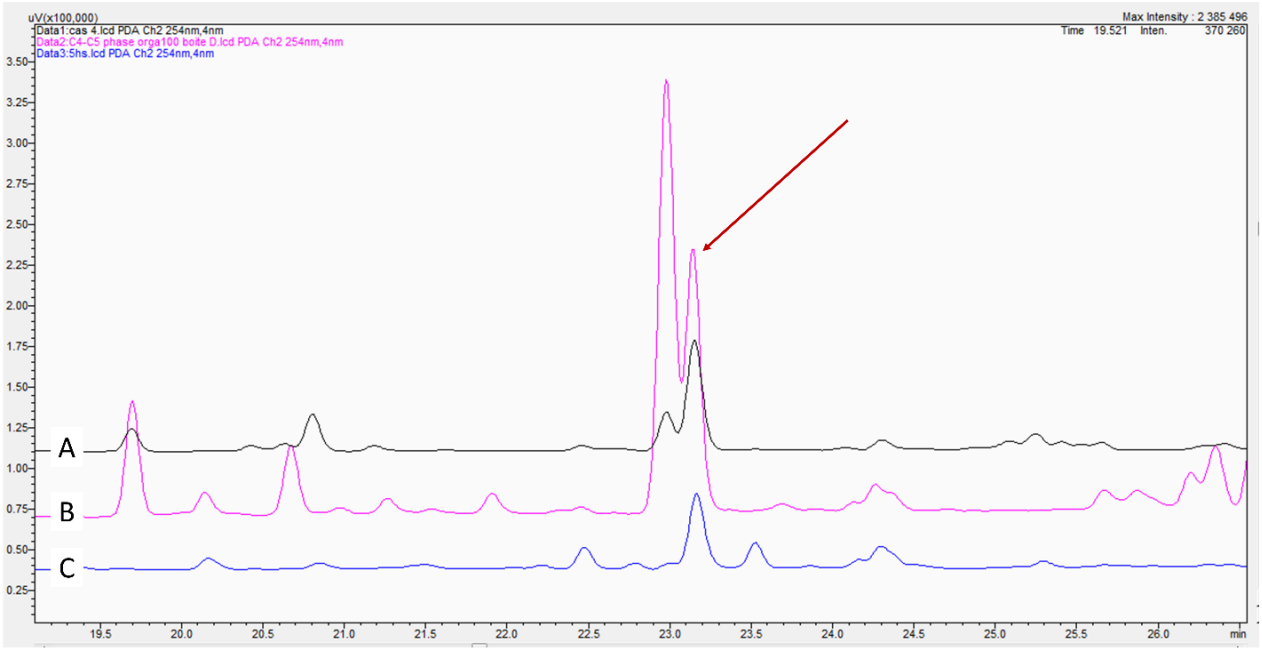


Figure 18 Chromatogrammes obtenus par HPLC analytique (observation à λ 254 nm) A) fraction F4; B) fraction D de l'extrait MeOH:CH2Cl2:AcOEt 1:2:3 + 0.1% d'AF de la série 2 et C) fraction Hs5 de l'extrait hexanique de la troisième série

Les fractions F11 et F12 ont quant à elles été regroupées d'après la comparaison de leurs chromatogrammes (Fig. 19). Le composé d'intérêt induit en co-culture et élué vers 18 min y est visible. La purification de cette fraction va prochainement être réalisé.

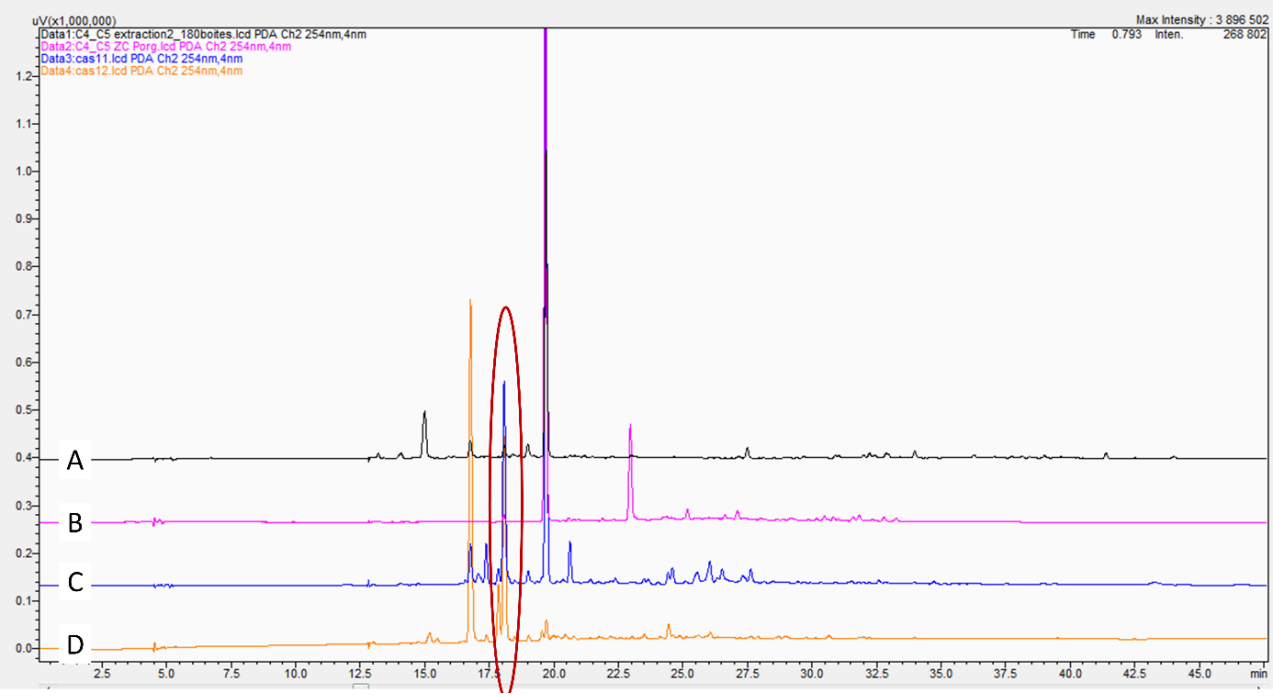


Figure 19 Comparaison des chromatogrammes : A) extrait CH2Cl2:AcOEt (v:v) de la co-culture C-C5 ; B) extrait CH2Cl2:AcOEt (v:v) de la zone de confrontation de la co-culture de C4-C5 ; C) fraction F11 et D) fraction F12

- *Fractionnement bioguidé*

Afin de valoriser malgré tout le lourd travail réalisé jusqu'à ces étapes de purification, les fractions F6-F8 présentant une activité antibactérienne intéressante (CMI entre 3,1 et 25 µg/mL) vis-à-vis de différentes bactéries GRAM + (Tab. 6) et ayant des profils UV similaires (Fig. 19) ont été combinées avec la fraction F7 (229,2 mg).

Tableau 6 Résultats de la CMI (µg/mL) de la fraction hexane et des fractions F6 et F8 sur différentes bactéries GRAM +.

	S. aureus **(CIP 53.156)**	***S. aureus*** **resistant (DSM 13661)**	***S. epidermidis*** **(CIP 53.124)**	***S. saprophyticus***	***S. agalactiae***
Fraction F6	3,1	50,0	25,0	25,0	-
Fraction F8	6,25	12,5	12,5	12,5	25,0
DMSO	-	-	-	-	-
Gentamicine	3,9	>250	>250	0,5	0,12

-: non active

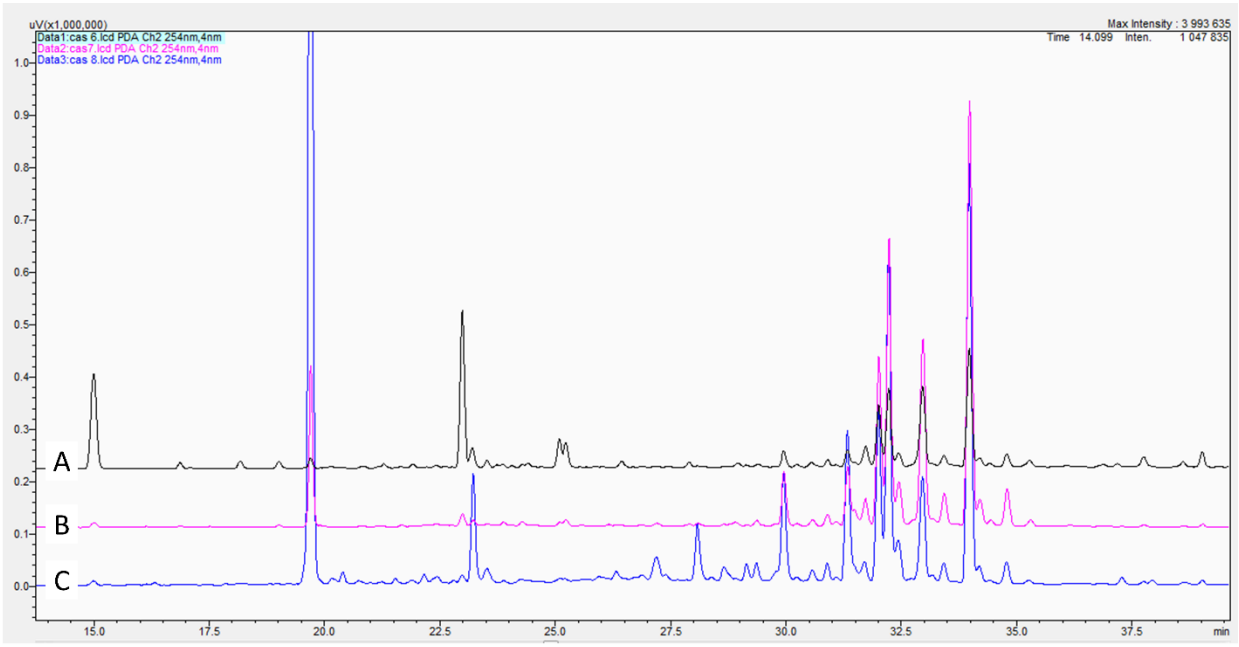


Figure 20 Chromatogrammes obtenus par HPLC analytique (observation à λ 254 nm) A) fraction F6; B) fraction F7 et C) fraction F8

L'extrait brut de la combinaison de ces trois fractions a été analysé en HPLC-ESI-MS. Les spectres de masse ont montré un fragment commun (*m/z* 261 et 215) (Fig. 21), nous faisant suspecter une série de molécules avec un même squelette.

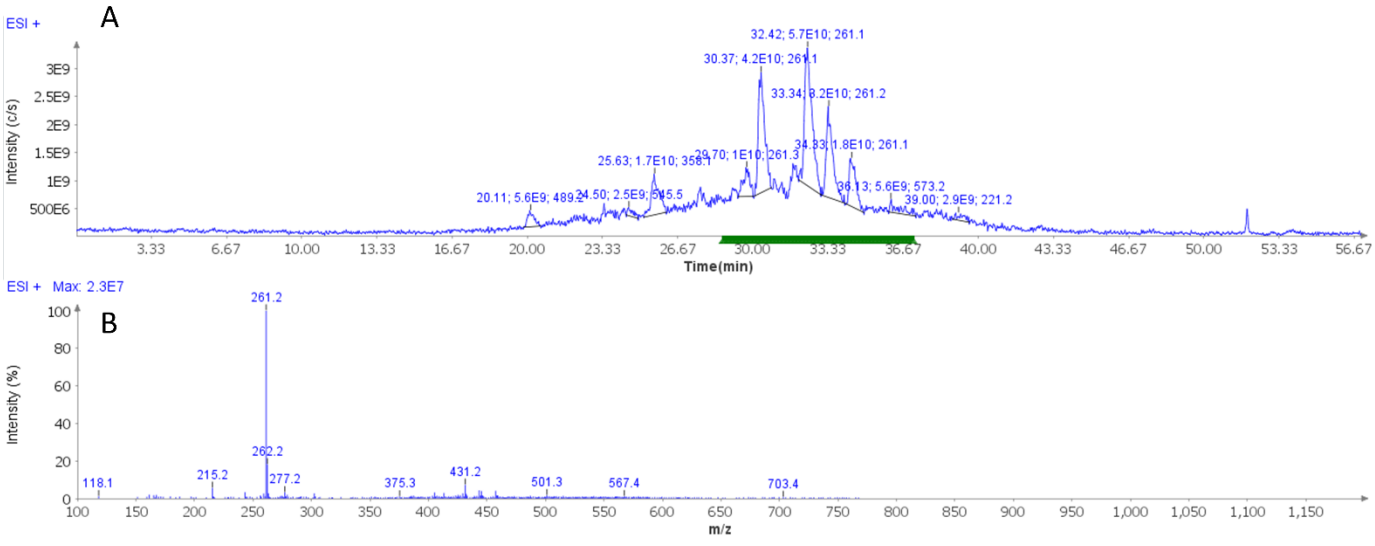


Figure 21 Analyse en HPLC-ESI-MS en mode positif de l'extrait brut de la fraction F6-8. A) chromatogramme de la fraction F6-8 et B) sprectre de masse

Le sepctre par RMN 2D de type de HSQC (Heteronuclear Single Quantum Correlation) de ce brut a été analysé par SMART : Small Molecule Accurate Recognition Technology, un outil basé sur l'intelligence artificielle pour générer des hypothèses de structures à partir de données RMN (Reher et al., 2020). Les trois premiers hits (cosine compris entre 85 et 88 %, score qui correspond à une haute similarité des spectres avec celui de l'extrait brut) comprennent un noyau érémophilane similaire à celui de l'acide intégrique (Singh et al., 1999), pointant cette fraction comme une très bonne candidate à une purification en vue d'obtenir de nouvelles molécules avec un squelette érémophilane. On a ainsi pu isoler, selon le protocole de purification 2 (cf p.171) par HPLC semi-préparative enphase inverse, sept nouvelles molécules sur lesquelles l'effort d'élucidation s'est concentré sur les variations de la chaîne latérale : **1** (5,33 mg, T_r 30,19 min), **2** (1,06 mg, T_r 31,7 min), **3** (20,03 mg, T_r 32,24 min), **4** (6,02 mg, T_r 32,37 min), **5** (1,39 mg, T_r 33,1), **6** (6,68 mg, T_r 33,31 min) et **7** (0,81 mg, T_r 34,05 min) (Fig. 22).

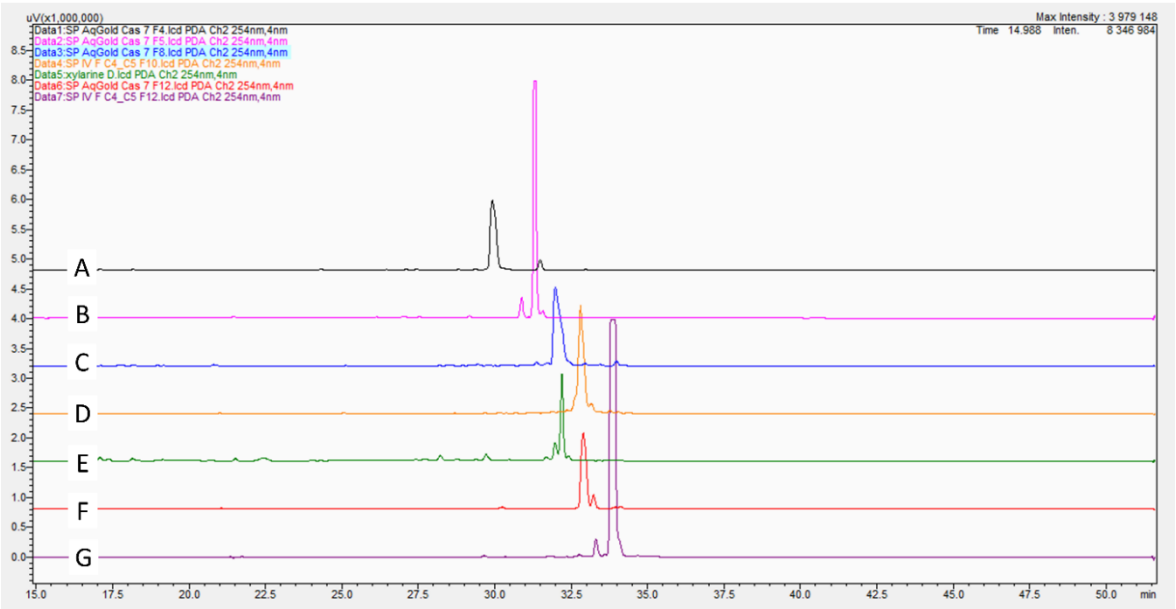


Figure 22 Chromatogrammes de la série de sept molécules obtenues après purification de la fraction F6-8. A) xylarine A 1 ; B) xylarine B 2 ; C) xylarine C 3 ; D) xylarine D 4 ; E) xylarine E 5 ; F) xylarine F 6 ; G) xylarine G 7

Comme indiqué dans le schéma de fractionnement et d'isolement, il est à préciser que ces composés sont produits de manière basale par *X. hypoxylon* en mono-culture (Fig. 23). Une culture de ce champignon est en cours afin d'apprécier leur production relative en mono-culture et d'isoler de plus grandes quantités de certains composés déjà obtenus mais en trop faible quantité pour une élucidation structurale.

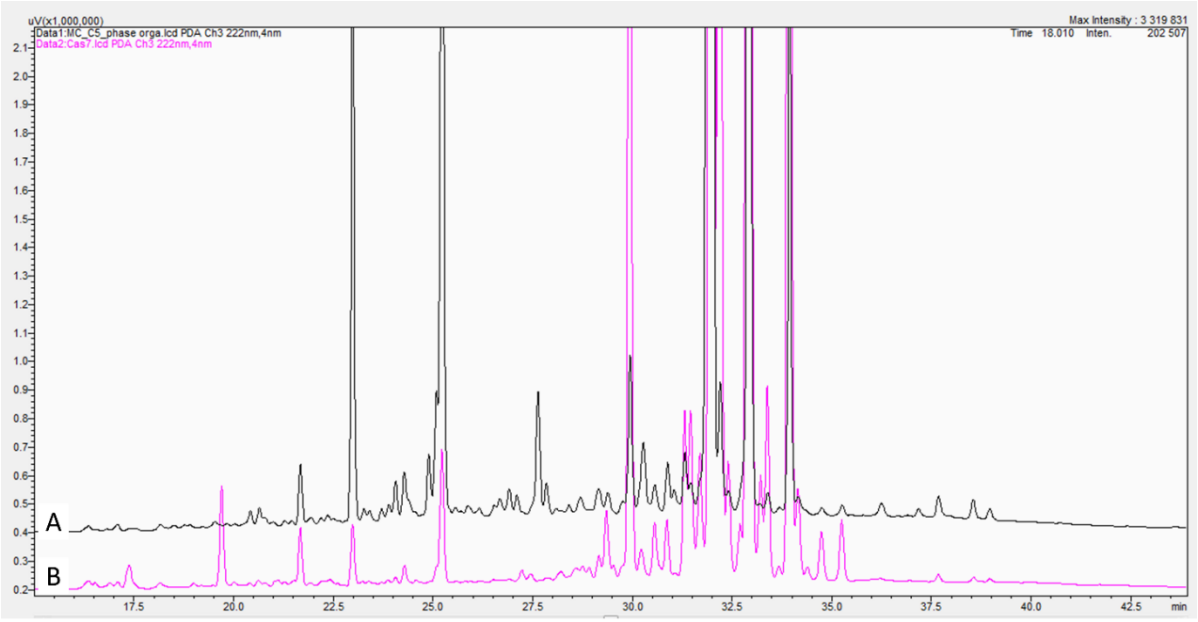


Figure 23 Comparaison des chromatogrammes entre A) l'extrait de monoculture de C5 et B) la fraction F6-8

De plus, les tests antibactériens menés sur cet extrait de mono-culture de C5 montrent bien une activité vis-à-vis de *S. aureus*.

c. Elucidation structurale des composés isolés

- *Composés isolés à partir de la Fraction F6-8*

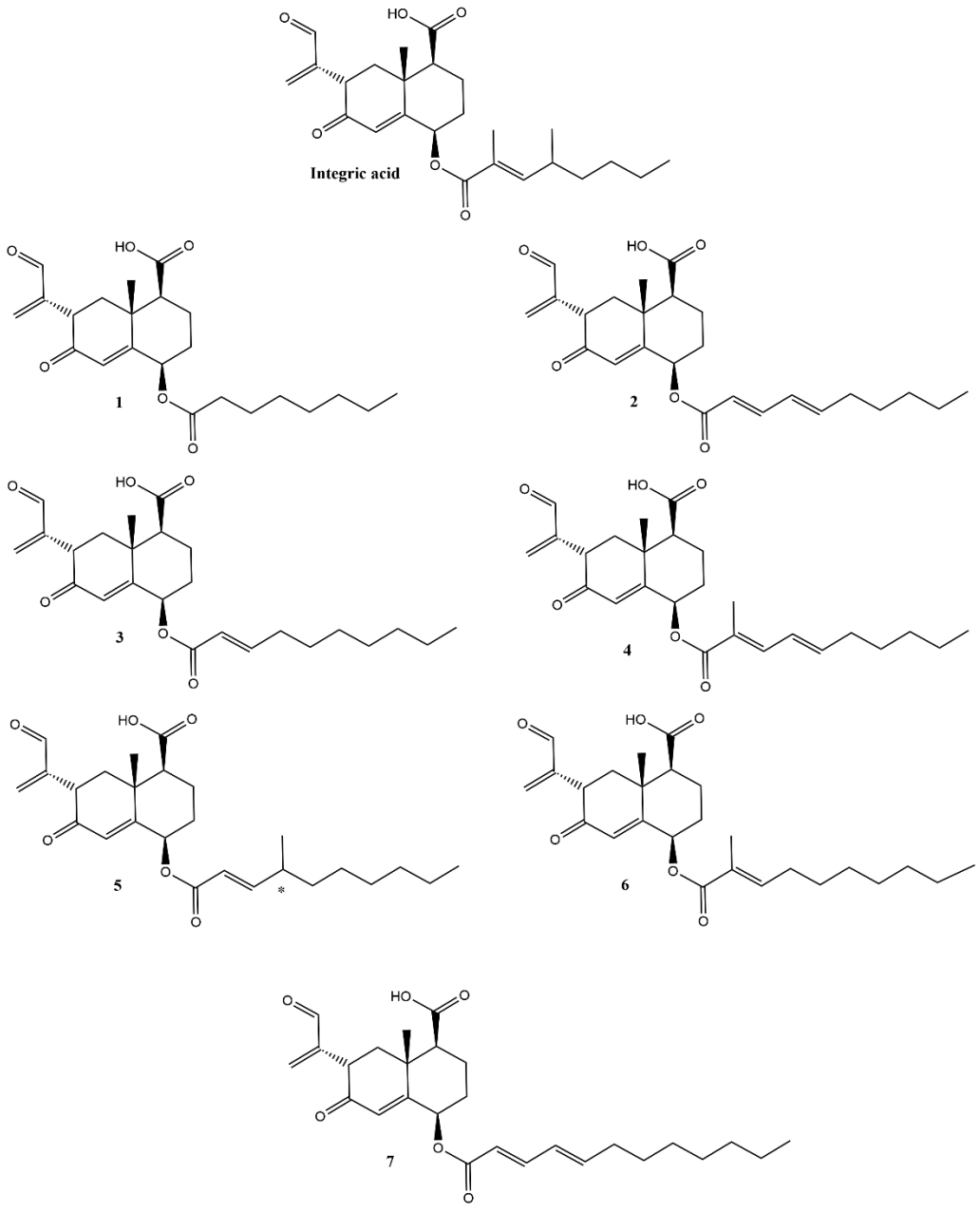


Figure 24 Structures de l'acide intégrique et des composés 1 -7

Les composés **1 – 7** ont pu être structuralement caractérisés (Fig. 24). Ces observations ont été corroborées par l'analyse spectrale MS-tandem de l'ensemble des sept molécules présentant un ion fragment commun à m/z 261 (pouvant correspondre à une formule brute de $C_{15}H_{17}O_4$), caractéristique de l'unité décaline, obtenue après clivage du groupe

ester suivi d'une déshydratation (Singh et al., 1999), et un fragment à m/z 215 (correspondant à une unité de $C_{14}H_{15}O_2$) provenant d'une perte ultérieure d'unité CH_2O_2. Le composé **1** a été obtenu sous forme de cristaux amorphes avec la formule moléculaire $C_{23}H_{32}O_6$ déduite par HR-ESIMS (*m/z* 403.2124 ($[M-H]^-$) suggérant 8 degrés d'insaturation. Ses données spectrales RMN ont été rapportées dans les figures S4 à S10, p. 47, Volume 2. Le spectre RMN ^1H présente (Tab. 6) des signaux distincts pour deux méthyles (un singulet à δ 1,50 et un triplet à δ 0,87), un méthine oxygéné (sous forme de doublet de doublet à δ 3,74), un méthine oléfinique (un singulet à δ 5,95), un groupe vinyle (sous forme de deux singulets à δ 6,44 et δ 6,31) et un groupe aldéhydique (un singulet à δ 9,51). Les autres signaux ont été attribués à 9 groupes de méthylène comme confirmé par le spectre RMN 1D Jmod qui présentait également un carbone quaternaire sp3 (à δ 38,93) et trois carbones de type carbonyle avec un δ supérieur à 170 ppm (à δ 172,48, 173,95, 197.0) Le dernier carbone de type carbonyle à δ 193.9 a été attribué grâce au spectre HSQC au groupe aldéhydique. Les corrélations 2D COSY (COrrelated SpectroscopY) et HMBC (Heteronuclear Multiple Bond Correlation) et la comparaison avec les données rapportées pour l'acide intégrique (Singh et al., 1999) ont confirmé la présence de l'unité érémophilane. De plus, les pics croisés HMBC pour le méthylène H-2' à δ 2,37 et H-3' à δ 1,67 avec le groupe carbonyle C-1' à δ 172,48 ont souligné la liaison de l'unité sesquiterpènique avec la chaîne latérale à travers un ester. Enfin, le spectre TOCSY 2D (Total Correlation SpectrocopY) a confirmé le système de spin correspondant à une chaîne latérale aliphatique contenant sept groupes méthylèniques. La stéréochimie relative du composé **1** est corroborée par la présence des constantes de couplage scalaire combinées aux données COSY, NOESY (Nuclear Overhauser Effect SpectroscopY) et TOCSY (Tab. S9, p. 73, Volume 2). Tout d'abord, les constantes de couplage de H-7 ($^3J_{H6ax}$ et $^3J_{H6eq}$ = 14,4 et 4,3 Hz, respectivement), ont révélé sa position axiale. De plus, les pics de corrélation NOESY entre H-7 et H-14 (groupe méthyle) et entre H-14 et H-3 (δ 2,27) et H-6 (à δ 2,13) ont conduit à leur localisation en face β de la conformation chaise correspondant à la position axiale pour H-14 et H-3 et à la position équatoriale pour H-6. L'assignation équatoriale de H-1 a été justifiée par sa constante de couplage de 4 Hz avec les deux protons H-2 et a été confirmée par sa relation NOESY avec H-9. Enfin, H-4 a été placé en position axiale grâce à ses constantes de couplage et ses données NOE avec H-2ax, H-3eq, H-6ax. Toutes ces preuves ont été représentées dans la figure 25.

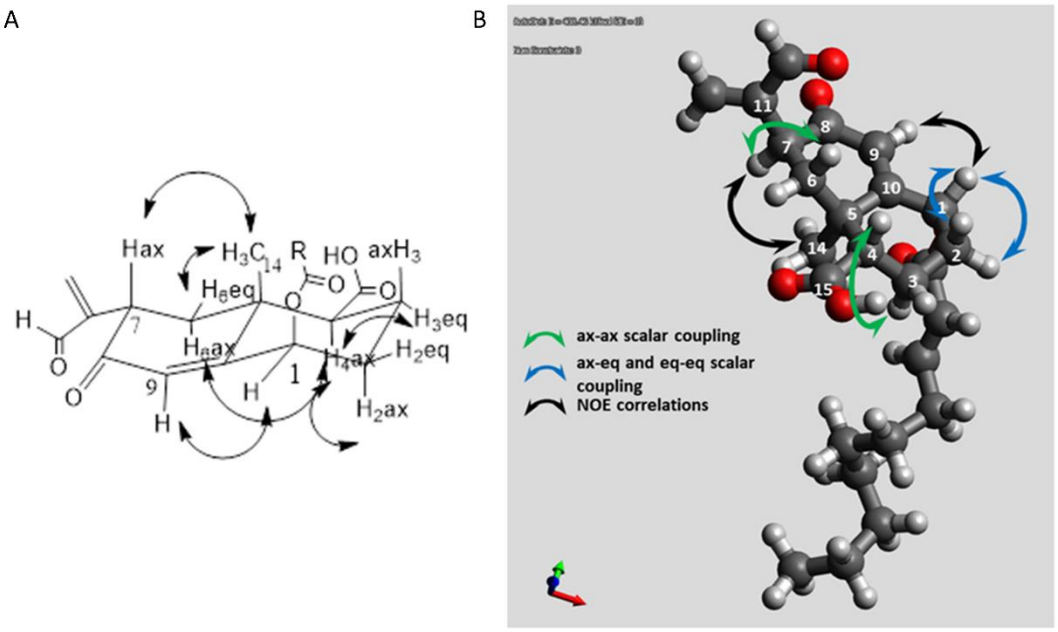


Figure 25 Conformation chaise du motif érémophilane et corrélations NOESY ; la Figure B représentant la structure 3D du composé 3 a été réalisée par le logiciel Avogadro 1.2 (http://avogadro.cc/)

L'analyse de tous les spectres RMN des composés **2**-**7** (Fig. S11 – S54, p. 51 - 72, Volume 2) a mis en évidence leur similarité structurelle avec celle du composé **1**. Ils possèdent le noyau érémophilane avec la même configuration relative que celle de **1**, étayée par les constantes de couplage (Tab. 7) et les corrélations clés NOESY (Tab. S1-S8) et ne diffèrent que par quelques modifications de la chaîne latérale. Le composé **2** de formule brute $C_{25}H_{32}O_6$ soutenue par HR-ESIMS (*m/z* 427.2125 ([M-H]$^-$) présente une chaîne plus longue de deux carbones et quatre carbones oléfiniques à δ 119.94, 139.31, 146.04, 146.45. La configuration de la liaison 2'-3' est attribuée grâce à la mesure de la constante de couplage du doublet correspondant au proton H-2'. En effet, la valeur de $^3J_{H2'\text{-}H3'}$ = 15.4 Hz permet sans ambiguïté de conclure à une configuration E. La configuration de la liaison 4'-5' en revanche est plus compliquée à déterminer car on se trouve face à un système ABX H-4' et H-5' étant presque superposés avec des signaux correspondant à des multiplets très complexes. Heureusement, l'acquisition du spectre RMN ^{1}H dans le benzène-*d6* a permis une levée de dégénérescence et par conséquent la mesure de la constante de couplage entre H-4' et H-5'. Celle-ci, d'une valeur de 15 Hz (Fig. S21, p. 56, Volume 2), nous permet d'attribuer également la configuration E à cette double liaison. Comparée au composé **2**, la formule brute $C_{25}H_{34}O_6$ (*m/z* 429.2282 [M-H]$^-$) du composé **3** suggère une perte d'insaturation révélée par les données RMN ^{13}C en position C-4' et C- 5'

(voir Fig. S20, p. 56, Volume 2 et Tab. 7). La liaison éthylénique subsistante conserve une configuration E ($^3J_{H2'-H3'}$= 15.6 Hz). Les données RMN et HR-EISMS ($C_{26}H_{34}O_6$) pour le composé **4** affichent un groupe méthyle supplémentaire (C-11 ') à δ 12,83 ppm situé sur le carbone 2'. Par rapport à la molécule **2** ne présentant pas ce groupe méthyle, 2' devient quaternaire et C-4', et surtout C-3', se trouvent blindés (respectivement -2.5 et -6.6 ppm). Les corrélations HMBC entre H-11' et C-1', C-2' et C-3' et entre H-3' et C-11', combinées aux signaux croisés NOESY entre H-3' et H-5' et le décalage dans le bas champ de C-2' (125,7) a conduit à la localisation de ce méthyle en C-2'. L'analyse de la constante de couplage ($^3J_{H4'-H5'}$ = 15 Hz) a révélé une configuration E de la liaison oléfinique 4'-5' et la corrélation NOESY entre H-4' et H-11'suggère que la liaison H2'-H3' est également de configuration E. Il est à noter que le composé **4** a été isolé en mélange avec le composé **3** dans un rapport 2/3-1/3. Les composés **5** et **6** possédant une formule brute similaire de $C_{26}H_{36}O_6$ (*m/z* 443.2439 ([M-H]$^-$) et 443.2438 ([M-H]$^-$), respectivement) sont des isomères et différent du composé **4** par une perte de deux hydrogènes soulignant la présence d'un seul degré d'insaturation sur la chaine de l'ester. Le groupe méthyle (CH_3-11') du composé **5** a été attribué en position C-4' en raison de sa forme de signal sous forme de doublet ($^3J_{H4'-H11'}$ = 6,7 Hz) ainsi que de ses corrélations HMBC avec C-3' et C- 4' alors qu'il est situé en C-2' dans le composé **6** comme le montrent ses corrélations HMBC avec C-1', C-2' et C-3'. La configuration E de la liaison oléfinique C-2'-C-3' a été établie par la longue constante de couplage ($^3J_{H2'-H3'}$ = 15,5 Hz) pour le composé **5** et par la forme du signal de H-3' sous forme de triplet quadruplet ($^3J_{H3'-H4'}$ = 7,5 et $^4J_{H3'-H11'}$ = 1,4 Hz) pour le **6**. Le dernier composé, le **7**, a présenté des données RMN similaires à celles du composé **2**, à l'exception de la présence supplémentaire de deux groupes méthylène également révélée par sa formule brute $C_{27}H_{36}O_6$ (avec m/z 455.2438 ([M-H]$^-$). La détermination de la configuration des deux insaturations a suivi le même processus et a abouti aux mêmes conclusions que pour la molécule **2**, à savoir qu'elles possèdent une configuration E.

Il est intéressant de souligner que, par rapport à l'acide intégrique, seul le composé **1** a la même longueur de chaîne latérale avec 8 carbones mais sans insaturation ni groupe méthyle. Il est à noter également que le composé **1** présente une analogie structurale forte avec le xylarénal A précédemment isolé de *Xylaria persicaria* (Smith et al., 2002) et qui possède une chaine aliphatique plus longue de 9 carbones. Les autres composés **2**-**7** présentent une longueur de chaîne C-10 avec une ou deux insaturations linéaires ou ramifiées par un méthyle. A notre connaissance, il s'agit du premier isolement de ces sept

dérivés d'erémophilanes et seuls deux champignons endolichéniques ont été décrits comme producteurs de ce type de sesquiterpènes (Le et al., 2013; Vo et al., 2022) mais ne présentant pas de chaîne latérale ester.

La configuration absolue des composés **1** à **7** (nommés finalement **xylarines A –F**) a été déterminée par comparaison des spectres de dichroïsme circulaire électronique (DCE) expérimentaux et calculés, par TD-DFT (théorie de la fonctionnelle de densité – dépendante du temps) pour la configuration 1*R*, 4*S*, *5R*, 7*S* (et identique à celle de l'acide intégrique (Singh et al., 1999)) (Fig. 23). Ces calculs ont été effectués par Pierre Le Pogam-Alluard (UMR CNRS 8076 BioCIS, Paris Saclay). La grande similarité des spectres théoriques avec les spectres expérimentaux valide la configuration absolue pour ces composés **1**-**7** comme étant 1*R,* 4*S, 5R*, 7S (Fig. 26).

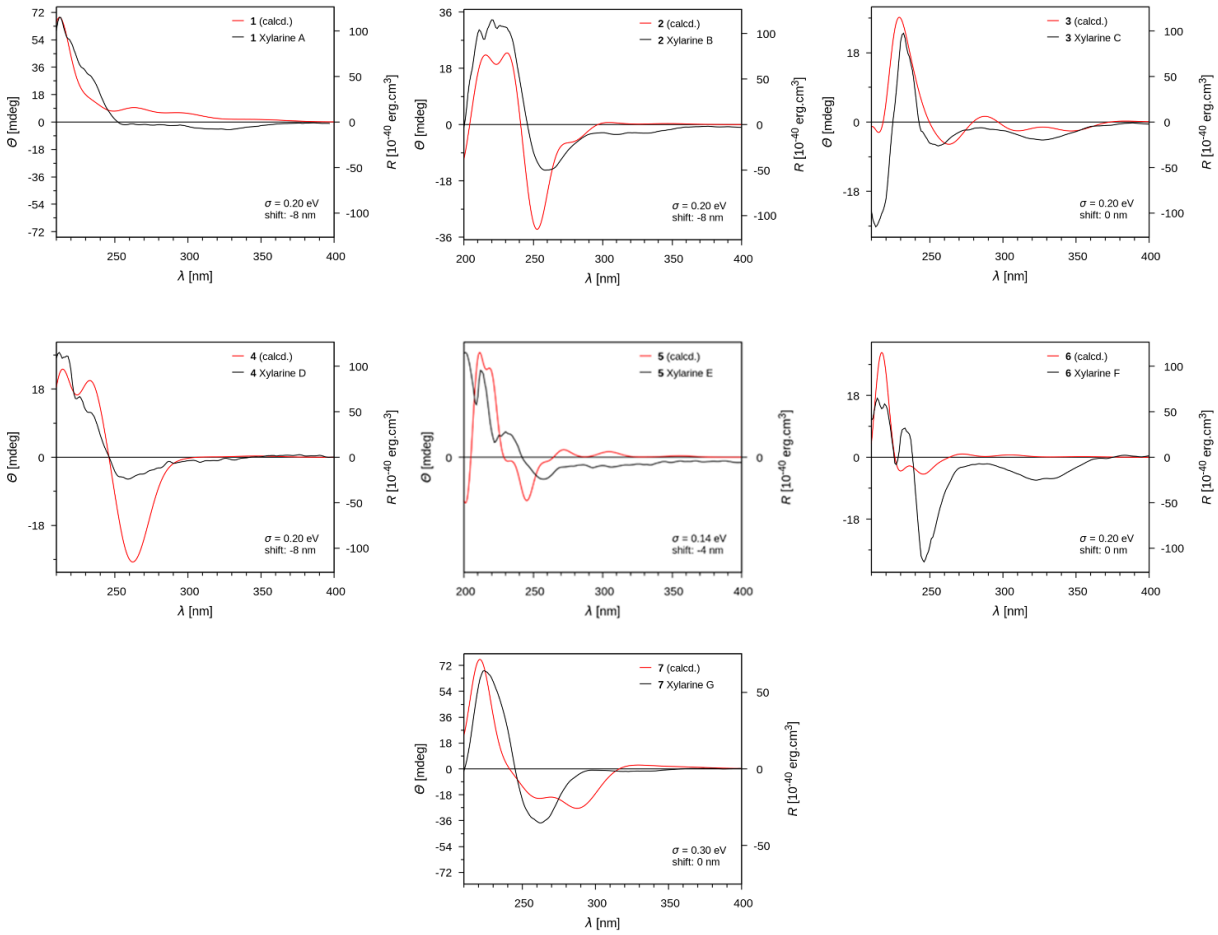


Figure 26 Superposition des spectres DCE expérimentaux et caclulés pour la configuration 1R, 4S, 5R, 7S pour les composés 1-7

Tableau 7 Données spectrales RMN pour les 7 composés à 500 MHz (RMN 1H) et 125 MHz (RMN 13C)

	1			2			3			4			5			6			7		
Position	δc (ppm)	δH (ppm)	*J* in Hz	δc (ppm)	δH (ppm)	*J* in Hz	δc (ppm)	δH (ppm)	*J* in Hz	δc (ppm)	δH (ppm)	*J* in Hz	δc (ppm)	δH (ppm)	*J* in Hz	δc (ppm)	δH (ppm)	*J* in Hz	δc (ppm)	δH (ppm)	*J* in Hz
1	73,7	5,46	bt (4.0)	73,7	5,51	bt (3.0)	73,8	5,49	bt (3.0)	74,1	5,52	bt (3.0)	73,6	5,5	bt	74,0	5,51	bt	73,7	5,5	bt (2.6)
2eq	30,4	2,08	m	30,5	2,12	m	30,5	2,1	m	30,5	2,13	m	30,5	2,1	m	30,6	2,09	m	30,5	2,1	m
2ax		1,83	m		1,89	tt (14.2, 3.9)		1,88	tt (14.1, 3.5)		1,89	m		1,87	m		1,86	m		1,88	tt (14.1, 3.7)
3ax	21,0	2,27	m	21,1	2,30	m	21,2	2,3	m	21,2	2,29	m	21,0	2,29	m	21,2	2,29	m	21,1	2,31	m
3eq		1,83	m		1,83	dq		1,82	dq (14, 2.9)		1,83	m		1,83	m		1,81	m		1,83	dq
4	53,8	2,48	dd (13. 3, 3.1)	53,9	2,5	dt (12.9, 3.2)	54,1	2,49	dd (12.4, 3.1)	53,9	2,5	dt (13.1, 3.4)	53,8		dd (13.4, 4.3)	54,0	2,49	dd (12.9, 2.8)	53,9	2,5	dd (12.9, 2.8)
5	38,9			39,0			38,9			38,9			39,0			38,9			38,9		
6ax	43,9	2,37	m	44,0	2,4	m	44,1	2,39	tapp (13.9)	44,0	2,4	m	44,0	2,4	m	44,0	2,4	tapp (13.9)	43,9	2,4	tapp (13.9)
6eq		2,13	dd (13.3, 4.3)		2,12	m		2,13	dd (13.3, 4.3)		2,13	m		2,14	m		2,15	dd (13.4, 4.4)		2,13	m
7	44,4	3,74	dd (14.4, 4.3)	44,5	3,75	dd (14.4, 4.3)	44,7	3,74	dd (14.5, 4.3)	44,5	3,75	dt (14.5, 4.7)	44,4	3,75	dd (14.5, 4.3)	44,5	3,75		44,5	3,75	dd (14.5, 4.3)
8	197,0			197,0			197,1			197,1			197,0			197,2		dd (14.4, 4.2)	197,0		
9	129,9	5,95	s	130,0	5,99	s	130,1	5,98	s	130,0	5,99	s	129,9	5,98	s	130,0	5,98	s	130,0	5,98	s
10	160,4			160,3			160,3			160,4			160,2			160,6			160,3		
11	149,7			149,7			149,6			149,7			149,6			149,7			149,7		
12a	136,7	6,44	s	136,7	6,44	s	136,8	6,44	s	136,7	6,44	s	136,7	6,44	s	136,8	6,44	s	136,7	6,44	s
12b		6,31	s		6,31	s		6,31	s		6,31	s		6,31	s		6,31	s		6,31	s
13	193,9	9,55	s	193,9	9,55	s	193,9	9,55	s	193,9	9,55	s	193,7	9,55	s	193,9	9,55	s	193,8	9,55	s
14	20,0	1,51	s	20,0	1,51	s	20,0	1,5	s	20,0	1,53	s	19,8	1,51	s	20,1	1,52	s	20,0	1,51	s
15	174,0			174,0			174,0			174,1			173,9			174,2			174,0		
1'	172,5			166,0			165,4			167,3			165,5			166,9			166,0		
2'	35,0	2,37	m	119,9	5,89	d (7.9)	122,1	5,88	dt (15.6, 1.5)	125,7			120,3	5,85	d (15.4)	128,5			119,9	5,89	d (15.4)
3'	25,6	1,62	m	146,5	7,29	dd (15.4, 10.0)	151,1	6,99	dt (15.6, 7.1)	140,0	7,2	d (11.3)	155,9	6,89	dd (15.7, 8.0)	143,8	6,82	tq (7.5, 1.3)	146,4	7,29	dd (15.3, 10.0)
4'	29,7	1.62 (4'a)	m	129,3	6,31	m	32,9	2,25	m	126,8	6,48	m	37,2	2,36	m	29,2	2,23	m	129,3	6,33	m
		1.30 (4'b)	m																		
5'	29,7	1.62 (5'a)	m	146,0	6,29	m	28,8	1,48	m	144,7	6,2	dt (14.6, 7.6)	36,7	1,37	m	29,8	1,32	m	146,0	6,29	m
		1.30 (5'b)	m																		
6'	32,4	1,3	m	33,6	2,19	m	29,7	1,32	m	33,8	2,22	m	27,9	1,3	m	29,3	2,23	m	33,6	2,21	m
7'	23,3	1,3	m	29,1	1,45	m	29,8	1,32	m	29,3	1,45	m	29,8	1,3	m	29,8	1,32	m	29,7	1,3	m
8'	14,3	0,87	m	32,1	1,31	m	32,5	1,32	m	32,1	1,33	m	32,5	1,3	m	32,5	1,29	m	29,4	1,45	m
9'				23,1	1,31	m	23,3	1,32	m	23,1	1,33	m	23,3	1,3	m	23,3	1,29	m	29,6	1,3	m
10'				14,3	0,88	m	14,3	0,87	t (7.0)	14,3	0,89	m	14,3	0,87	m	14,3	0,88	t (7.0)	32,5	1,3	m
11'										12,8	1,94	s	19,5	1,05	d (6.7)	12,6	1,84	s	23,3	1,3	m
12'																			14,3	0,87	t (7.0)

Les données ont été enregistrées à 500 MHz pour le proton et à 125 MHz pour le carbone dans l'acétone-d_6.

tapp : triplet apparent

- *Composé issu de la fraction F10*

L'analyse de la fraction F10 a été réalisée par RMN et comparée aux données spectrales de la littérature. Ainsi, la massarilactone H (**8**) a pu être identifiée (Cimmino et al., 2021) et est produite de manière basale par *D. variisporum* (Fig. 27) comme précédemment décrit.

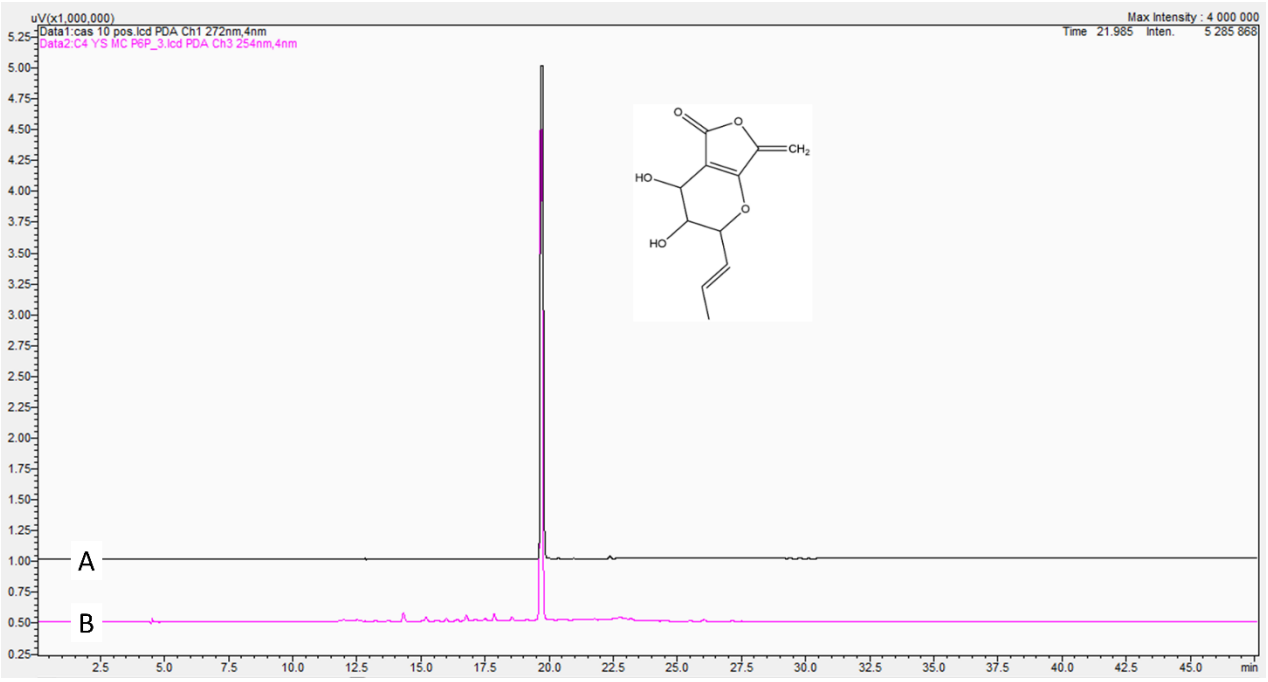


Figure 27 Comparaison des chromatogrammes pour le composé 8. A) Massarilactone H (8) et B) chromatogramme de l'extrait de monocuture de C4

d. Evaluation des activités biologiques des composés isolés

Les activités antibactériennes des composés isolés ont été déterminées vis-à-vis d'un panel de pathogènes bactériens humains (Tab. 8) et comparées à la gentamicine comme témoin positif et au DMSO comme contrôle négatif. Les nouveaux sesquiterpènes érémophilanes **2**, **5** et **7** n'ont pas été testés du fait de leur faible quantité. Les composés **1**, **3**, **4** et **6** ont montré une activité antibactérienne vis-à-vis de *S. aureus, S. epidermidis* et *S. saprophyticus* avec une CMI comprise entre 0,78 et 25,00 µg/mL. Les dérivés érémophilanes **3** et **6** présentent les activités les plus fortes avec des CMI comprises entre 0,78 et 6,25 µg/mL contre tous les pathogènes testés sauf *E. coli* et *P. aeruginosa* qui n'ont montré aucune sensibilité à aucun des composés testés. Ces résultats mettent en lumière

l'incapacité de ces composés à traverser la paroi des bactéries GRAM – en raison putativement de la taille du noyau érémophilane. Cependant, leur activité contre *S. aureus* et *S. epidermidis* est supérieure à celle du témoin positif. Enfin, le composé **1** présentant la chaine aliphatique la plus courte est le composé le moins actif soulignant l'importance de la longueur de cette chaîne et/ou de sa rigidité dans l'activité antibactérienne.

Tableau 8 Evaluation de l'activité antibactérienne des composés 1, 3, 4 et 6 vis-à-vis de six bactéries pathogènes de l'homme (CMI exprimée en µg/mL)

Souches bactériennes	**1**	**3**	**4**	**6**	**8**	**Gentamicine**	**DMSO**
Staphyloccocus aureus (CIP 53.156)	6.25	0.78	1.56	0.39	-	3.91	-
S. aureus resistant (DSM 13661)	25.00	1.56	3.10	0.78	-		-
S. epidermidis (CIP 53.124)	12.50	0.78	3.10	1.56	-	125	-
S. saprophyticus	6.25	3.10	6.25	1.56	-	0.25	-
Streptococcus agalactiae	25.00	3.10	6.25	1.56	-	0.12	-
Escherichia coli (CIP 54.8)	-	-	-	-	-	3.91	-
Pseudomonas aeruginosa (CIP A22)	-	-	-	-	-	1.95	-

Du fait de l'activité de l'acide intégrique vis-à-vis de l'intégrase du VIH, les composés **1**, **3**, **4** et **6** sont en cours d'évaluation biologique vis à vis de coronavirus à l'Institut Pasteur de Lille dans l'équipe de Karin Séron. De plus, l'antibiose initiale ayant été observée entre deux souches fongiques, ces quatre composés seront testés ultérieurement sur des phytopathogènes dans l'équipe de Claudia Bartoli, INRAE, Le Rheu.

Le composé basalement produit par *D. variisporium*, la massarilactone H (**8**), n'a présenté quant à elle, aucune activité antibactérienne, comme précédemment décrit (Teponno et al., 2017).

e. Etude métabolomique des extraits de mono et co-cultures

Une analyse par UHPLC-Orbitrap de l'extrait brut organique de la **série 3**, des mono-cultures et des fractions F4-6, F11, F13 et F24 obtenues après chromatographie flash sur silice sphérique (Fig. 16) a été réalisée en mode négatif et positif dans l'équipe de Mohamed Mehiri à l'Université de Nice. Cette analyse a permis, après traitement par MZmine, de pouvoir utiliser un outil récemment développé par Damien Olivier,

MolNotator (Olivier-Jimenez et al., 2021). La nature de l'espèce ionique est rarement prise en compte et entraîne une redondance car un seul composé peut être présent sous différentes formes dans tout le réseau. Cette méthode innovante utilise les différentes espèces d'ions produites pour une même molécule lors de l'ionisation (adduits, dimères, trimères, fragments dans la source) et permet de créer par triangulation un nœud de molécule (ou nœud neutre). Ainsi, l'estimation de la masse calculée de la molécule associée est permise. Ces nœuds neutres offrent plusieurs avantages : i) chaque molécule est représentée dans son contexte d'ionisation, connectée à tous les ions produits et indirectement à certains composés co-élués, mettant ainsi également en évidence des espèces d'adduits largement présentes et inattendues ii) les neutres prédits servent d'ancre pour fusionner les modes d'ionisation positifs et négatifs complémentaires en un seul réseau, iii) la déréplication est améliorée par l'utilisation de tous les ions disponibles connectés aux nœuds neutres, et les masses moléculaires calculées peuvent être utilisées pour une déréplication de masse exacte.

Le réseau généré sur les données de HRMS^2 (Fig. 28) a permis de faire ressortir 70 nœuds spécifiques à la co-culture de C4-C5 qui ont été regroupés dans le tableau S16, p.80, Volume 2 et qui seront recherchés par la suite. Une dizaine de nœuds ont pu être retrouvés dans la fraction F4 et pourraient correspondre au composé induit à 23.2 min que l'on ciblait au préalable mais qui a finalement été perdu. De même, les nœuds retrouvés dans les fractions F11 et F13 peuvent correspondre au composé induit à 18.2 min. Ces molécules seront à rechercher en priorité lors d'une prochaine étude réalisée sur la co-culture de ces deux champignons.

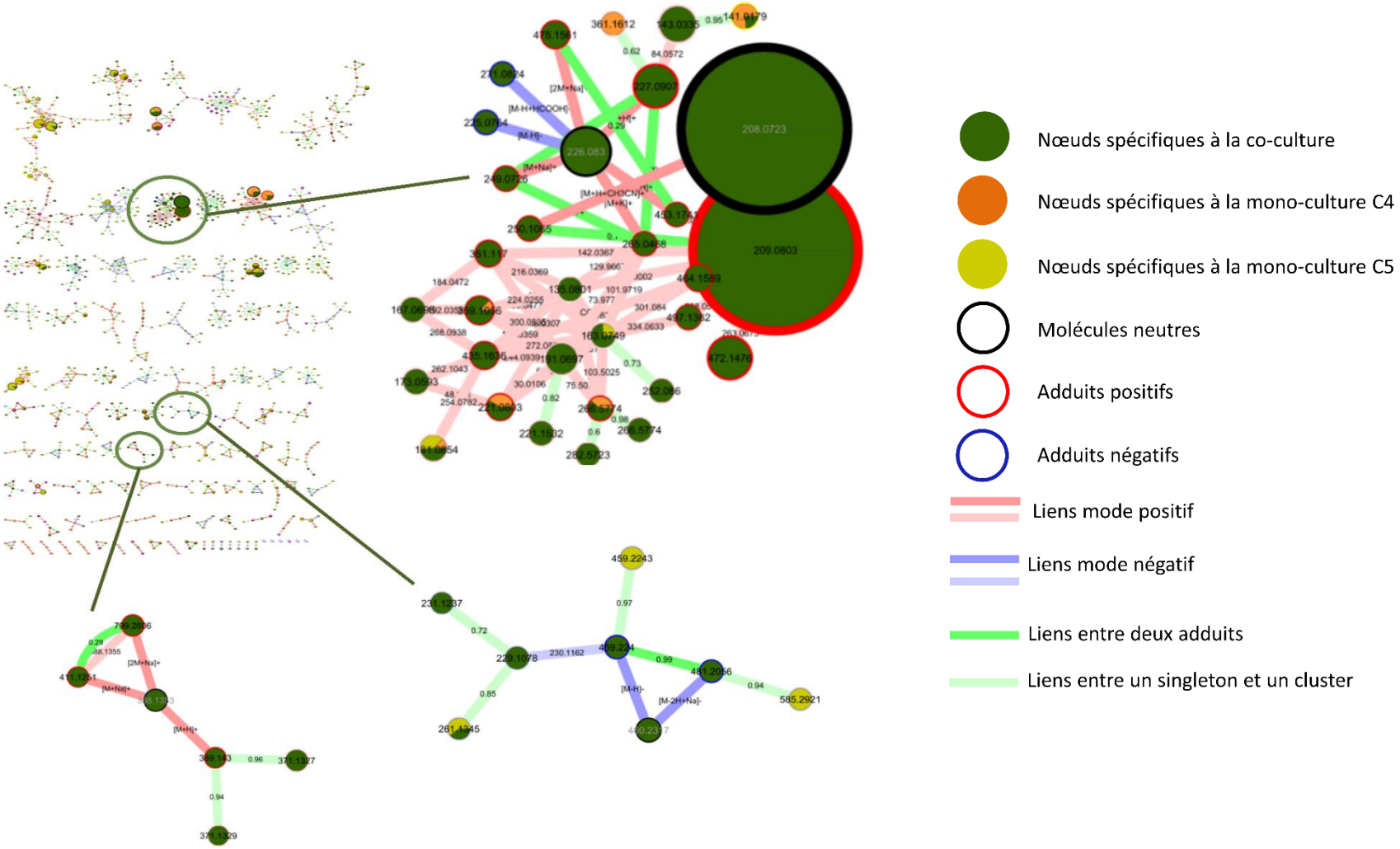


Figure 28 Réseaux moléculaires générés par l'outil MolNotator (Olivier-Jimenez et al., 2021) montrant les nœuds spécifiquement retrouvés dans les co-cultures et ceux présents en mono-culture

2. Etude mycochimique du couple *M. phragmitis* (C44) et *Tolypocladium* sp (C45).

A. Etat de l'art bibliographique

Les espèces de *Microdochium* sont, pour certaines, connues comme étant des phytopathogènes (Xu and Nicholson, 2009) alors que d'autres ont été isolées de plantes sans provoquer de maladie (Ernst et al., 2011). De plus, des études ont montré que certaines espèces de *Microdochium* étaient capables de produire des molécules à large spectre d'activité antibiotique. En effet, *Microdochium nivale*, un champignon marin, a été décrit comme producteur de cyclosporine A, molécule possédant des propriétés antifongiques très prometteuses (Bhosale et al., 2011). L'étude d'une autre espèce elle, *Microdochium bolleyi*, a montré que ce champignon produisait des isocoumarines également actives vis-à-vis de pathogènes fongiques, bactériens ainsi que des algues (Zhang et al., 2008). Peu d'études ont été menées sur le champignon *Microdochium phragmitis* et son métabolome. Il a cependant été rapporté que son extrait brut présentait une intéressante activité cytotoxique (Santiago et al., 2012) et une activité antifongique (Liu et al., 2016).

Contrairement à *M. phragmitis*, pléthore d'articles scientifiques relatent l'isolement et le métabolisme secondaire des espèces de *Tolypocladium*. Certaines espèces ont même déjà été isolées de lichens pour lesquelles des métabolites ont été identifiés (Hu et al., 2017; Li et al., 2015). Les molécules issues de la culture des champignons du genre *Tolypocladium* possèdent de nombreuses activités biologiques telles que : antimalariale modérée (Fukasawa et al., 2018), inhibition de l'activité tyrosinase (Khan et al., 2021) antimicrobienne (Xu et al., 2019), antitumorale (Leung et al., 2006), immunosuppressive (Balaraman et al., 1991), anticancéreuse (Hayakawa et al., 2008; Kebede et al., 2017).

B. Résultats

Pour l'étude chimique de ces deux champignons en mono et co-culture, nous avons réalisé une succession de trois séries de culture à plus grande échelle sur, respectivement 20 ou 30 boites de Petri (pour les mono- et co-cultures) afin de comparer les chromatogrammes

et puis 100 et enfin 440 boîtes en co-culture. Sur 20 boîtes de cette troisième série, la zone de confrontation a été spécifiquement extraite (Fig. 29).

Figure 29 Co-culture de Tolypocladium sp. *et* M. phragmitis

a. Analyses des extraits

Comme précédemment présenté pour le couple de champignons C4 et C5, les deux premières séries de cultures fongiques pour C44 – C45 ont été extraites avec un mélange 1, de solvants composé de MeOH:CH_2Cl_2:AcOEt 1:2:3 + 0.1% d'AF alors que la troisième série (440 boîtes de co-culture) et sa zone de confrontation (20 boites) ont été extraits avec un mélange 2, CH_2Cl_2:AcOEt (v:v). Les quantités des extraits obtenues sont indiquées dans le tableau ci-dessous. Les quantités des extraits obtenues sont indiquées dans le tableau 9 ci-dessous.

Tableau 9 Quantités d'extrait en mg obtenues pour les différentes séries de culture de C44-C45

Solvant d'extraction		Extrait	Série	Quantité obtenue (g)
Mélange 1		C44_MC	1 : 20 boîtes	55.5
Mélange 1		C45_MC	1 : 20 boîtes	30.7
Mélange 1		C44-C45_CC	1 : 30 boîtes	275.8
Mélange 1		C44-C45_ZC	3 : 30 boîtes	2.4
Mélange 1	Phase organique	C44-C45_CC	2 : 100 boîtes	2.4
Mélange 1	Phase aqueuse	C44-C45_CC	2 : 100 boîtes	1.1
Mélange 2		C44-C45_CC	3 : 440 boîtes	3500

Les analyses HPLC-DAD (extraits préparés à 2 mg/mL dans l'acétone) des extraits issus des mono- et co-cultures et de la zone de confrontation de la coculture des 440 boîtes ont été comparées (Fig. 30) et indiquent l'apparition de certains composés relatifs à la co-culture et notamment au niveau de la zone de confrontation.

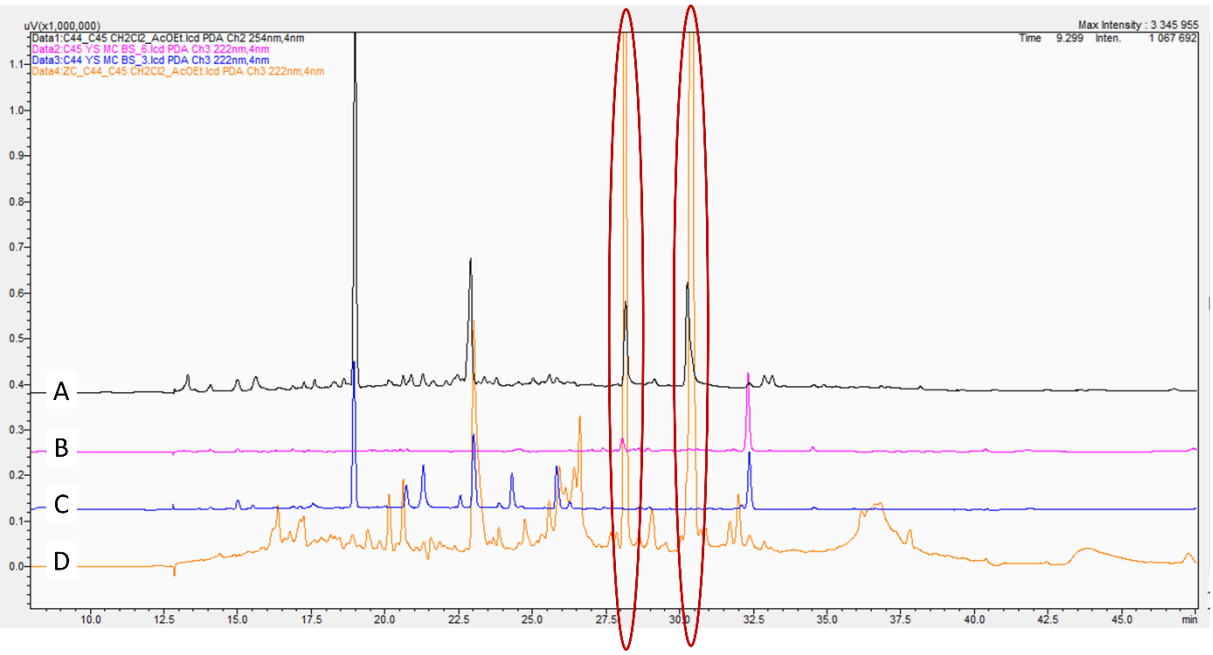


Figure 30 Comparaison des chromatogrammes entre : A) l'extrait brut de la co-culture de la 3ème série ; B) l'extrait de la mono-culture de C45 ; C) l'extrait de la mono-culture de C44 et D) l'extrait de la zone de confrontation entre C44 et C45

b. Fractionnement des extraits de co-culture et isolement

Les schémas de fractionnement et de purification des différentes séries de co-culture du couple C44-C45 sont présentés dans la figure suivante (Fig. 31).

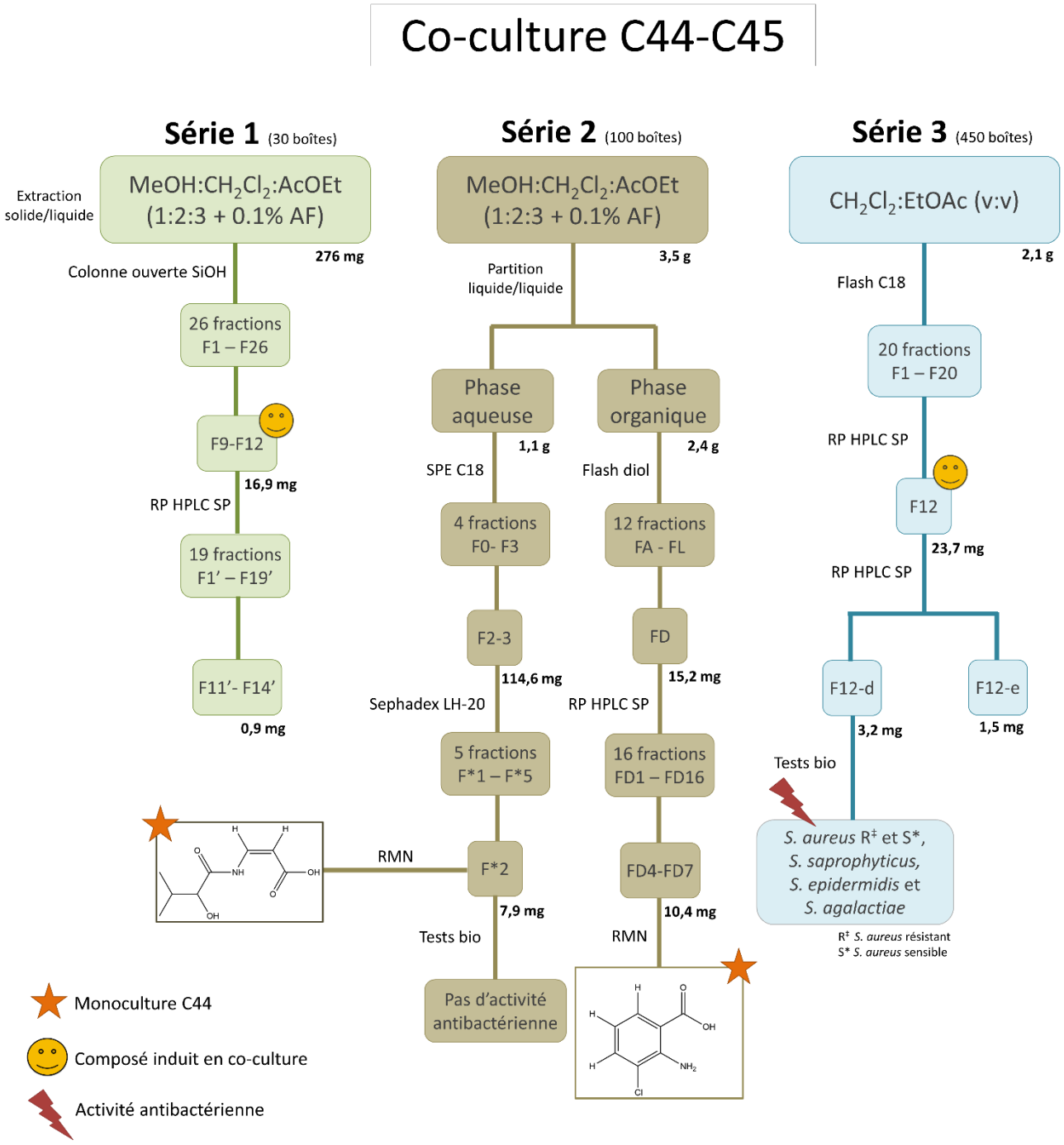


Figure 31 Fractionnements et purification des extraits bruts de la co-culture C44 – 45 et évaluation biologique de certaines fractions

- *Fractionnement et isolement des composés dont certains repérés comme spécifiques de la co-culture C44-C45*

L'extrait brut obtenu après extraction de la première série de co-culture a été fractionné en réalisant une colonne ouverte silice suivant le protocole décrit en page **170** donnant 149 fractions. Parallèlement à l'élution, le suivi par chromatographie sur couche mince (CCM) a permis de regrouper ces dernières en 26 fractions. Après préparation et analyse par HPLC-DAD de ces fractions, la comparaison des chromatogrammes obtenus à λ 272 nm a permis le rassemblement des fractions F9 à F12 en vue d'une purification par HPLC semi-préparative suivant le protocole 3, page 172 et permettant d'obtenir 19 sous-fractions. Toujours grâce à un suivi en HPLC-DAD, les fractions F11' à F14' ont pu être regroupées par comparaison de leurs chromatogrammes (Fig. 31).

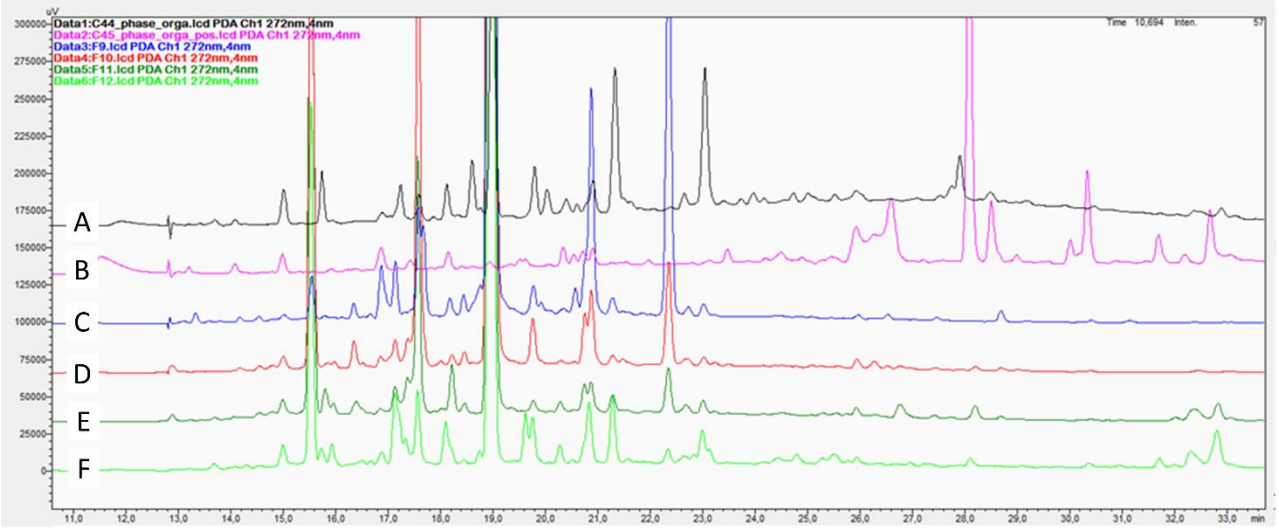


Figure 32 Chromatogrammes des fractionnements obtenus après purification par HPLC-semipréparative. A) extrait brut organique de la monoculture de C44 ; B) extrait brut organique de la monoculture de C45 ; C) fraction F9 ; D) fraction F10 ; E) fraction F11 et F) fraction F12

L'exploration chimique s'est arrêtée là pour cette série du fait des faibles quantités obtenues et de la richesse des fractions malgré les étapes de purification.

Le fractionnement (Fig. 31), de **l'extrait brut organique (2.4 g) de la deuxième série**, obtenu après partition liquide/liquide a été réalisé par chromatographie flash sur phase diol selon le gradient 2 (cf p. 169), et a permis d'obtenir 12 fractions de FA à FL. A partir de la fraction FD (15.2 mg) et après purification sur HPLC semi-préparative en phase

inverse (selon le protocole 4), 16 sous-fractions ont été recueillies de FD1 à FD16. Les sous-fractions FD4 à FD7 apparues pures ont été regroupées (10.4 mg) du fait de la similitude de leurs profils chromatographiques (Fig. 33). La molécule correspondante a également été retrouvée dans la mono-culture de C44.

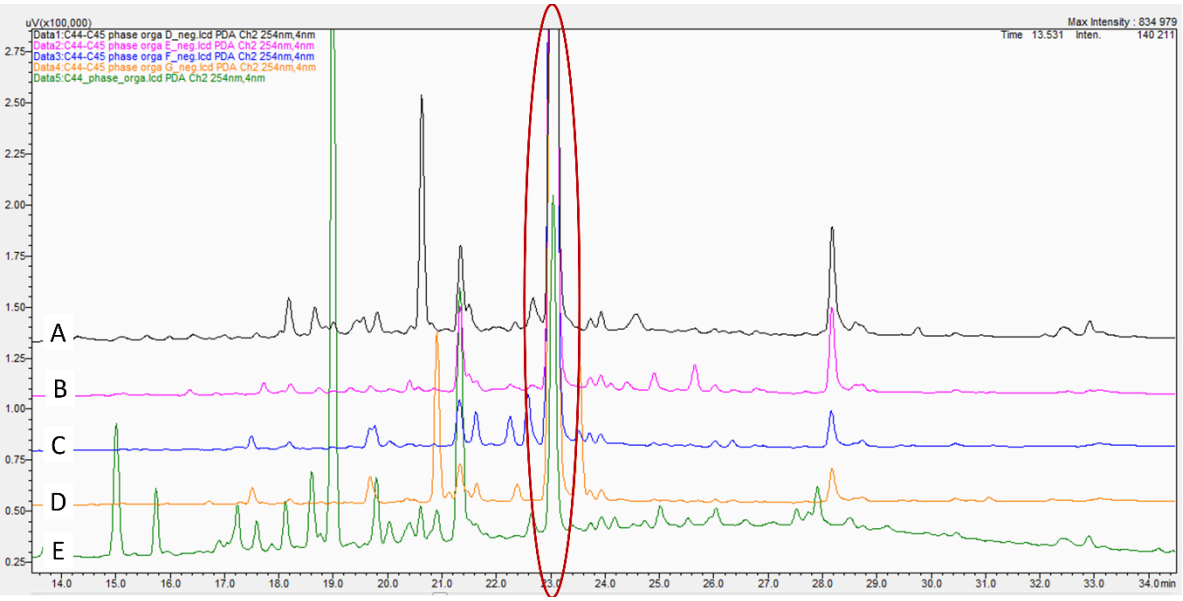


Figure 33 Chromatogrammes A) fraction 4 de l'extrait brut organique de la co-culture C44-C45 ; B) fraction 5 de l'extrait brut organique de la co-culture C44-C45 ; C) fraction 6 de l'extrait brut organique de la co-culture C44-C45 et D) fraction 7 de l'extrait brut organique de la co-culture C44-C45 et E) extrait brut organique de la mono-culture C44

Le fractionnement de l'extrait brut aqueux de la deuxième série (1.1 g) à l'aide de cartouches SPE CHROMABOND® C_{18}ec, élué par un gradient de solvant (H_2O, MeOH puis AcOEt) suivant le protocole 1 (cf p. 171) a permis l'obtention de 4 fractions de F0 à F3. Les deux dernières ont été combinées (114.6 mg) pour une nouvelle purification par Sephadex LH20 et 5 sous-fractions ont pu être recueillies : F*1 à F*5 dont la seconde F*2 (7.91 mg), pure. Cette molécule est aussi retrouvée produite de manière basale par C44 en mono-culture (Fig. 34).

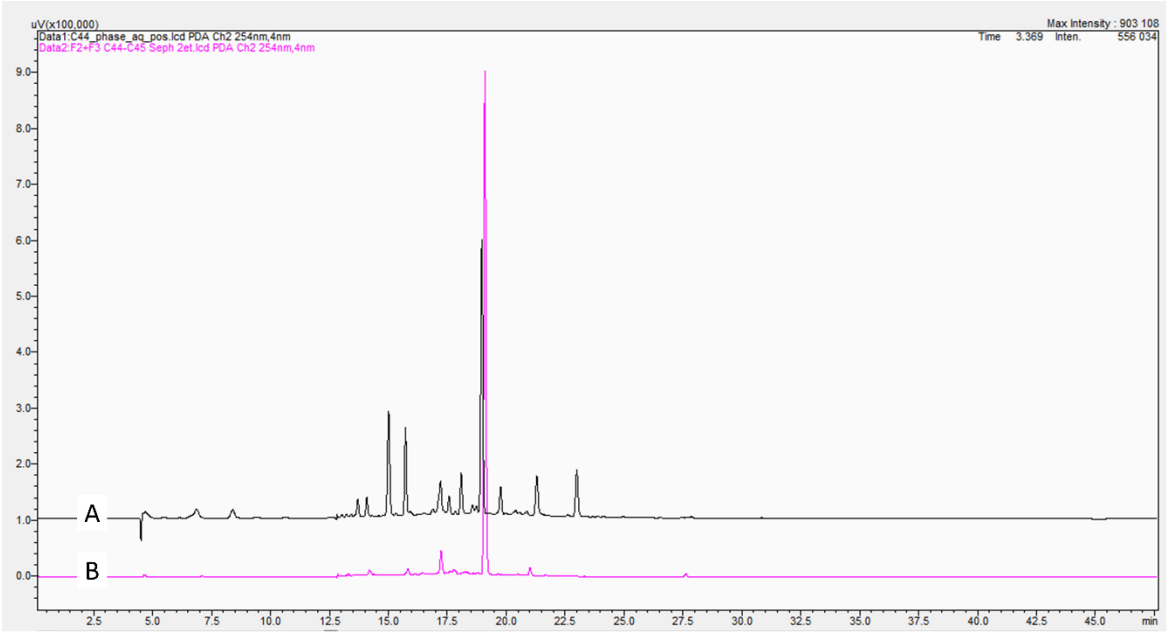


*Figure 34 Chromatogrammes de : A) extrait brut aqueux de la mono-culture de C44 et B) fraction F*2 de la co-culture de C44-C45*

Pour une meilleure optimisation de la purification, le fractionnement **de l'extrait organique brut la troisième série de co-culture** (2.1 g) (Fig. 30) a été réalisé par chromatographie flash en phase inverse, l'utilisation d'une phase normale n'ayant précédemment pas été satisfaisante, avec un mélange d'éluant ACN/H_2O suivant le gradient 6 (cf p. 170) et a permis l'obtention de 20 fractions de F1 à F20. La comparaison des chromatogrammes UV à 220 nm des extraits totaux de la co-culture et de la zone spécifiquement ciblée au niveau de la compétition a permis de mettre en évidence des pics uniquement présents en co-culture et en proportions plus importantes dans cette zone de confrontation (Fig.29). La fraction correspondant à ces pics, F12 (23.67 mg) a donc été purifiée par HPLC semi-préparative selon le protocole 5 pour obtenir 9 nouvelles sous-fractions F12-a à F12-i. Les sous-fractions F12-d (3.19 mg) et F12-e (1.5 mg) ont été observées comme étant celles contenant des produits d'intérêt (Fig. 35).

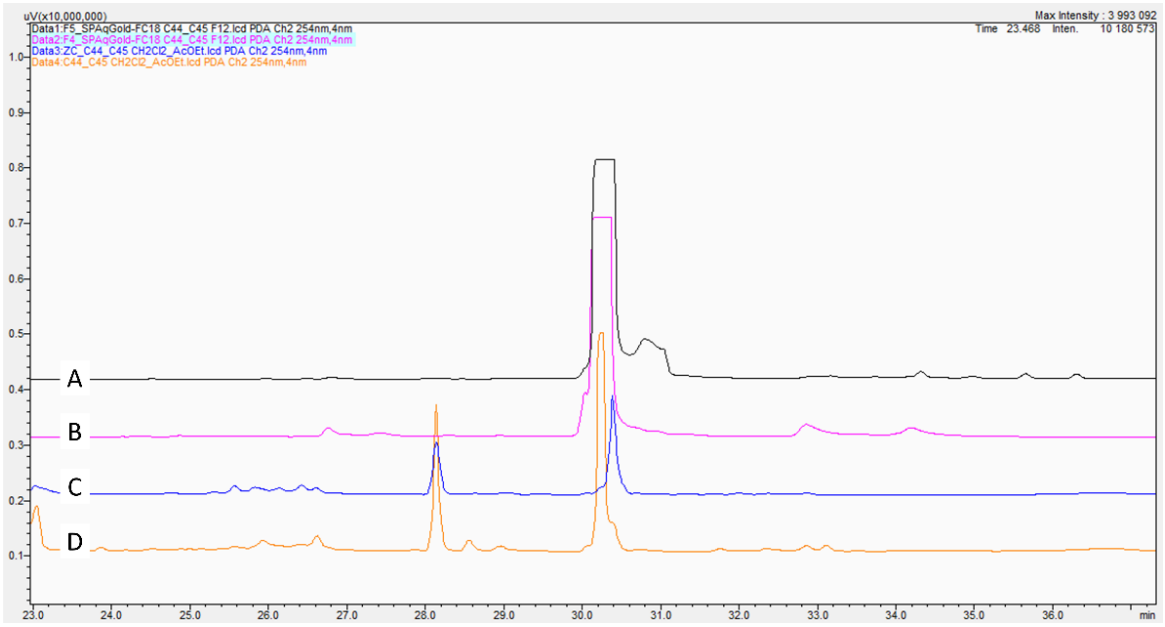


Figure 35 Chromatogrammes UV, λ à 254 nm : A) sous fraction F12-e ; B) sous-fraction F12-d : C) extrait brut de la zone de confrontation de la troisème série de co-culture C44-C45 et D) extrait brut total de la troisième série

La sous-fractinon F12-e nécessite une étape supplémentaire de purification avant une analyse pour déterminer sa structure chimique.

c. Elucidation structurale

- *Composé issu de la fraction FD4 - FD7*

L'analyse de spectre de masse basse résolution de type ESI en mode négatif indiquant la présence d'un atome de chlore (*m/z* 171.1 $[M - H]^-$ et *m/z* 365.0 $[2M + Na-2H]^-$) (Fig. 36) et suggérant une formule brute de $C_7H_6ClO_2N$ ainsi que l'analyse des spectres RMN ont permis d'identifier le composé comme étant l'acide-3-chloroanthranilique (T_r 23.3 min) (Fig. 37).

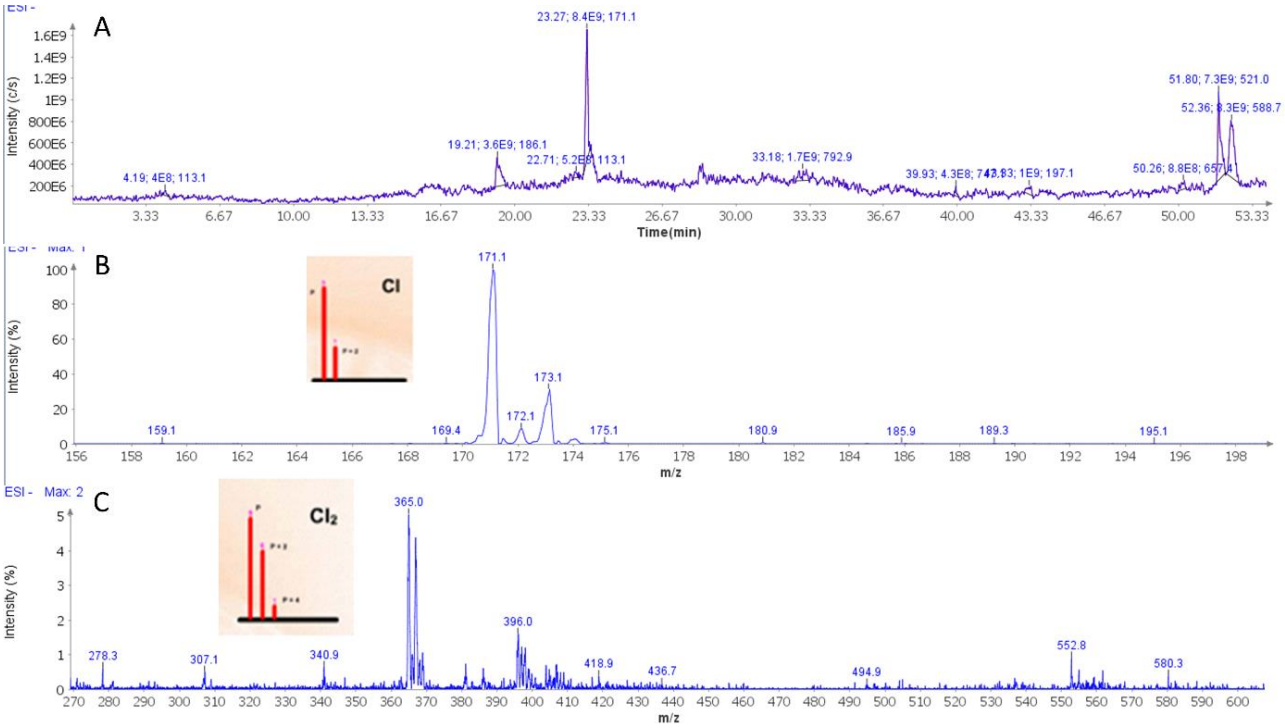


Figure 36 A) Chromatogramme et B) TIC de spectromètre de masse basse résolution de l'acide 3-chloroanthranilique

- *Composé issu de la fraction F*2*

Le composé de cette fraction a pu être caractérisé après comparaison des données avec la littérature comme étant l'énamidine (T_r 18.9 min) (Fig. 37) (Mandavid et al., 2015; Noriler et al., 2019; Yu et al., 2019).

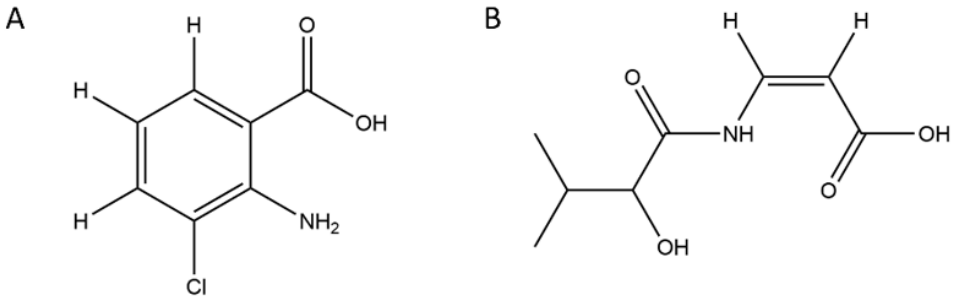


Figure 37 Structure de l'acide chloroanthranilique (9) et de l'énamidine (10)

- *Composé issu de la fraction F12-d*

Par manque de disponibilité de la RMN 500 MHz, le composé issu de la fraction F12-d n'a pas été encore analysée et le sera prochainement.

d. Evaluation des activités biologiques des composés isolés

Testée, l'énamidine, comme précédemment décrit ne présente pas de bioactivité envers les bactéries GRAM +. Dépeinte comme une molécule présentant une activité antibactérienne vis-à-vis de bactéries GRAM – (Mandavid et al., 2015), elle n'a ici, eu aucun effet sur *E. coli*. Au contraire, le composé issu de la fraction F12-d présente une activité antibactérienne intéressante vis-à-vis des GRAM + mais n'a pas d'effet sur les GRAM – (Tab. 10).

Tableau 10 Evaluation de l'activité antibactérienne de l'énamidine et du composé F12-d vis-à-vis de six bactéries pathogènes de l'homme (CMI exprimée en µg/mL)

Souches bactériennes	**Enamidine**	**F12-d**	**Gentamicine**	**DMSO**
Staphyloccocus aureus (CIP 53.156)	-	1.3356	3.91	-
S. aureus resistant (DSM 13661)	-	3.10	250	-
S. epidermidis (CIP 53.124)	-	0.39	125	-
S. saprophyticus	-	3.10	0.50	-
Streptococcus agalactiae	-	3.10	0.25	-
Escherichia coli (CIP 54.8)	-	-	3.91	-
Pseudomonas aeruginosa (CIP A22)	-	-	1.95	-

3. Etude mycochimique du couple *C. rusci* (C9) et *M. heredicola* (C11)

A. Etat de l'art bibliographique sur ces champignons

L'abondance de références bibliographiques concernant le champignon *Coccinectria ruscii* est très limitée voire inexistante : seulement trois publications sur la souche dont deux la décrivent comme un champignon possédant une activité ligninase augmentée (Anthony et al., 2021; Whalen et al., 2018). Concernant le nombre d'études trouvées portant sur le genre *Coccinectria*, le résultat est assez surprenant : seulement 25 références. Aucune d'entre elles ne traite de l'aspect chimique de ce genre.

Pour son partenaire de culture, là aussi, la recherche est bien maigre. Néanmoins, le genre *Melanconium* donne de meilleurs résultats. Si certaines espèces sont décrites comme des phytopathogènes (Mubarik et al., 2018; Robinson et al., 1978) d'autres sont décrites comme pouvant produire l'acide 3-hydroxypropionique possédant une activité nématicide (Schwarz et al., 2004).

B. Résultats

a. Analyse des extraits

Comme présenté pour les deux couples de champignons précédents, les deux séries de cultures fongiques ont été extraites avec un mélange de solvants composé de MeOH:CH_2Cl_2:AcOEt 1:2:3 + 0.1% d'AF. Les quantités des extraits obtenues sont indiquées dans le tableau 10 ci-dessous.

Une partition liquide/liquide a été faite sur l'extrait brut de la CC C9-C11 comme première étape de séparation. Au vu de la quantité d'extrait brut obtenu à partir de la phase aqueuse (400 mg), cette dernière a donc été choisie pour poursuivre les étapes de fractionnement. En effet, la masse de cet extrait est 50 fois plus importante que celle de l'extrait organique (8 mg) (Tab. 11).

Tableau 11 Quantités d'extraits (en mg) obtenues selon les séries de culture pour C9 -C11

	Extraits	Série	Quantité obtenue (mg)
Phase organique	C9_MC	1 : 20 boîtes	1.9
	C11_MC	1 : 20 boîtes	2.4
	C9-C11_CC	1 : 40 boîtes	8.0
	C9-C11_ZC	1 : 40 boîtes	10.3
	C9-C11_CC	2 : 100 boîtes	500
Phase aqueuse	C9_MC	1 : 20 boîtes	1.5
	C11_MC	1 : 20 boîtes	9.5
	C9-C11_CC	1 : 40 boîtes	400
	C9-C11_ZC	1 : 40 boîtes	129.6
	C9-C11_CC	2 : 100 boîtes	3400

b. Fractionnement des extraits de co-culture C9-C11

Comme indiqué sur le schéma de fractionnement (Fig. 38), l'**extrait brut aqueux de la première série** (400 mg) a été fractionné par chromatographie flash sur phase diol selon le gradient 3 permettant d'obtenir 23 fractions (F1 – F23).

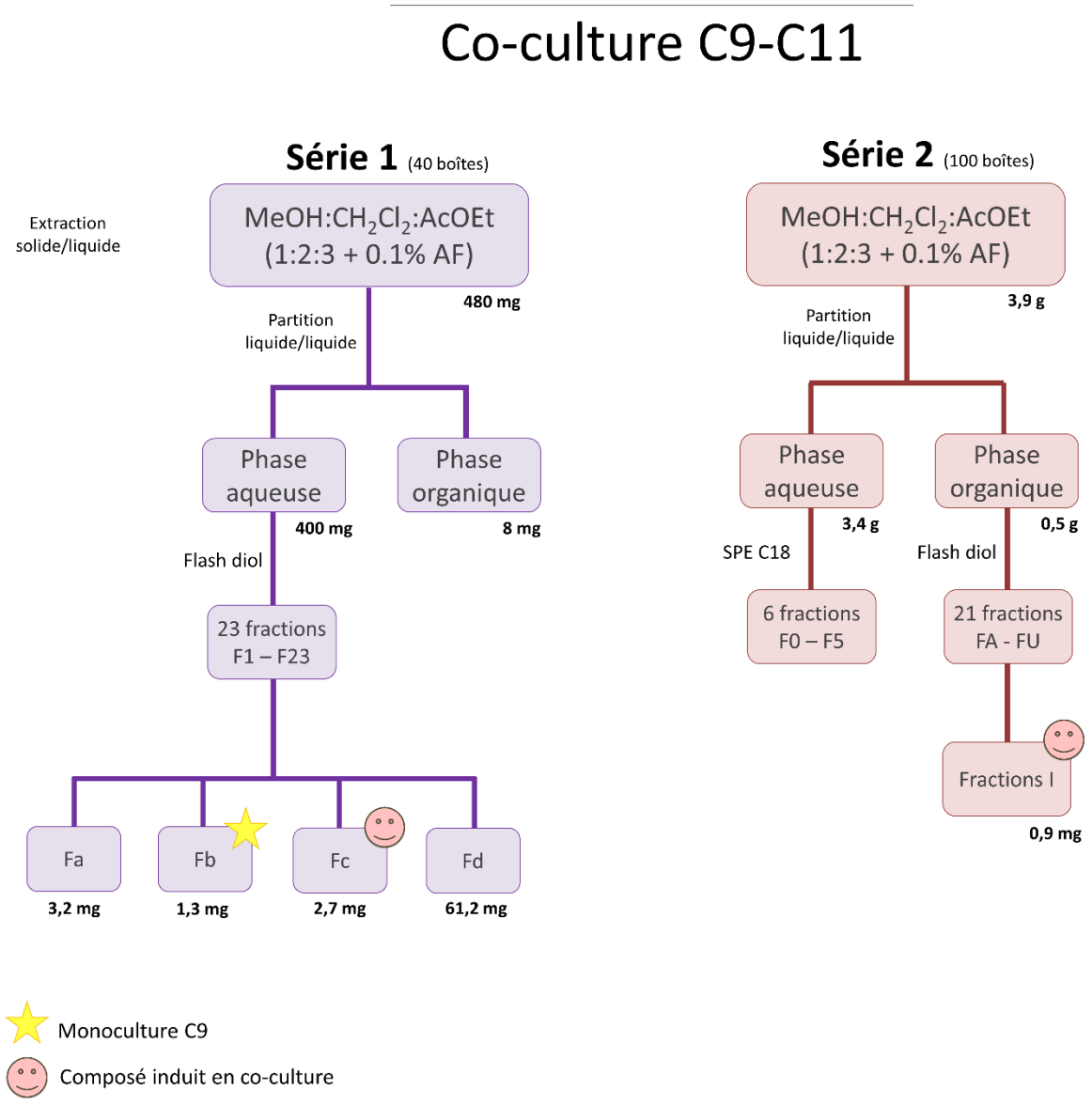


Figure 38 Fractionnement des extraits bruts de la co-culture C9-C11

Le suivi en HPLC-DAD nous a permis de regrouper des fractions et d'obtenir une fraction d'intérêt, Fc, dont le composé majoritaire ne semble être produit qu'en coculture (Fig. 39).

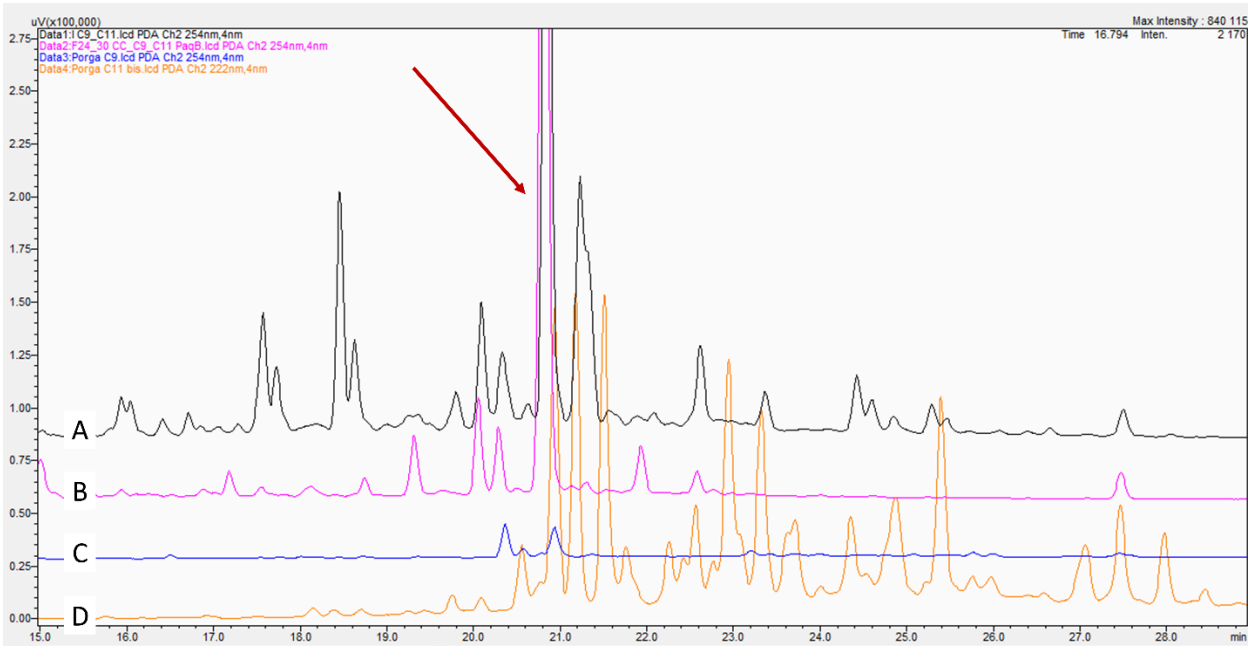


Figure 39 Comparaison des chromatogrammes : A) extrait de la co-culture de C9-C11 ; B) fraction Fc ; C) extrait brut de la mono-culture de C9 et D) extrait brut de la mono-culture de C11

Les autres fractions n'ont pu être plus exploitées toujours du fait des faibles quantités obtenues.

Ainsi, pour ce couple, **une deuxième série de co-culture** a été également réalisée, dont l'extrait brut s'élevait à 3.9 g. Un fractionnement par SPE C_{18}, selon le protocole 2 (cf p. 171), de l'extrait aqueux représentant la quasi-totalité de l'extrait brut total, a été entrepris menant à 6 fractions (FO à F5). Les 500 mg de l'extrait organique ont également été fractionnés une première fois grâce à une chromatographie flash sur phase diol, selon le gradient 2 (cf p. 169) donnant 21 fractions (FA à FU) dont la neuvième, en très faible quantité et riche, semble contenir le composé d'intérêt.

Par manque de temps, l'exploration chimique de ce couple de champignons n'a pas été plus approfondie.

iv. Conclusion

Les études préliminaires, motivées par des observations nous paraissant intéressantes, ont mis en évidence l'influence des conditions de culture sur les aspects phénotypiques et métaboliques des différentes souches fongiques étudiées. Une fois les différents paramètres sélectionnés (souches, milieux et support), des cultures à grande échelle ont été menées afin d'obtenir des quantités d'extraits bruts suffisants pour les étapes successives de fractionnement et de purification. Une dizaine de métabolites spécialisés ont été isolés de la co-culture C4-C5, pour la majorité basalement produits en mono-culture et non encore décrits. Ces molécules ont présenté des activités antibactériennes vis-à-vis de souches GRAM + et dans l'attente de résultats, de potentielles bioactivités antimicrobiennes à l'encontre de souches fongiques et bactériennes phytopathogènes et également des propriétés antivirales. L'isolement et la caractérisation structurale de ces molécules a été facilitée par l'utilisation d'outils innovants comme SMART ou encore MolNotator. La détermination de la configuration absolue a pu être finalisée par des outils de calculs de type TD-DFT pour prédire les spectres de CDE. Les études mycochimiques des deux autres couples fongiques étant moins avancées, il sera intéressant de continuer les isolements, caractérisations et tests d'activité biologique.
En perspective de ce travail, il serait également intéressant de pousser les investigations au niveau génomique en séquençant les génomes complets de ces champignons. Le genome mining permettrait une meilleure compréhension des produits naturels isolés dans cette étude, de leur évolution et de leurs potentiels rôles au sein de l'holobionte lichénique.

v. Matériels et méthodes

a. Solvants et réactifs

Tous les réactifs commerciaux ont été achetés chez Sigma Aldrich (Val de Reuil, France et St. Quentin Fallavier, France). Un système de purification d'eau EasyPure (Barnstead™, ThermoFisher Waltham, MA, USA) a été utilisé pour obtenir de l'eau de qualité HPLC et LC/MS pour l'analyse chromatographique. Les solvants deutérés ont été achetés chez Euriso-top (Gif-sur-Yvette, France).

b. Cultures fongiques et extraction

Les champignons *Dendrothyrium variisporum* (C4 ; numéro d'accession GenBank OL891605), *Xylaria hypoxylon* (C5 ; numéro d'accession GenBank OL891603), *Coccinonectria rusci* (C9 ; numéro d'accession GenBank OL891609), *Melanconium hedericola* (C11 ; numéro d'accession GenBank OL891612), *Michrodochium phragmitis* (C44 ; numéro d'accession GenBank OL891632) et *Tolypocladium* sp (C45 ; numéro d'accession GenBank OL891615) ont été précédemment isolés du lichen *R. geographicum* récolté dans le Finistère (Miral et al., 2022), France. Sous forme de précultures pendant 14 jours, *D. variisporum* et *X. hypoxylon* ont été maintenus individuellement sur milieu gélosé YS, *C. rusci* et *M. hedericola* sur milieu gélosé ISP2 et *M. phragmitis* et *Tolypocladium* sp. sur milieu gélosé YS.

Pour les co-cultures, des « plugs » de 5 mm de chaque culture pure ont été coupés et inoculés sur les côtés opposés de la même boîte de Pétri de 9 cm contenant environ 20 mL de milieu correspondant puis incubées à température ambiante pendant 14 jours. Les monocultures ont été menées de la même façon à la différence qu'un seul « plug » de préculture a été inoculé sur les boîtes.
Pour les essais en 6 puits comme pour les cultures à plus grande échelle ainsi que les zones de confrontation prélevées individuellement, les géloses ont été broyées et extraites deux fois avec un mélange de solvants (à raison de 250 mL de mélange pour 5 boîtes de Pétri) puis placées sous agitation à 120 tr/min pendant 60 min suivie de 60 min au bain à ultrasons. Le mélange a été filtré sous aspiration à travers un entonnoir Büchner en utilisant une étamine. L'extrait brut a été recueilli, séché avec du sulfate de sodium anhydre et filtré à travers un papier filtre, puis la phase organique a été évaporée sous vide afin d'obtenir un extrait sec organique.
Les mélanges de solvant utilisés ont été : soit MeOH:CH_2Cl_2:AcOEt 1:2:3 + 0.1% d'AF soit un mélange CH_2Cl_2:AcOEt (v:v).

c. Méthodes séparatives

- *Chromatographie de type flash*

Au cours des étapes de fractionnements, les séparations ont été effectuées par chromatographie flash sur un appareil PuriFlash de chez Interchim (Montluçon, France). Les phases stationnaires utilisées étaient des colonnes prépackées (CHROMABOND® Flash 15, 25 ou 40 g, Macherey-Nagel, Hoerdt, France) constituées de silice ou de silice greffée sous forme diol ou en C18 (phase inverse). Pour les séparations sur phase normale, les solvants utilisés étaient du *n*-hexane ou cyclohexane, du dichlorométhane, de l'acétate d'éthyle et du méthanol de qualité analytique. Pour les séparations sur phase inverse la phase mobile utilisée était constituée d'acétonitrile de qualité analytique et d'eau ultra-pure. Différents gradients ont été utilisés et sont indiqués ci-dessous. Chaque fraction obtenue après séparation a été analysée pour sa composition par analyse LC-ESI-MS selon les conditions décrites dans la partie méthode analytique.

Les gradients utilisés pour les fractionnements sur **phase diol** sont les suivants :

Gradient 1 : Phase mobile composée de A (*n*-hexane), B (CH_2Cl_2), C (EtOAc) et D (MeOH). Le gradient suivant a été appliqué à un débit de 20 mL/min : de 0 à 8 min : 100 % (A), de 8 à 16 min : 60 % (B)/40 % (A), de 16 à 24 min : 100% (B), de 24 à 32 min : 80% (B)/20% (C) ; de 32 à 40 min : 50 % (B)/50% (C) ; de 40 à 48 min : 20 % (B)/80 % (C) ; de 48 min à 56 min : 100% (C) ; de 56 à 64 min : 80 % (C)/20 % (D) ; de 64 à 72 min : 50 % (C)/50% (D) ; de 72 à 80 min : 20 % (C)/80 % (D) ; de 80 à 95 min : 100 % (D).

Gradient 2 : Phase mobile composée de A (*n*-hexane), B (CH_2Cl_2), C (EtOAc) et D (MeOH). Le gradient suivant a été appliqué à un débit de 20 mL/min : de 0 à 2 min : 100 % (A), de 2 à 8 min : 40 % (B)/60 % (A), de 8 à 16 min : 60% (B)/40% (A), de 16 à 24 min : 100% (B); de 24 à 32 min : 80 % (B) /20%(C); de 32 à 40 min : 50 % (B)/50 % (C) ; de 40 min à 48 min : 20% (B)/80% (C) ; de 48 à 56 min : 100 % (C) ; de 56 à 64 min : 80 % (C)/20 % (D) ; de 64 à 72 min : 50 % (C)/50 % (D) ; de 72 à 80 min : 20 % (C)/80 % (D) et de 80 à 90 min : 100% (D).

Gradient 3 : Phase mobile composée de A (CH_2Cl_2), B (EtOAc) et C (MeOH) et D (H_2O). Le gradient suivant a été appliqué à un débit de 20 mL/min : de 0 à 8 min : 100 % (A), de 8 à 16 min : 60 % (A)/40 % (B), de 16 à 24 min : 60% (A)/40% (B), de 24 à 32 min : 100% (B); de 32 à 40 min : 80 % (B) /20%(C); de 40 à 48 min : 50 % (B)/50 % (C) ; de 48 min à 56 min : 20% (B)/80% (C) ; de 56 à 64 min : 100 % (C) ; de 64 à 72 min : 80 % (C)/20 % (D) ; de 70 à 80 min : 50 % (C)/50 % (D) ; de 80 à 88 min : 20 % (C)/80 % (D) et de 88 à 96 min : 100% (D).

Le gradient utilisé pour les fractionnements sur **silice sphérique** est le suivant :

Gradient 4 : Phase mobile composée de A (cyclohexane), B (EtOAc), C (MeOH) et D (H_2O). Le gradient suivant a été appliqué à un débit de 20 mL/min : de 0 à 5 min : 100 % (A), de 5 à 6 min : 10 % (B)/90 % (A), de 6 à 10 min : 40% (B)/60% (A), de 10 à 16 min : 60% (B)/40% (A) ; de 16 à 24 min : 100 % (B) ; de 24 à 32 min : 80 % (B)/20 % (C) ; de 32 min à 40 min : 50% (B)/50% (C) ; de 40 à 48 min : 20 % (B)/80 % (C) ; de 48 à 56 min : 100 % (C) ; de 56 à 64 min : 80 % (C)/20 % (D) ; de 64 à 80 min : 50 % (C)/50 % (D).

Gradient 5 : Phase mobile composée de A (CH_2Cl_2), B (EtOAc), C (MeOH) et D (H_2O). Le gradient suivant a été appliqué à un débit de 20 mL/min : de 0 à 5 min : 100 % (A), de 5 à 6 min : 10 % (B)/90 % (A), de 6 à 10 min : 40% (B)/60% (A), de 10 à 16 min : 60% (B)/40% (A) ; de 16 à 24 min : 100 % (B) ; de 24 à 32 min : 80 % (B)/20 % (C) ; de 32 min à 40 min : 50% (B)/50% (C) ; de 40 à 48 min : 20 % (B)/80 % (C) ; de 48 à 56 min : 100 % (C) ; de 56 à 64 min : 80 % (C)/20 % (D) ; de 64 à 80 min : 50 % (C)/50 % (D).

Pour la **phase inverse** les cartouches CHROMABOND® Flash RS 80 C_{18}ec (Macherey-Nagel, Allemagne) ont été utilisés avec le gradient 6 suivant : phase mobile composée de A (H_2O), B (ACN). Le gradient suivant a été appliqué à un débit de 35 mL/min : de 0 à 5 min : 90 %(A)/10%(B), de 5 à 45 min : 100 % (B), de 45 à 105 min : 100% (B).

- *Chromatographie sur colonne de silice ouverte*

Afin de préparer la silice en vue d'un fractionnement sur colonne ouverte, préparer 40 mg de silice, préalablement séchée à l'étuve, pour 1 mg d'extrait. La colonne a été

conditionnée en mode gradient en passant de 100% de dichlorométhane en jusqu'à 100% de MeOH. De l'ammoniaque est ajoutée à la fin jusqu'à l'obtention d'un mélange de 95% MeOH/ 5% NH_4OH.

- *Séparation par extraction sur phase solide (SPE)*

Les cartouches SPE CHROMABOND® C_{18}ec 6 mL, 1 g (Macherey-Nagel, Allemagne) ont été utilisées. Chaque fraction a été analysée pour sa composition par analyse HPLC-ESI-MS selon les conditions décrites dans la partie méthode analytique.

Protocole1 : Les cartouches ont été conditionnées avec 6 mL de MeOH puis 6 mL d'H_2O. L'échantillon est chargé sur la colonne puis élué avec 15 mL d'eau milliQ suivi d'une deuxième élution avec un mélange de 10 mL H_2O:MeOH (v:v), une troisième élution avec 10 mL de MeOH puis une dernière élution avec 10 mL d'AcOEt. Ces étapes sont répétées une nouvelle fois.

Protocole 2 : Les cartouches ont été conditionnées avec 20 mL de MeOH puis 20 mL d'H_2O. L'échantillon est chargé sur la colonne puis élué avec 35 mL d'eau milliQ suivi d'une deuxième élution avec un mélange de 60 mL H_2O:MeOH (1:1), une troisième élution avec 45 mL de MeOH. Ces étapes sont répétées une nouvelle fois.

- *Chromatographie par filtration sur gel*

Une colonne Sephadex™ LH-20 a été utilisée avec pour mélange de solvants d'élution CH_2Cl_2:AcOEt:MeOH:AF (70:20:5:1 ; v:v:v:v). Chaque fraction a été analysée pour sa composition par analyse LC-ESI-MS selon les conditions décrites dans la partie méthode analytique.

- *HPLC semi-préparative*

La purification des composés issus des fractionnements précédents a été menée sur une chaîne HPLC - Diode Array Detector (LC-DAD) (Shimadzu, Marne La Vallée, France)

Différents protocoles ont été utilisés. Chaque fraction a été analysée pour sa composition par analyse HPLC-ESI-MS selon les conditions décrites dans la partie méthode analytique.

Protocole 1 : Séparation sur colonne Grace en phase inverse Prevail C18-Select (250 x 3.0, 5 µm) et un système de gradient a été appliqué : A (H_2O + 0,1 % AF) et B (ACN + 0,1 % AF) à un débit de 2.5 mL/min dans le système HPLC initial : 100 % (A) ; de 0 à 5 min : 100 % (A) ; de 5 à 35 min : de 100 %(A) à 100 % (B) ; de 35 à 45 min 100 % (B), de 45 à 50min 100%(B) à 100%(A), de 50 min jusqu'à 60 min 100 % (A).

Protocole 2 : Séparation sur ThermoFisher Scientific en phase inverse Hypersil GOLD aQ (250 x 20, 5 µm) et un système de gradient a été appliqué : A (H_2O + 0,1 % AF) et B (ACN + 0,1 % AF) à un débit de 8 mL/min dans le système HPLC : initial : 100 % (A) ; de 0 à 5 min : 100 % (A) ; de 5 à 10 min : de 100 % (A)/0 % (B) à 40 %(A)/60%(B), de 10 à 48 min : de 40 %(A)/60 %(B) 30 % (A)/70 % (B) ; de 48 à 50 min : 30 % (A)/70 % (B) à 0 %(A)/100%(B); de 50 à 55 min : 100 % (B), de 55 à 60 min : 100 % (B) à 100%(A) ; de 60 à 70 min : 100 % (A).

Protocole 3 : Séparation sur colonne Grace en phase inverse Prevail C18-Select (250 x 3.0, 5 µm) et un système de gradient a été appliqué : A (H_2O + 0,1 % AF) et B (ACN + 0,1 % AF) à un débit de 2 mL/min dans le système HPLC : initial : 100 % (A) ; de 0 à 5 min : 100 % (A) ; de 5 à 30 min : de 100 % (A)/0 %(B) à 50 % (A)/50% (B) ; de 30 à 35 min 50 % (A)/50 % (B); de 35 à 45 min : 50 % (A)/50 % (B) à 100 % (B); de 45 à 55 min : 100 % (B), de 55 à 60 min : 100 % (B) à 100% (A), de 60 à 65 min 100% de (A).

Protocole 4 : Séparation sur colonne Grace en phase inverse Prevail C18-Select (250 x 3.0, 5 µm) et un système de gradient a été appliqué : A (H_2O + 0,1 % AF) et B (ACN + 0,1 % AF) à un débit de 2 mL/min dans le système HPLC : initial : 100 % (A) ; de 0 à 8 min : 100 % (A) ; de 8 à 20 min : de 100 %(A) à 80 % (A)/20%(B) ; de 20 à 30 min : 80 % (A)/20 % (B); de 30 à 32 min : de 80 % (A)/20 % (B) à 75%(A)/25%(B) ; de 32 à 40 min : de 75%(A)/25%(B) à 55%(A)/45%(B) ; de 40 à 45 min : 55 % (A)/45%(B) , de 45 à 50 min : de 55 % (A)/45%(B) à 0%(A)/100 % (B) ; de 50 à 55 min 100% de (B) ; de 55 à 60 min : 100%(B) à 100%(A), de 60 à 65 min 100%(A).

Protocole 5 : Séparation sur colonne ThermoFisher Scientific en phase inverse Hypersil GOLD aQ (250 x 20, 5 µm) et un système de gradient a été appliqué : A (H_2O + 0,1 % AF) et B (ACN + 0,1 % AF) à un débit de 8 mL/min dans le système HPLC : initial : 100 % (A) ; de 0 à 5 min : 100 % (A) ; de 5 à 10 min : 100 % (A)/0 % (B) à 45 % (A)/55 % (B) ; de 10 à 38 min : 45 % (A)/55 % (B) à 30 % (A)/70 % (B) ; de 38 à 43 min : 30 % (A)/70 % (B) à 100 % (B) ; de 43 à 48 min : 100 % (B) ; de 48 à 50 min : de 100 %(B) à 100% (A) et de 50 à 65 min 100 %(A).

d. Méthodes analytiques

- *HPLC-ESI-MS*

Les analyses HPLC ont été réalisées sur un système HPLC – Diode Array Detector (LC-DAD) (Shimadzu, Marne La Vallée, France). à l'aide d'une colonne ThermoFisher Scientific en phase inverse Hypersil GOLD aQ (250 x 4,6, 5 µm) et un système de gradient a été appliqué : A (H_2O + 0,1 % AF) et B (ACN + 0,1 % AF). Le gradient suivant a été appliqué à un débit de 0,8 mL/min dans le système HPLC : initial : 100 % (A) ; de 0 à 5 min : 100 % (A) ; de 5 à 35 min : 100 % (A)/0 % (B) à 0 % (A)/100 % (B) ; de 35 à 45 min : 100 % B ; de 45 à 50 min : 100 % (A)/0 % (B) à 0 % (A)/100 % (B) ; de 50 à 55 min : 100 % (A). Vingt microlitres d'échantillons de concentration à 2 mg. ml^{-1} pour les analyses des mono- ou co-cultures à petite échelle (plaques 6 puits, boîte séparée et co-cultures sur 40 boites et 100) et à grande échelle (350 ou 440 boites) à 5 $mg.ml^{-1}$ ont été injectés.
Pour les analyses en spectrométrie de masse de l'acide formique a été ajouté à hauteur de 0,1% dans chaque solvant. Le débit utilisé est de 0,8 $mL.min^{-1}$ et un split en sortie du détecteur UV permet de limiter le débit arrivant dans le spectromètre de masse à 0,2 $mL.min^{-1}$. L'analyse par spectrométrie de masse est effectuée en mode ESI (+ ou -) par un appareil expression CMS de chez Advion (Ithaca, NY, USA). Le traitement des données est réalisé sur le logiciel LabSolutions pour les données de chromatographie et le logiciel Advion pour les données de masse.

- *UHPLC-MS²*

Les extraits bruts ont été analysés à l'aide d'un UHPLC Vanquish couplé à un spectromètre de masse (MS) Thermo Q-Exactive (Thermo Fisher Scientific GmbH, Brême, Allemagne) et à une source ESI fonctionnant avec le logiciel Xcalibur (version 2.2, ThermoFisher Scientific). La séparation des métabolites a été réalisée sur une colonne ThermoFisher Scientific Hypersil GOLD (150 x 2,1 mm, 1,9 µm) avec un volume d'injection de 5 µL et un débit de 0,2 $mLmin^{-1}$. La phase mobile était composée de H_2O + 0,1 % AF et d'ACN + 0,1 % AF et le gradient suivant a été utilisé : H_2O:ACN 90:10 pendant 5 min, H_2O:ACN 90:10 à 0:100 pendant 30 min, 0:100 pendant 5 min, 0:100 à 90:10 pendant 15 min. L'acquisition des données a été réalisée sous ionisation en mode de commutation de balayage complet (positif et négatif) de *m/z* 1200 à 70000 à une résolution de 70 000.

- *RMN*

Les spectres RMN 1D (1H et 13C) et 2D (COSY, HSQC, HMBC et NOESY) ont été obtenus sur un spectromètre Bruker® 500 MHz (Bruker® Billerica,MA, USA) avec une cryosonde TCI, présent sur la plate-forme centrale de PRISM (Biosit, Rennes, France). Tous les spectres ont été acquis dans de l'acétone-d_6 et les spectres additionnels avec du benzène-*d6*. Les déplacements chimiques (□) et les constantes de couplage (*J*) sont exprimés en partie par million (ppm) et en Hertz (Hz) respectivement. La multiplicité des signaux est indiquée comme suit : s (singulet), bs (singulet large), d (doublet), t (triplet), m (multiplet), dd (doublet dédoublé), tapp (triplet apparent) etc. Les données ont été traitées avec le logiciel MestReNova.

- *Pouvoir rotatoire*

Les pouvoirs rotatoires ont été enregistrés à l'aide d'un polarimètre automatique PerkinElmer 341 à 293 K à la raie D du sodium (589 nm). Ce pouvoir rotatoire est ensuite déterminé à partir de la formule ci-dessous : $\mathbf{[\alpha_D^{20}] = \alpha\ mesuré\ /\ (l \times c)}$
Avec l : longueur de la cuve en dm ; *c* : concentration de la solution en $g.ml^{-1}$; $[\alpha_D^{20}]$ en $°.dm^{-1}.g^{-1}.ml$

- *Dichroïsme circulaire électronique*

Le dichroïsme circulaire a été réalisée dans le MeOH sur un spectromètre JASCO J-815 ECD avec une longueur de trajet de cuvette de 0, 5 mm. Les moyennes des scans ont été acquises en triple et le signal CD du MeOH a été soustrait par la suite.

- *Spectrométrie de masse Haute résolution (HRMS)*

Les mesures HRMS pour la détermination exacte de la masse ont été réalisées sur le spectromètre de masse de type Q-Exactive de chez ThermoFisher (Waltham, MA, USA) qui est un hybride entre un quadripôle et un orbitrap pour l'ionisation par électrospray au CRMPO (Centre Régional de Mesures Physiques de l'Ouest), Université de Rennes 1.

e. Descriptif des produits isolés

(**1**), **xylarine A** : structure cristalline amorphe ; $[\alpha]_D$ + 70.3 (*c* 0.2, MeOH); les données ^{1}H et ^{13}C (500 MHz) sont reportées dans le tableau 7 ; HR-ESIMS *m/z* 403.2124 $[M-H]^-$ (calcd pour $C_{23}H_{31}O_6$ 403.21261).

(**2**), **xylarine B** : structure cristalline amorphe ; $[\alpha]_D$ + 20.0 (*c* 0.1, MeOH); les données ^{1}H et ^{13}C (500 MHz) sont reportées dans le tableau 7 ; HR-ESIMS *m/z* 427.2125 $[M-H]^-$ (calcd pour $C_{25}H_{31}O_6$ 427.21261).

(**3**), **xylarine C** : poudre amorphe ; $[\alpha]_D$ + 26.3 (*c* 0.8, MeOH) ; les données ^{1}H et ^{13}C (500 MHz) sont reportées dans le tableau 7 ; HR-ESIMS *m/z* 429.2282 $[M-H]^-$ (calcd pour $C_{25}H_{33}O_6$ 427.22826).

(**4**), **xylarine D** : structure cristalline amorphe ; $[\alpha]_D$ + 20.0 (*c* 0.3, MeOH) ; les données ^{1}H et ^{13}C (500 MHz) sont reportées dans le tableau 7 ; HR-ESIMS *m/z* 441.2282 $[M-H]^-$ (calcd pour $C_{26}H_{33}O_6$ 441.22826).

(**5**), **xylarine E** : structure cristalline amorphe ; $[\alpha]_D$ + 14.0 (*c* 0.1, MeOH) ; les données ^1H et ^{13}C (500 MHz) sont reportées dans le tableau 7 ; HR-ESIMS *m/z* 443.2352 $[M-H]^-$ (calcd pour $C_{26}H_{35}O_6$ 443.24391).
(**6**), **xylarine F** : poudre amorphe ; $[\alpha]_D$ + 100.9 (*c* 0.2, MeOH) ; les données ^1H et ^{13}C (500 MHz) sont reportées dans le tableau 7 ; HR-ESIMS *m/z* 443.2439 $[M-H]^-$ (calcd pour $C_{26}H_{35}O_6$ 443.24391).
(**7**), **xylarine G** : poudre amorphe $[\alpha]_D$ + 10.0 (*c* 0.1, MeOH) ; es données ^1H et ^{13}C (500 MHz) sont reportées dans le tableau 7 ; HR-ESIMS *m/z* 455.2438 $[M-H]^-$ (calcd pour $C_{27}H_{35}O_6$ 455.24391).

(**8**) **F10: massarilactone H** : poudre amorphe ; 1H NMR (300 MHz, DMSO) δ 5.82 (m, 1H), 5.62 (d, *J* = 5.2. Hz, 1H), 5.53 (d, *J* = 4.3 Hz, 1H), 5.19 (d, *J* = 3.2. Hz, 1H), 5.07 (d, *J* = 3.2 Hz, 1H), 4.90 (dd, *J* = 8.2, 1.6 Hz, 1H), 4.17 (brs, 1H), 3.72 (q, *J* = 4.6 Hz, 1H), 1.67 (d, *J* = 5.6 Hz, 3H). ^{13}C RMN (75 MHz, DMSO) δ 18.7, 61.4, 71.2, 85.1, 93.4, 103.1, 126.0, 131.8, 147.9, 163.2.

(**9**) **acide chloroanthranilique** : Tr à 23.1 min ; ESI mode négatif basse résolution *m/z* 171 ^1H RMN (500 MHz, CD_3OD) δ 7.77 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.47 (dd, *J* = 7.9, 3.5 Hz, 1H), 6.79 (t, *J* = 7.9, 7.9 Hz, 1H).

(**10**) **enamidine** : Tr à 18.9 min ; ESI mode négatif basse résolution *m/z* 186 ; ^1H RMN (500 MHz, CD_3OD) δ 7.38 (d, *J* = 9.0 Hz, 1H), 5.14 (d, *J* = 9.0 Hz, 1H), 3.98 (*d*, J = 3.4 Hz, 1H), 2.14 (m, 1H), 1.25 (m, 1H), 0.98 (d, *J* = 6.8 Hz, 1H), 0.82 (d, *J* = 6.8 Hz, 1H).

f. Traitement de données UHPLC-Orbitrap par MZmine et réalisation de réseaux moléculaires

L'acquisition des données a été réalisée en mode de commutation de balayage complet (positif et négatif) de m/z 130 à 1200 à une résolution de 70 000. Les fichiers bruts obtenus par UHPLC-ESI-MS^2 du format propriétaire de Thermo ont d'abord été convertis en mzXML (Pedrioli et al., 2004) à l'aide du module MSConvert (Chambers et al., 2012) de Proteowizard (Kessner et al., 2008) puis importés et traités à l'aide de MZmine 2.53 (Pluskal et al., 2010).

Les paramètres MZmine utilisés, pour les deux modes, étaient les suivants, que ce soit pour les données en mode positif ou négatif.

Module	**Paramètres**
Raw data import	Importation de tous les fichiers mzXML
Raw data methods > Mass detection	Scans: MS level: 1 Mass detector: centroid Noise level: 2.0E4 Mass list name: masses
Raw data methods > Mass detection	Scans: MS level: 2 Mass detector: centroid Noise level: 0 Mass list name: masses
Peak list methods > Peak detection > ADAP Chromatogram builder (Myers et al., 2017)	Scans: MS level: 1 Mass list: masses Min group size in # of scans: 5 Group intensity threshold: 5.0E5 Min highest intensity: 5.0E5 m/z tolerance: 15 ppm
Peak list methods > Peak detection > Chromatogram deconvolution	Algorithm: Wavelets (ADAP) S/N threshold: 50 S/N estimator: Intensity window SN Min feature height: 5.0E5 Coefficient/area threshold: 150 Peak duration range: 0.01 – 10.00 RT wavelet range: 0.01 – 0.30 m/z center calculation: MEDIAN m/z range for MS2 scan pairing (Da): 0.002 Rt range for MS2 scan pairing (min): 0.3
Peak list methods > Isotopes > Isotopic peak grouper	m/z tolerance: 0.002 m/z or 15.0 ppm

	Retention time tolerance (min): 0.3 absolute (min) Maximum charge: 4 Representative isotope: lowest m/z
Peak list methods > Aligment > Join aligner	m/z tolerance: 0.002 or 15 ppm Weight for m/z: 1 Retention time tolerance: 0.3 absolute (min) Weight for RT: 1 Require same charge state: checked
Peak list methods > Gap filling > Peak finder	Intensity tolerance: 90% m/z tolerance: 0.02 m/z or 15 ppm Retention time tolerance: 0.2 absolute (min) Rt correction: checked
Peak list methods > Filtering > Duplicate peak filter	Filter mode: new average m/z tolerance: 0.002 m/z or 15 ppm Retention time tolerance: 0.2 absolute (min)
Peak list methods > Filtering > Peak list rows filter	Keep only peaks with MS2 scan: checked Reset the peak number ID: checked
Peak list methods > Export/Submit to GNPS-FBMN	Merge MS/MS: checked Select spectra to merge: across samples m/z merge mode: weight average (remove outliers) intensity merge mode: sum intensities Expected mass deviation: 0.002 m/z or 15 ppm Cosine threshold: 60% Peak count treshold: 40% Isolation window offset: 0.000 m/z Isolation window width: 3.00 m/z Filter rows: all
Peak list methods > Export for SIRIUS	Merge MS/MS: checked

Select spectra to merge: across samples
m/z merge mode: weight average (remove outliers)
intensity merge mode: sum intensities
Expected mass deviation: 0.002 m/z or 15 ppm
Cosine threshold: 60%
Peak count treshold: 40%
Isolation window offset: 0.000 m/z
Isolation window width: 3.00 m/z
Filter rows: all

A l'issue du traitement, les fichiers MGF sont exportés de MZmine et importés dans SIRIUS 4.9.5 (Dührkop et al., 2019) et traités pour les formules moléculaires à l'aide des paramètres suivants : Adduct = [M+ ?], SIRIUS : Instrument = Orbitrap, Filter by isotope pattern = unchecked, MS/MS isotope scorer = Ignore, MS2 MassDev (ppm) = 5, Candidates = 3, Candidats par ion = 1, Consider only formulas in DBs = All but combinatorial DBs.

Les fichiers MGF et CSV exportés de MZmine avec la sortie SIRIUS ont été traités à l'aide de la bibliothèque Python MolNotator (Olivier-Jimenez et al., 2021). La déréplication a été réalisée à l'aide des bibliothèques spectrales GNPS et des bases de données COCONUT (Sorokina et al., 2021) et LOTUS (Rutz et al., 2021).

g. Analyses computationnelles

Les coordonnées du conformère le plus faible en énergie des composés **1**-**7** ont été optimisées par TD-DFT de type B3LYP/6-31G(d) (Becke, 1993; Lee et al., 1988; Shimkevich, 2014). L'analyse vibrationnelle dans l'approximation harmonique a été effectuée au même niveau de théorie après convergence de l'optimisation géométrique, et le minimum local a été caractérisé par l'absence de fréquence imaginaire. Les énergies d'excitation et les forces rotationnelles correspondantes pour les 60 premières transitions électroniques ont ensuite été calculées par la méthode TD-DFT (Cai and

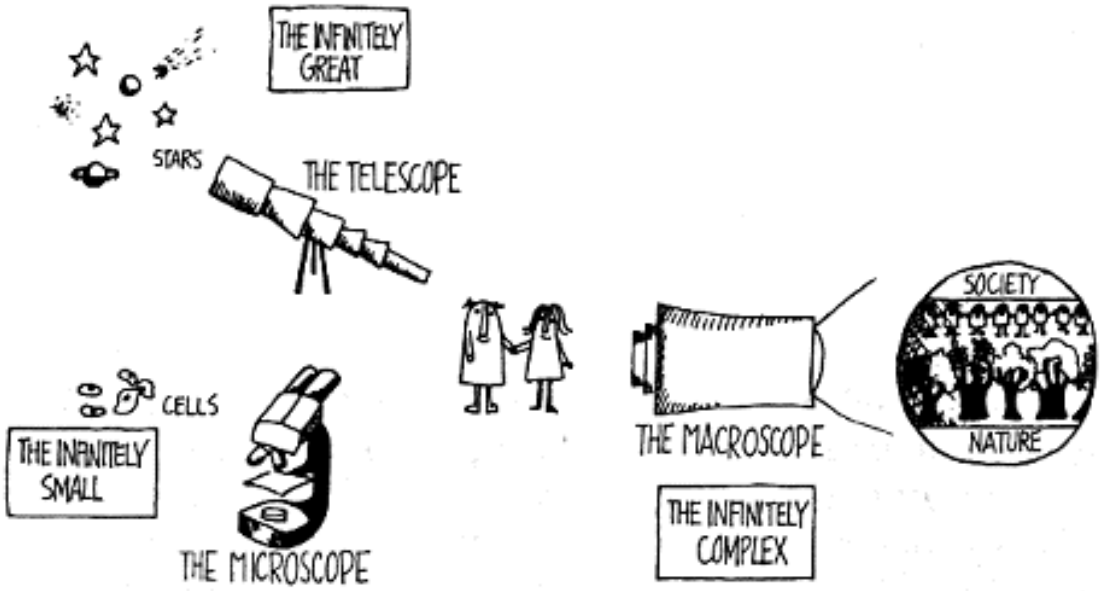
Reimers, 2000) au niveau B3LYP/6-31G*. Le spectre DCE a été calculé par la sommation de fonctions gaussiennes (Stephens and Harada, 2010) à l'aide du logiciel SpecDis v1.64 (Bruhn et al., 2013). Les valeurs de bandes passantes (□) et les éventuels décalages hypsochromiques ou bathochromiques (le cas échéant) sont indiqués sur les graphiques montrant la superposition des spectres DCE expérimentaux et calculés par la méthode TD-DFT pour tous les composés **1**-**7**.

h. Tests biologiques

- *Activités antibactériennes*

Les tests d'activité antibactérienne ont été effectués en utilisant la technique de dilution en bouillon dans des microplaques à 96 puits en triple exemplaire. Toutes les souches de *Staphyloccocus aureus* sensibles et résistantes (CIP 53.156 et DSM 13661), *S. epidermidis* (CIP 53.124), *S. saprophyticus*, *Escherichia coli* (CIP 54.8), *Streptococcus agalactiae* et *Pseudomonas aeruginosa* (CIP A22) ont été fournies par l'équipe NuMeCan (Nutrition, Métabolismes et Cancer), Université de Rennes 1. Elles ont été cultivées à 37°C en utilisant le milieu Luria Broth (LB) (2,5 g de peptone, 2,5 g de NaCl, 1,5 g d'extrait de levure et 500 mL d'eau distillée). Les composés ont été préparés dans du DMSO biologique (D2650 Sigma Aldrich) à 5 mg/mL pour les composés testés et 10 mg/mL pour la gentamicine (G1272 – 10 mL Sigma Aldrich) comme témoin positif. Selon le Clinical and Laboratory Standards Institute (CLSI) (National Committee for Clinical Laboratory Standards, 2004), les composés ont été dilués en série 1 : 2 dans du LB dans une plaque stérile à 96 puits. Chaque puits a ensuite été inoculé avec 10^6 UFC/mL de chaque souche pendant 24 h à 37 °C. Les solvants utilisés pour préparer les composés ont également été testés sur les bactéries comme contrôle négatif. Tous les puits ont ensuite été étalés sur gélose LB et incubés pendant 24h à 37°C. Des mesures de densité optique ont été effectuées à 630 nm à l'aide d'un lecteur Allsheng AMR100 (Hangzhou Allsheng instruments Co., Ltd., Chine) avant et après incubation pour obtenir des valeurs d'inhibition de la croissance. Par la suite, la concentration minimale inhibitrice (CMI), définie comme la concentration minimale capable d'inhiber la croissance bactérienne visible, a été déterminée comme le puits clair ayant la plus petite concentration.

DISCUSSION & PERSPECTIVES



DISCUSSION & PERSPECTIVES

Les lichens sont des organismes complexes et représentent des écosystèmes autonomes, définis comme étant des holobiontes (Hawksworth and Grube, 2020) et retrouvés dans tous les habitats terrestres. Ils contribuent de manière significative au cycle minéral et au flux d'énergie à l'échelle mondiale. Le microbiote associé à la symbiose, isolé dès le début du XX^{ème} siècle et étudié de manière approfondie depuis peu, se dévoile comme étant très riche et diversifié, comptant en son sein bactéries, champignons et même virus, et présentant de potentielles activités biologiques (Grimm et al., 2021).

Au cours de ces trois années de travaux de thèse, les études menées ont étayé le fait que les lichens sont des niches prospères dans lesquelles il reste encore de nombreuses zones d'ombre qui méritent d'être mises en exergue. Nous nous sommes intéressés à l'étude de la microflore associée à un lichen encroutant, *Rhizocarpon geographicum,* communément retrouvé dans des habitats très différents (environnements montagneux, littoraux) (Armstrong, 2011) et très peu étudié, notamment du fait de son adhérence forte au substrat qui rend l'obtention de quantités substantielles de thalle lichénique très difficile.

Retranscrite dans le Chapitre II, l'étude de la communauté microbienne et notamment fongique associée à *R. geographicum*, même si vraisemblablement encore très parcellaire, a révélé une importante richesse et variété, et ce malgré le faible échantillon étudié. Nous avons ainsi isolé 68 isolats fongiques répartis dans 43 groupes phylogénétiques, 15 isolats bactériens répartis dans 5 groupes taxonomiques et 3 microalgues appartenant à 2 espèces. Il est intéressant de souligner, d'un côté, que 12 isolats fongiques sont apparus nouveaux (aucune correspondance dans GenBank) et d'autre part, les souches bactériennes ont été majoritairement représentées par *Paenibacillus etheri*, une bactérie précédemment décrite comme pouvant dégrader des dérivés *tert*-butyl ether (Guisado et al., 2016). Un second isolement, suivant une approche culturomique (neuf différents milieux ; classiques, supplémentés en lichen et/ou en algue et en antibiotiques ; étalement après enrichissement et dilutions) a permis de confirmer cette abondance et cette diversité à travers l'isolement massif de plus de 1900 bactéries à partir de 24 échantillons provenant de différents écosystèmes, maritimes, terrestres et de basse montagne. Parmi toutes ces bactéries, 1063 ont pu être amplifiées et caractérisées dont 696 ont été

identifiées comme étant uniques et 3 n'ayant aucune correspondance sur la base de l'ARN 16S dans GenBank. Fait intéressant, parmi les bactéries isolées lors de ces travaux, une seule est commune avec l'étude précédemment décrite, *Caballeronia mineralivorans.* Il est à noter qu'elle n'a pas été retrouvée dans la population de Crozon mais celles de Trégastel *et Baulon* Les genres *Paenibacillus* et *Lichenibacterium* sont également retrouvés partagés. Les milieux spécialement conçus avec de l'extrait de lichen et d'algues ont permis d'isoler des bactéries rarement isolées habituellement. De plus, les milieux supplémentés en antibactériens ont mené à l'isolement de 126 bactéries résistantes aux antibiotiques dont 87 ont présenté une qualité de séquence 16S correcte. Deux d'entre elles, *Pseudomonas gessardii* et *Pseudomonas frederiksbergensis,* ont aussi montré une activité contre une bactérie pathogène de l'homme, *S. aureus* lors de tests préliminaires. Afin de mieux comprendre les bases génétiques de l'activité de cette résistance aux antibiotiques, après le séquençage complet du génome de onze bactéries, nous avons étudié la présence de protéines de résistance aux antimicrobiens (AMR) chez 9 des bactéries en utilisant AMRFinder Plus (Feldgarden et al., 2021) permettant une évaluation précise du contenu en gènes AMR. Contre toute attente, le profil de AMR a été défini par la provenance des lichens en suggérant que des stratégies antimicrobiennes spécifiques sont adoptées au niveau local/habitat.

D'autre part, trois des sites d'échantillonage, Crozon, Trégastel et Carolles, ont subi d'importantes marées noires, le Torrey Canyon en 1967, l'Amoco Cadiz en 1978, le Tanio en 1980 ou encore l'Amazzone en 1988 ("Ces marées noires qui ont marqué la Bretagne," n.d.). Ainsi, des tests de tolérance aux polluants organiques persistants sont en cours. Si ces tests préliminaires déjà probants se confirment par des répétitions et des approfondissements alors, dans le contexte d'une pollution anthropogénique intense, une potentielle valorisation de ces souches en biotechnologie pourra être envisagée. En effet, 60% de la pollution des sols en France est due aux hydrocarbones polycycliques aromatiques, et depuis peu l'inquiétude concernant les substances per- et polyfluoroalkyles est grandissante (Ministère de la transition écologique et solidaire, n.d.). Les lichens représentent une niche sous-explorée pour les micro-organismes très diversifiés (Grube et al., 2009; Suzuki et al., 2016) adaptés aux conditions extrêmes, y compris les environnements contenant des métaux (McLean et al., 1998) et des hydrocarbures. En raison de leur grande tolérance à des taux élevés de métaux lourds, comme l'uranium, diverses études ont exploité les lichens comme bioindicateurs (Conti

and Cecchetti, 2001). De plus, une étude récente (Nahar et al., 2019) a démontré la capacité de biodégradation d'une espèce de *Pseudomonas* isolée d'un lichen à biodégrader deux naphtalènes et anthracènes HAP industriels omniprésents, soulignant le potentiel de la microflore associée aux lichens dans la bioremédiation. À la lumière de cela, notre hypothèse majeure est que la microflore des lichens peut développer une résistance à divers polluants par la mise en place de divers mécanismes tels que la dégradation, la métabolisation et la transformation.

Parmi la souchothèque bactérienne et fongique constituée lors des différents isolements, et notamment de précédents travaux au laboratoire, une bactérie, *P. etheri*, apparue abondante lors d'isolement à partir d'échantillons prélevés à Crozon, a été choisie pour l'étude de son métabolome, plus précisément de son volatolome, et de son activité sur des nématodes, parasites de cultures. La présence de nématodes bactériophages sur *R. geographicum* et les références bibliographiques relatant les effets nématicides de composés organiques volatils issus de *P. polymyxa* (Cheng et al., 2017), nous ont encouragés à évaluer les composés organiques volatils de *P. etheri* sur deux types de nématodes phytoparasitaires (NPP) à kyste, *Heterodera schachtii* et *Globodera pallida*. Ces NPP sont l'une des contraintes majeures à la production agricole et en particulier dans les cultures maraîchères et fruitières à haute valeur ajoutée (Bernard et al., 2017). Ils peuvent entraîner une perte de rendement économique importante estimée à plus de 100 milliards de dollars américains par an (Jones et al., 2013). Les expérimentations menées ont permis de mettre en évidence une activité nématicide provenant des composés organiques volatils (COV) produits par la bactérie. Parmi eux, l'isoamyl acétate déjà décrit comme antimicrobien (Rybakova et al., 2017), et le 2-phenylethyl ester, décrit comme antifongique (Ruiz-Moyano et al., 2020), ont été identifiés comme les principaux composés volatils produits par la bactérie, le premier étant celui responsable de l'activité. D'une manière intéressante, les COV ont produit un effet significatif sur les deux genres de nématodes à kystes utilisés dans notre étude. Cet effet pourrait reposer sur la nature hydrophobe et non chargée des molécules qui leur permettrait de pénétrer facilement les membranes cellulaires et d'induire par la suite des perturbations telles qu'une perméabilité accrue et une fuite de composants intracellulaires (Schulz-Bohm et al., 2017).

Malgré le « bien vivre ensemble » apparent au cœur de l'holobionte lichénique, la microflore fongique isolée à partir du lichen *R. geographicu*m, comme décrit dans la première partie du Chapitre II, a révélé une certaine dualité en conditions de laboratoire. En effet, lors de l'isolement des champignons associés au lichen, des zones de confrontation ont été observées permettant ainsi la sélection de dix souches fongiques interagissant deux à deux. Cette interaction par un phénomène d'antibiose a été décrite en 1889 par Vuillemin (Klein, 2012) pour qui la vie oscille toujours entre une tendance à la destruction et une tendance à la production, entre une tendance antibiotique et une tendance symbiotique. Une multitude d'interactions microbiennes se produisent dans la nature et l'impact de l'intimité microbienne est souvent sous-estimé (Schroeckh et al., 2009) notamment parce que de nombreux clusters de gènes (BGC) restent silencieux dans des conditions standard de laboratoire. Nous avons alors cherché à imiter les conditions écologiques naturelles, où les micro-organismes coexistent au sein de réseaux microbiens complexes, et où la compétition pour des ressources limitées et l'antagonisme sont les caractéristiques de ces microhabitats qui favorisent divers mécanismes de défense reposant principalement sur la production de métabolites spécialisés bioactifs (Marmann et al., 2014). Ainsi, en conditions de laboratoire, l'approche OSMAC (Palma Esposito et al., 2021; Vieira et al., 2021) et la co-culture (Bader et al., 2021) de plusieurs micro-organismes permettent une compétition entre eux et l'activation et la transcription de gènes biosynthétiques aboutissant à la production de molécules non produites dans des conditions de culture axéniques (Bertrand et al., 2013; Chagas and Pupo, 2018; Rutledge and Challis, 2015). Dans le cadre de nos travaux, la valorisation de la souchothèque fongique a nécessité différentes étapes allant de la sélection de souches d'intérêt à la valorisation des composés isolés. Le choix des souches fongiques étudiées, s'est basée sur une observation et une étude bibliographique des différents champignons impliqués dans des confrontations. Une fois les souches fongiques choisies, les conditions de culture selon une approche OSMAC ont été mises en place pour 5 couples de champignons (sur différents supports : boîtes 6 puits, boîtes de Petri compartimentées ou classiques ; et en utilisant 7 milieux différents) puis les profils chimiques obtenus pour chacune des conditions ont été analysés par HPLC-DAD. Ainsi, après cette étude préliminaire, champignons et conditions de culture sur boîtes de Petri classique adaptées pour chaque couple ont été sélectionnés pour une mise en culture à plus grande échelle. Ces études mycochimiques, et plus particulièrement celle du couple *D. variisporum* (C4) et *X.*

Hypoxylon (C5), champignons peu étudiés, tout particulièrement le premier, a permis l'isolement de 10 molécules dont 7 nouveaux sesquiterpènes nommés xylarines A-G et dérivant de l'acide intégrique décrit en 1999 par Singh et al. Ces sesquiterpènes possèdent une même unité de type érémophilane reliée par une liaison ester à une chaîne aliphatique variable de 7 à 11 carbones, saturée ou insaturée avec une ou deux insaturations, linéaire ou ramifiée par un méthyle. L'élucidation structurale de ces composés a été réalisée par l'analyse complète des spectres RMN 1D, 2D et la configuration absolue de type 1*R,* 4*S, 5R*, 7S déterminée (sauf pour un C asymétrique de la xylarine E) par similarité des spectres de dichroïsme circulaire électronique expérimentaux et ceux obtenus par TD-DFT. Les sesquiterpènes érémophilanes étant décrits comme possédant des activités biologiques intéressantes (Yuyama et al., 2017), six de ces molécules, isolées en quantité suffisante, ont été testées pour leur activité antibactérienne, avec des résultats positifs pour 4 d'entre elles. Ces résultats ont mis en lumière l'incapacité de ces composés à traverser la paroi des bactéries GRAM – en raison putativement de la taille du noyau érémophilane mais également l'importance de la longueur de cette chaîne et/ou de sa rigidité dans l'activité antibactérienne. D'autres tests, antiviraux et antifongiques vont être menés dans les jours, voire semaines à venir. Malheureusement, aucune de ces molécules isolées ne sont le fruit d'une induction en co-culture, elles sont basalement produites par les champignons en conditions axéniques. En activant certains gènes par divers moyens, grâce au genome mining par exemple, la production de métabolites induits ou surexprimés pourrait être optimiser. Cependant, l'élucidation de la voie de biosynthèse serait nécessaire. Plus simplement, l'appréciation des mécanismes de régulation moléculaire pourrait améliorer la production de ces molécules.

L'étude mycochimique, encore en cours pour le couple *Michrodochium phragmitis* (C44) et *Tolypocladium* sp. (C45), a permis l'isolement de deux molécules induites en co-culture cette fois-ci mais non encore identifiées. Une des molécules présente une activité antibactérienne significative vis-à-vis de *S. aureus* résistant. Cette étude, ainsi que celle du couple *Coccinonectria rusci* (C9) et *Melanconium heredicola* (C11) sont encore à l'état d'ébauche et mériteront une exploration plus approfondie. Une dernière étape serait l'identification des déclencheurs moléculaires responsables de l'induction observée.

L'isolement de nouvelles molécules est dépendant de la stratégie de fractionnement, qui dans notre cas s'est plutôt tournée vers des molécules hydrophobes. Ainsi, en envisageant

une stratégie plus orientée vers le fractionnement des composés polaires, de nombreuses molécules supplémentaires pourraient certainement être caractérisées et donc potentiellement de nouvelles activités biologiques identifiées.

Au même titre que certaines molécules sont difficilement isolables, de nombreux microorganismes restent récalcitrants à l'isolement et à la cultivabilité dans des conditions de laboratoires (Epstein, 2013). Des stratégies innovantes d'isolement et de culture *in vitro* ont été développées afin de pouvoir pallier cette non cultivabilité (Bollmann et al., 2007; Epstein et al., 2010; Kaeberlein et al., 2002; Nichols et al., 2010). Elles ont d'ailleurs permis l'isolement d'une nouvelle bactérie, *Eleftheria terrae,* capable de produire la teixobactine, dernier antibiotique découvert depuis les années 60 et actuellement en cours d'essais cliniques (Ling et al., 2015). Nos essais d'isolement avec la méthodologie de l'iChip et des traps n'ont pas abouti du fait de la nécessité d'importantes mises au point. En effet, classiquement les iChips, dispositifs utilisant une membrane semi-perméable sont installés dans des environnements humides, voire immergés (MacIntyre et al., 2019), contexte écologique bien éloigné des conditions que nous avons mis en place. Cependant, la mise au point d'une telle stratégie pourrait être une idée intéressante à creuser dans l'optique de rechercher les espèces les plus difficiles à cultiver.

Enfin, dans un but de meilleure compréhension de l'écologie de l'holobionte, nous envisagerons d'étudier la biotransformation du métabolite lichénique majoritaire de *R. geographicum*, à savoir l'acide rhizocarpique possédant différentes activités (Giez et al., 1994; Joshi et al., 2020; Kokubun et al., 2007), par des bactéries sélectionnées dans la souchothèque de façon à augmenter la diversité métabolique produite par ces microorganismes.

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"And it is significant. For animals, as well as plants, there have never been individuals. This new paradigm for biology asks new questions and seeks new relationships among the different living entities on Earth. We are all lichens."[1]
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[1] Gilbert, S. F., Sapp, J. & Tauber, A. I. A Symbiotic View of Life: We Have Never Been Individuals. *The Quarterly Review of Biology* **87**, 325–341 (2012).

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UNIVERSITÉ DE RENNES 1

Titre : Microbiote de l'holobionte lichénique *Rhizocarpon geographicum* : étude de la diversité et applications biotechnologiques

Mots clés : *Rhizocarpon geographicum*, holobionte lichénique, microbiote, produits naturels

Résumé : Les lichens, organismes symbiotiques complexes considérés comme « holobionte », représentent des niches d'une richesse exceptionnelle d'un point de vue microbien et constituent une ressource privilégiée pour la découverte de métabolites spécialisés d'intérêt. Les travaux réalisés lors de cette thèse ont porté, d'une part, sur l'étude de la diversité fongique et bactérienne arborée au sein du lichen *Rhizocarpon geographicum* et d'autre part, sur la valorisation de la souchothèque d'un point de vue biotechnologique. L'étude de la communauté microbienne de *R. geographicum,* issu d'environnements côtiers et terrestres, a été étudiée par des approches culture-dépendantes classiques et à haut débit et a permis la constitution d'une souchothèque d'environ 700 bactéries uniques. Au sein de la souchothèque, une bactérie, *Paenibacillus etheri*; a été selectionnée pour l'étude, dans le domaine du biocontrôle de l'effet de son volatolome sur des nématodes à kystes. De plus, des souches fongiques interagissant deux à deux par un phénomène d'antibiose ont été étudiées suivant les méthodologies OSMAC et de co-culture. L'analyse de leurs profils métaboliques et la mise en place de stratégies de fractionnement ont permis d'isoler, entre autres, sept nouvelles molécules de type érémophilane. Ces molécules ont été caractérisées d'un point de vue chimique et quatre d'entre elles présentent d'intéressantes activités antibactériennes vis-à-vis de *Staphylococcus aureus* sensible et résistant.

Title: Microbiota of the lichen holobiont *Rhizocarpon geographicum*: study of diversity and biotechnological applications

Keywords: *Rhizocarpon geographicum,* lichen holobiont, microbiota, natural products

Abstract: Lichens, complex symbiotic organisms considered as "holobionts", represent niches of exceptional richness from a microbial point of view and constitute a privileged resource for the discovery of specialized metabolites of interest. The work carried out during this thesis focused, on the one hand, on the study of fungal and bacterial diversity within the lichen *Rhizocarpon geographicum* and on the other hand, on the valorization of the strain collection from a biotechnological point of view. The study of the microbial community of *R. geographicum*, from coastal and terrestrial environments, has been studied by classical and high-throughput culture-dependent approaches and has allowed the constitution of a collection including approximatively 700 unique bacteria. Among the strains, a bacterium, *Paenibacillus etheri*; has been selected for the study, in the field of biocontrol of the effect of its volatolome on cyst nematodes. Moreover, fungal strains interacting in pairs through an antibiosis phenomenon have been studied using OSMAC and co-culture methodologies. Analysis of their metabolic profiles and development of fractionation strategies have led to the isolation of seven new eremophilane molecules among other molecules. These molecules have been characterized from a chemical point of view, and four of them have shown interesting antibacterial activities towards sensitive and resistant *Staphylococcus aureus.*