Universidade de Aveiro Departamento de Biologia
2018

DIANA DA CONCEIÇÃO MARINHO PEREIRA

Bacteria from sub-explored environments: potential to fight lung cancer and associated infections

Bactérias de ambientes sub-explorados: potencial para combater o cancro do pulmão e infeções microbianas assoacidas

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutora Catarina Pires Ribeiro Ramos Marques, Cientista Convidada do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus pais e irmã.

o júri

presidente

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palavras-chave

Bactéria, compostos naturais, potencial biotecnológico, atividade antimicrobiana, atividade anticancerígena, indústria, ambiente, saúde, cancro pulmonar, infeções bacterianas do trato-respiratório, ensaio MTT

resumo

O cancro do pulmão lidera atualmente a taxa de mortalidade associada a cancro, à escala mundial. É um cancro altamente incidente, invasivo e metastizante. O tratamento do cancro é normalmente assegurado pela combinação de cirurgia, e terapias adjuvantes (quimio- e radioterapia). No entanto, esta abordagem apresenta problemas em termos de eficácia, toxicidade para tecidos periféricos e resistência. Estas limitações associadas à ocorrência de infeções bacterianas do trato respiratório (IBTR) fomentam a ineficácia do tratamento do cancro, conduzindo a elevada mortalidade e morbidade. Nos últimos anos tem havido um esforço para produzir uma nova geração de compostos que colmatem estas lacunas clínicas, mas os resultados ainda não são satisfatórios. Assim, a busca de novos compostos naturais que evidenciem atividade anticancerígena e/ou antimicrobiana é premente. Neste contexto, os microorganismos constituem uma rica fonte de compostos bioativos, graças ao seu metabolismo secundário desenvolvido em resposta a condições de *stress*, para garantir a sua sobrevivência/resiliência. Ambientes extremos e/ou sub-explorados, como as grutas, sustentam microorganismos com elevado potencial biotecnológico. Assim, os objetivos deste trabalho consistiram em: (1) fazer o estado da arte sobre o cancro do pulmão, IBTR a ele associadas, tratamentos disponíveis e a descoberta de novos compostos naturais com valor terapêutico; (2) apresentar uma inédita revisão acerca de grutas Cársicas calcárias, os habitats microbianos que sustentam e o poder biotecnológico desses microorganismos para aplicações industriais, ambientais e médicas; (3) realizar o rastreio da atividade antimicrobiana e anticancerígena de bactérias isoladas de nichos ambientais sub-explorados. Archaea e Bacteria em grutas Cársicas sofreram uma evolução sob pressões seletivas, desenvolvendo assim um metabolismo biossintético altamente especializado e sustentado por uma diversidade genética peculiar. Em particular, têm capacidade de sintetizar enzimas adaptadas a temperaturas baixas, assim como possuem um resistoma e parvoma muito atraentes para aplicações biotecnológicas. O rastreio da bioatividade de bactérias isoladas de grutas calcárias demonstrou o seu potencial antimicrobiano contra bactérias promotoras de IBTR (ensaios com colónia viva e extratos brutos). Além disso, alguns dos extratos de diferentes frações de cultura bacteriana (*Pseudomonas* spp.) apresentaram atividade anticancerígena, uma vez que a atividade de células de cancro do pulmão de rato (*Lewis lung cancer*) foi diminuída/inibida. Este estudo constitui um contributo na procura de compostos naturais que futuramente possam servir no combate ao cancro do pulmão.

keywords

Bacteria, natural compounds, biotechnological potential, antimicrobial activity, anticancer activity, industry, environment, health, lung cancer, bacterial infections of the respiratory-tract, MTT assay

abstract

Lung cancer currently leads the cancer-related mortality rate worldwide. It is a highly incident, invasive and metastatic cancer. Cancer treatment is usually ensured by the combination of surgery, and adjuvant therapies (chemo- and radiotherapy). However, this approach presents problems in terms of efficacy, toxicity to peripheral tissues and resistance. These limitations associated with the occurrence of bacterial respiratory tract infections (IBTR) promote the ineffectiveness of cancer treatment, leading to high mortality and morbidity. In recent years there has been an effort to produce a new generation of compounds that fill these clinical gaps, but the results are still not satisfactory. Thus, the search for new natural compounds that evidence anticancer and/or antimicrobial activity is crucial. In this context, microorganisms constitute a rich source of bioactive compounds, due to their secondary metabolism developed in response to stress conditions, ensuring their survival/resilience. Extreme and/or under-exploited environments, such as caves, sustain microorganisms with high biotechnological potential. Thus, the objectives of this work were: (1) to make the state of the art on lung cancer, BTRI associated with it, available treatments and the discovery of new natural compounds with therapeutic value; (2) present an unprecedented review of calcareous karstic caves, the microbial habitats it sustain and the biotechnological power of these microorganisms for industrial, environmental and medical applications; (3) screening the antimicrobial and anticancer activity of bacteria isolated from under-exploited environmental niches. Archaea and Bacteria in karst caves underwent an evolution under selective pressures, thus developing a highly specialized biosynthetic metabolism and sustained by a peculiar genetic diversity. In particular, karst cave bacteria and archaea have the ability to synthesize enzymes adapted to low temperatures, as well as have a very attractive resistome and parvome for biotechnological applications. The screening for the bioactivity of bacteria isolated from limestone caves demonstrated its antimicrobial potential against BTRI-promoting bacteria (live colony and crude extract assays). In addition, some of the extracts from different fractions of bacterial culture (mostly *Pseudomonas* spp.) showed anticancer activity, since the cellular activity of lung cancer cells was decreased/inhibited. This study contributes to the search for natural compounds that may be useful in the fight against lung cancer in the future.

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Abbreviations

- **ALK -** anaplastic lymphoma kinase
- **BRTI** - bacteria associated with respiratory tract infections
- **CD74-NRG1 -** CD74-Neuregulin1
- **CF** – Cell fraction
- **CFF** – Cell-free fraction
- **DMEM** - Dulbecco's Modified Eagle Medium
- **DMSO -** Dimethyl Sulfoxide
- **EGFR -** epidermal growth factor receptor
- **FBS –** Fetal bovine serum
- **GWAS –** Genome wide association study
- **LLC -** Lewis lung cancer
- **MLSA -** multilocus sequence typing analysis
- **MTT -** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- **NRPS –** non-ribosomal peptide synthase
- **NSCLC -** non-small cell lung carcinoma
- **NTRK -** TRKA receptor tyrosine kinase
- **PD-L1** - death ligand 1
- **PKS –** polyketide synthase
- **RET -** ret proto-oncogene
- **ROS1** - ROS proto-oncogene 1, receptor tyrosine kinase
- **SCLC -** small cell lung carcinoma
- **TNM** - Tumour Node Metastasis
- **TOC -** total organic carbon
- **VEGF** – vascular endothelial growth factors
- **WHO -** World Health Organization

Chapter I - General Introduction

1. Lung cancer

1.1. Epidemiology

Although lung cancer was an infrequent disease before the 20th century, it has been since then a major issue in public health[1]. It is the most common cancer within the population worldwide (11.6% incidence)[2], and it has been triggering a social and economic burden to the healthcare systems[3,4]. Lung cancer is more prevalent in men[2], although its incidence is decreasing in men and increasing in women[1,5]. Its incidence may also vary between sexes depending on the age, as in men the age standardized rate of incidence is 31.5 (%) and in women of 14.6 (%)[2]. It was recently estimated that in 2018 there will be 2.09 million cases of lung cancer diagnosed, which may end up in 1.76 million deaths[2]. Moreover, only 18.1% of patients survive 5 years after diagnosis[6].

Lung cancer is one of most incident, highly invasive and metastasizing cancers[7], gaining resistance to several drugs along the treatment[8,9]. The carcinogenic effects in normal lung cells contributing to the culmination of lung cancer are attributed to many factors[1,7,10,11]. A major culprit is the habit of smoking[1,3,5–7,10,11]. An increase in tobacco consumption is followed decades later by a rise in the incidence of this cancer[1]. The substances in tobacco can transform normal cells due to their carcinogenic effects[1,4]. The vulnerability of individuals to tobacco-induced lung cancer might be dependent on competitive gene/enzyme interactions that activate precursors of carcinogens (*i.e.*, procarcinogens) and the integrity of endogenous mechanisms for repairing lesions in DNA[7]. Consequently, lung neoplasia may develop, which can occur both in smoking and non- or passive-smoking individuals, since there are many other co-contributing factors and interactions between them[1,3]. Other risk factors may also be associated, pollution exposure, work-related exposure (*e.g.*, asbestos, coal tar, x-ray and gamma radiation, arsenic, chromium, plutonium), prior lung disease and genetic susceptibility[1,3,11,12]. There are indeed interfering intrinsic factors, for example, age (higher occurrence beyond 50 years of age)[3], gender[3], race/ethnicity[11] and social-economic situation[3]. The heritability of respiratory-tract cancers is estimated in 17.5% occurrences in a cohort study with twins from Nordic countries[13] and 12.3% in a genome-wide association study (GWAS)[12], being the associated *loci* identified the CHRNA3/5 (α-nicotinic acetylcholine receptor), TERT (telomerase reverse transcriptase), HLA (human leukocyte antigen), BRCA2 (breast cancer 1) and CHEK2 (checkpoint kinase 2)[12]. The heritability of lung cancer is still poorly understood, but McKay *et al.* (2017)[12] recently identified novel *loci* associated with lung cancer heritability, such as RNASET2 (ribonuclease T2), SECISBP2L (SECIS binding protein

2 like), NRG1 (neuregulin 1), CHRNA2 (cholinergic receptor nicotinic alpha 2 subunit) and RTEL (regulator of telomere elongation helicase 1)[12]. This study also highlights the heterogeneity in host genetic susceptibility to the disease throughout histological subtypes, with some *loci* related with lung cancer in general and others specifically with lung adenocarcinoma[12] (*cf.* 1.2). Lung cancer development and epidemiology can also be constrained, or in turn favour, the occurrence of viral infections, specific immune responses[14], inflammatory reactions[11] and bacterial infections[11,15,16].

1.2. Carcinogenesis and lung cancer types

The phenotype of lung cancer is a multistep series of genetic and epigenetic mutations evolving into an invasive tumour by clonal expansion[7]. These mutations, called oncogenic driver mutations[8], are crucial for malignant cells growth and division[17], as well as for the continuous accumulation of mutations, assimilated during clonal expansion, which affect tumour invasion, metastasis, and resistance[7]. The identification of these molecular changes improves prevention, early diagnostic and success of treatment[7,10,17]. Several genetic alterations have already been identified[7,8,18], such as EGFR[8,18] (epidermal growth factor receptor), ALK[8,18] (anaplastic lymphoma kinase), ROS1[8,18] (ROS proto-oncogene 1 receptor), RET[8,18] (ret proto-oncogene), NTRK1[18,19] (TRKA receptor tyrosine kinase) and CD74–NRG1[18,20,21] (CD74- Neuregulin1). These driver mutations can occur by several mechanisms, like rearrangements , fusions or point mutations[8,18–21]. Carcinogenic cells then disrupt regulatory pathways, what prevents cell proliferation and homeostasis regulation[7].

Lung cancer has, in a biological perspective, a comprehensive classification[17], in line with its cellular heterogeneity[7,18]. It can arise in many locations of the bronchial tree, therefore inducing a high panoply of symptoms[7] that can occasionally cause misdiagnosis[10]. The classification of primary tumours is broadly divided in two large histological types: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC)[5,7,18]. Their frequency in the population is also different, as far as NSCLCs accounts for 85% of all lung cancers and the remaining 15% are SCLCs[18]. NSCLCs are subdivided into adenocarcinoma, large cell carcinoma, and squamous cell carcinomas[5,10,18]. The continuous advances in this area led to the update of the classification of lung cancers by the World Health Organization (WHO) in 2015[18,22]. With this update, various subtypes of lung cancer were displaced in the classification due to recently identified molecular profiles and targetable genetic mutations in lung cancer[18]. For example, the distinction between adenocarcinoma and squamous cell carcinoma was manly owed to microscopic structural

differences, that could lead to errors because of poorly differentiation of these structures. Nowadays, with the update of WHO, the distinction is supported by genetic markers associated with each subtype of tumour, hence decreasing identification and characterization errors[18]. Even though, there has been a need to improve subtyping of the tumour, as to safeguard correct therapeutic choices once different subtypes respond differently to the same chemotherapeutic drugs[18,22]. For example, the chemotherapeutic agent bevacizumab, which targets EGF, can provoke life-threatening internal bleeding when administered to lung cancer patients with squamous cell carcinoma[22].

1.3. Options of treatment for lung cancer

Usually, patients are treated following several approaches[6], which selection may be based on different criteria and decisions undertaken by multidisciplinary teams[5,22]. Multidisciplinary teams must consider the most appropriate treatment in regard to the neoplasia histological type, genetic alterations, cancer staging and patients' fitness, age, clinical history and prior diseases[5,7]. Yet, the first step is to know the correct subtyping of the tumour and its staging[6,22].

SCLCs are classified into limited or extensive[6]. Limited SCLCs means that the tumour is restricted to one side of the chest; in extensive SCLCs, the cancer is spread to the other side of the chest or other parts of the body[6]. Regarding NSCLCs, the staging of the tumour is done by the evaluation of the location, proliferation and metastatic degree of the tumour[22], and can assume level I to IV[6]. A stage I tumour is only present in the lung; in stage II and III it can also extend to lymph nodes; in stage IV there is a high level of metastasis along the respiratory system and even in distant organs[6]. Stages I to III are considered for surgery, which may be combined or not with adjuvant therapies. The therapeutic decision for stage IV lung cancers usually only regards palliative care (though a certain curative treatment can be applied through a palliative chemotherapy) as to improve the comfort of the patient[6], as normally it had already affected vital body organs, and no current therapies can counteract such advanced clinical scenario[5]. In summary, this classification of tumours (in general) is associated with the degree of metastasis and the number of nodes, constituting the TNM (Tumour Node Metastasis) classification system, one of the most used systems in cancer staging[5,7,10].

Among the two types of lung cancer, SCLCs behave more aggressively[17] due to its fast growth; however, it is more reactive to chemotherapy[6,7] and radiotherapy[7] and, therefore, resection is typically not the first option[5,7,22]. NSCLCs are normally treated with a combination of surgery[5,17]

and adjuvant therapies[7,17]. Generally, adjuvant therapies for lung cancer include chemotherapy, radiation therapy and targeted therapy, with curative and/or palliative purpose[5,6]. Chemotherapeutic agents are cytotoxic and have as ultimate goal apoptosis induction[23,24]. The usual chemotherapeutic drugs for lung cancer are etoposide and a platinum-based therapy (cisplatin or carboplatin)[6]. Etoposide is a drug that inhibits the DNA topoisomerase II complex, ultimately causing double-strand DNA breaks[23,25], and is mostly used in SCLCs. Cisplatin is able to interfere with DNA repair mechanisms, causing DNA damage due to its ability to interact with purine residues on the DNA strand[24] by covalent binding[25]. Carboplatin has a similar mechanism of action, being an analogue of Cisplatin, but less potent than it[24]. These chemotherapeutic drugs have, however, drawbacks related with toxicity[6,25] and resistance[24]. Patients treated with Cisplatin acquired resistance and presented hepatotoxicity (liver toxicity)[24], nephrotoxicity (kidney toxicity)[24,25], and cardiotoxicity (heart-tissue toxicity)[24] effects. Moreover, the patients can suffer from nausea and vomiting[25]. This led to the search of analogues like Carboplatin[25], and the potential application of alternative therapeutic approaches through the combination of other anticancer drugs, like Paclitaxel, Osthole and Metformin[24], though the latter are still in test phase. Carboplatin and Etoposide induce myelosuppression as a major side effect[6,25], causing neutropenia and thrombocytopenia[6]. Etoposide can also induce mast cell degranulation[25]. In turn, ionising radiation techniques have improved in the last few years, having now reduced its toxic effects and enhanced tissue integrity preservation[6], what makes them increasingly welcomed therapeutic strategies.

As the understanding of the molecular and genetic features of lung tumour increases[18], target therapies have been developed, and have gradually become more efficacious, feasible and tailored for each patient and lung cancer subtype[22]. In targeted therapies for lung cancer, the targets already covered were mutations in EGF receptor[6,8], ALK[6,8] and vascular endothelial growth factors (VEGF)[6,7]. Recently, it has been approved drugs targeting alterations in death ligand 1 (PD-L1)[22], its receptor (PD1)[22], c-ros oncogene 1 (ROS-1)[6,8] and B-Raf (BRAF)[6,8]. Among the target therapies, immunotherapy is growing and have been showing good results in some cases[6,8,22]. Despite the promising results, targeted therapies have drawbacks namely regarding the development of resistance[7,8] after *ca.* 12 months of treatment[6]. This resistance could be due to secondary mutations on the molecular targets[8] or activation of another molecular pathways[7,8].

Despite all therapeutic efforts, lung cancer remains one of the most deadliest cancers worldwide[6,26]. As aforementioned, chemotherapeutic agents have several side effects[6,7,24] and patients tend to develop resistance, being this resistance the cause of many relapses of the

disease[24]. In targeted drugs, however, resistance is also a major problem[7,8], regardless of the reduced side effects[6]. Hence, it is highlighted the need for different or alternative complementary treatment drugs and/or strategies[7], what may require the discovery and isolation of new bioactive compounds with anticancer properties[27], to put us a step ahead in the fight against lung cancer.

1.4. Respiratory tract infections in lung cancer patients

Infections are quite common in immunocompromised patients, such as lung patients under chemotherapy treatment[28] and immunosuppressive treatment[29]. In particular, respiratory tract infections, resistance to antibiotics and drug toxicity are two of the major reasons of treatment failure[28], which can ultimately end up in high mortality[28,30,31] and morbidity of immunocompromised patients[28,29]. On the other hand, the respiratory tract has areas that are not reachable by antimicrobials[32,33].

According to the risk sources, these infections can be divided into hospital-acquired (nosocomial)[28,34], community-acquired[28,30] and ventilator/catheter treatment-related infections[28,35]. The occurrence of such bacterial infections in patients with cancer are a serious issue, resulting in death events in more than 50% of the cases[31]. In lung cancer patients, this problem is an even worst scenario and, because of this, it needs further and deeper attention as to prevent the occurrence of synergistic overwhelming damages. According to Berghmans *et al.*[31], infections of the respiratory tract have been accounted in up to 84% lung cancer patients; although it has yet to be shed some light more on the understanding of their impact on cancer progression and patients' survival[31]. One of the pioneer studies about this matter, which was performed by Perlin *et al.*[36], showed that pulmonary infections may affect the rates of survival in a negative manner, and that pulmonary infections are recurrent in lung cancer patients. Perlin and colleagues also hypothesized that the causes of these respiratory infections could be bronchial obstruction, myelosuppression, immunosuppression, tumour invasion and necrosis[36]. In fact, the risk of infection in cancer patients is higher primarily because of defects in important cells involved in immune system responses, such as neutrophils[37], B and T cells[29]. Chemotherapy treatment, as aforementioned, causes immunosuppression[37], like neutropenia[38], hence further compromising the protective function of neutrophils against pulmonary bacterial infections[29,37]. Also, the disruption of lung epithelial barriers by treatments or tumour invasion/obstruction can indeed increment the risk of infection[29,31].

In immunocompromised patients, the bacteria usually isolated in respiratory tract-infections are Gram-negative (*e.g., Pseudomonas aeruginosa*, *Klebsiella* spp., *Escherichia coli*, *Moraxella catarrhalis*, *Stenotrophomonas maltophilia*) and Gram-positive (*e.g.*, *Staphylococcus aureus* and *Streptococcus pneumoniae*) bacteria[29]. In ventilator treatment-related pneumonia, infections are mainly due to Gram-negative bacteria like *Klebsiella pneumoniae*, *P. aeruginosa*, *E. coli*, *Acinetobacter* and the Gram-positive *Staphylococcus aureus*[28]. In lung cancer patients, though, have been also associated several bacterial species with respiratory tract infections. In such pulmonary infections, Gram-negative bacteria like *K. pneumoniae*, *Haemophilus influenzae*, *P. aeruginosa* and *Enterobacter cloacae* have been recognized in more than 68% of the lung cancer cases[31]. As for the tracheobronchial tree, some of the bacteria isolated were *S. pneumoniae*, *S. aureus*, *H. influenzae*, *E. coli*, *P. aeruginosa*, and *M. catarrhalis*[31]. Respiratory infections in lung cancer patients have also been addressed by nontuberculous Mycobacteria[15,16]. It must be underlined that the respiratory tract has an indigenous microbiome, which can undergo changes and shifts depending on many factors, as well as it can simultaneously play a role on the development of infections in the respiratory tract[32,39].

Other human pathogens of interest are *Aeromonas hydrophila* and *Micrococcus luteus*. *A. hydrophila* is not a frequent human pathogen[40], but it is more often isolated in immunocompromised patients[40,41], usually associated with gastroenteritis[41–43]. This bacterium has been linked occasionally to respiratory infections[41,43]. The first time *A. hydrophila* was isolated from an immunocompromised patient was in 1954, from several tissues, including lungs[42]. It was also reported a lung abscess in an immunocompromised patient due to *Aeromonas hydrophila* infection, being this strain resistant to ampicillin[40]. There was a study, in several French hospitals, that reported numerous infections by *Aeromonas spp*., and 6% of them were respiratory tract infections[44]. In this study, there were nosocomial infections induced by aeromonads also in cancer patients, being *A. hydrophila* the second most commonly isolated bacteria[44]. Like *A. hydrophila*, *M. luteus* is not a usual human pathogen[45,46], but it can be an opportunistic microorganism[47] in immunocompromised patients[45]. There were at least two reported cases of catheter infection by *Micrococcus luteus*[45,46]. This opportunistic pathogen was also identified in bacteraemia infections of cancer patients[48]. Likewise, pneumonia lead by *M. luteus* infection had been reported in leukaemia patients[46,49], resulting in pulmonary haemorrhage in some cases[49].

In the following sections are provided more details on the major characteristics of the infections driven by the bacteria included in the present study.

i. Klebsiella pneumoniae

K. pneumoniae is a gram-negative bacterium[50] superficially covered by a capsule that is responsible for its virulence[51] and a facultative anaerobe[52]. It belongs to Enterobacteriaceae family[53] and it is an opportunistic pathogen that can be found in the environment, skin, intestines and mouth of humans[50]. Actually, *K. pneumoniae* is one of the most harmful human pathogens[50]. *K. pneumoniae* produces many virulence factors, although their function is not entirely clear[53]. These virulence factors can be structural bacterial proteins like capsular polysaccharides and lipopolysaccharides[50] (also associated with its capsule) to avoid phagocytosis and serum bactericidal activity[53], or adherence factors such as *fimbriae*, which helps adhering to host epithelial cells[50]. This bacterium can form biofilms, what further promotes their self-protection and maintenance[50]. The virulence character of *K. pneumoniae* is worsen by the development of resistance to several antibiotics, including β-lactams (*e.g.*, penicillin, cephalosporin)[54] and cephalosporins[50]. This is associated with the emerging number of strains showing ability to produce broad spectrum β-lactamases (ESBLs) enzymes[54], given that their encoding genes are usually located in transferable plasmids[55].

ii. Escherichia coli

Escherichia coli is a gram-negative bacterium and it belongs to Enterobacteriaceae family[56]. It is a facultative aerobic bacterium and its optimum temperature is 37ºC[56]. *E. coli* is a commensal microbe present in different biological niches of the human body (*e.g.*, gut), but there are strains with different virulent grades, responsible for enteric[56] and extra-enteric infections[56,57]. In this context, *E. coli* produces several virulence factors[56], and they can be encoded in the genome or organized in clusters, called pathogenicity islands, inside of transferable plasmids[56,57]. The degree of the virulence depends on the number of pathogenicity islands (it shows a cumulative effect) and the strain genetic background, plus the infection location[57]. *E. coli* nosocomial pneumonias are becoming a more frequent issue[57], such as bacteraemia developed by resistant *E. coli*[58].

iii. Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative rod-like flagellated bacterium with an encapsulated cover, from the Pseudomonadaceae bacterial family. *P. aeruginosa* is found in soil,

plants, water and in humans (in equilibrium and dysbiotic states)[50]. It grows under aerobic conditions, but it can also be facultative anaerobic in the presence of nitrate[50]. It is optimal temperature of growth is 37ºC, but it can withstand up to 42ºC without inactivation of the bacterial cells metabolism[50]. *P. aeruginosa* produces numerous virulence factors, like extracellular enzymes and toxins[50,59]. This opportunistic pathogen is one of the most identified in hospital-acquired pneumonia[50,59,60], infecting immunocompromised patients and being a major problem in cancer patients, as far as it can cause chronic lung infections in addition to acute tissue infections[59]. *P. aeruginosa* produces two pigments, pyoverdin and pyocyanin[50]. A pyocyanin-derived molecule is siderophores, which gives them the ability to capture iron from the host or the environment[50]. The risk for *P. aeruginosa* infections are related with its resistance to antibiotics, because it has structural proteins, like capsule polysaccharides and lipopolysaccharides that block phagocytosis and serum-killing factors[50]. This is particularly worrying for multi-drug resistant strains of *P. aeruginosa*[60]. Its capacity to form biofilms, enhances its protection against antibiotics and phagocytosis[59]. Moreover, *P. aeruginosa* resistance can also be improved through the acquisition of transferable resistance plasmids, denominated R-factors[50].

iv. Aeromonas hydrophila

Aeromonas hydrophila is a gram-negative and rod-like uniflagellate bacterium[41]. It belongs to Aeromonadaceae family and are facultative anaerobic organisms[42], growing at temperature range of 15-38ºC[61]. It is found mainly in the environment, with special focus in water sources[43]. *A. hydrophila* is an opportunistic pathogen, infecting principally immunocompromised patients[41]. These infections may be led by several virulence factors, such as polysaccharides, proteases, elastase, DNase, lipases, amylase, adhesins, agglutinins and enterotoxins[42,43]. The treatment of *A. hydrophila*-driven infections has been raising difficulties due to the gaining of resistance against drugs, what is thought to be a consequence of the extensive use of antimicrobial drugs in livestock, fish (aquaculture) and humans[43]. Usually resistance genes are encoded in *A. hydrophila* genome, however, they can acquire transferable plasmids[43]. The expression of these genes triggers the production of β-lactamases, which guarantees resistance to several antibiotics of the β-lactams group[43].

v. Micrococcus luteus

Micrococcus luteus is a gram-positive spherical-like bacterium, belonging to the Micrococcaceae family[47]. They are strict aerobic microbes, with an optimum temperature of growth of 25-37ºC[47]. *M. luteus* are found in the environment, both terrestrial and aquatic habitats, and in humans, particularly in skin[47]. It is considered a commensal organism but it can be an opportunistic pathogen, especially in immunocompromised individuals[45,46]. There is a case reported of *Micrococcus luteus* pneumonia in a patient with acute leukemia[46]. There is a reduced knowledge about susceptibility/resistance in the genera *Micrococcus spp.*, but infections had been usually treated successfully with a combination of antimicrobial drugs, such as vancomycin and penicillin [45,47].

1.5. Natural compounds with anticancer activity

Current lung cancer therapeutics present limitations and drawbacks (*cf.* section 1.3), and the failure in bringing new effective and less toxic synthetic products lead the investigators to search for natural compounds[62], as natural anticancer products can present less adverse side effects[63]. Hence, scientists have been trying to discover and isolate new natural compounds with anticancer activity. Slowly, the framework of anticancer research, which was been relying on the synthesis of small molecules, is entering in an era of targeted therapy with natural products[62–64]. These natural agents are (secondary) metabolites, that can be isolated from microorganisms, invertebrates, plants and animals[65]. They have appealing properties and are bioactive, which renders them the capacity to bind and interact with enzymes and biological receptors, thus triggering a cascade of reactions in specific metabolic pathways, similarly to synthetic drugs [65,66].

Some challenges or steps have to be accomplished and taken into consideration in what regards natural products discovery, namely isolation, bioactivity screening and synthesis[66]. Although the testing of crude extracts is a first approach allowing the screening and selection of those with potential interest for anticancer treatments, the extracts cannot be directly applied in clinics[65]. Also, only limited amounts of the compounds of interest can usually be extracted from the available organisms biomass[62], what difficult their use as anticancer drugs, besides the high costs normally required for compounds extraction, isolation and purification. At this point, chemical synthesis has been essential to overcome such limitations[66]. The last issue is the activity

evaluation of these natural compounds. Classically, this is preliminarily based on the analysis of its cytotoxicity against human or murine cancer cell lines in *in vitro* assays[62]. These cell lines could be of different tissues or altered to be resistant/sensitive to some anticancer drugs, lacking genes encoding a specific enzyme or with altered metabolic pathways[62]. Currently, it has been made an effort to understand the molecular mechanisms involved in cancer and the development of anticancer drugs has following a trend of target-based approaches for the refinement of the discovery process of novel drugs[62]. In this context, he use of new techniques such as combinatorial synthesis and high-throughput screening has increased the discovery of anticancer drugs based on natural compounds[66].

One of the most interesting sources are bacteria. There are many antitumoral antibiotics used in chemotherapy, being these drugs refined products of bacterial metabolism. Examples of them are Actinomycin, Streptozocin, Bleomycin, Mitomycin C, Daunomycin, Idarubicin, Doxorubicin and Epirubicin[63]. The most-known are Doxorubicin, Actinomycin D and Mitoxantrone (Doxorubicin analogue)[62]. Doxorubicin was isolated from *Streptomyces peucetius* and it is used in several cancers, such as lung cancer[67]. Doxorubicin interacts with DNA molecule, intercalating with the double strand and inhibits DNA/RNA polymerases and topoisomerase II[25,67]. It also produces reactive oxygen species and interacts with components of cellular membrane[67]. Mitoxantrone has similar anti-tumour activity like its analogue, Doxorubicin[25]. The advantage of this drug is that it does not produce oxygen reactive species, thus not causing oxidative stress in cells[25]. Actinomycin was also isolated from a *Streptomyces* spp.[68], enters tumour cells by diffusion, and its activity inhibits transcription and protein synthesis, interacting with double or single-stranded DNA[25].

1.6. Objectives and structure of the dissertation

At the light of the above, lung cancer is a disease with high incidence and associated mortality, that urgently requires new therapeutics and treatment strategies that may complement current ones. Besides, lung cancer patients frequently have an immunocompromised system that greatly enhances their susceptibility to bacterial infections. As such, respiratory-tract infections are a major problem in lung cancer patients affecting their survival, what is aggravated by the rising of antimicrobial resistance, thereby posing a huge challenge to leverage the impact of these infections. In this context, new effective anticancer and antimicrobial compounds, capable of inducing fewer undesirable side-effects and toxicity for the patient, are hence claimed by clinics and pharmaceutical industry.

In order to contribute for overcoming all these matters it was hypothesized that bacteria isolated from under-explored environments, such as caves, may offer greater opportunities to discovering new bioactive compounds, given their unusual secondary metabolism to withstand selective pressures existing in cave habitats. Therefore, the main goal of this dissertation was to assess the biotechnological potential of those bacteria, through the screening of the bioactivity of their crude extracts against lung bacterial infections (antimicrobial activity) and lung cancer proliferation (anticancer activity).

This broader aim was developed throughout four chapters of this dissertation.

Chapter I is the general introduction of the dissertation, describing the state-of-the-art. It starts with a description of lung cancer, its epidemiology and carcinogenesis, and an overview of the available treatment options. It approaches also the infections in lung cancer patients and the need for the discovery of new natural compounds.

Chapter II is a review paper, entitled “Light from the darkness: cave microbes as biotechnological tools for industry, environment & health”. The aim of this chapter is the review of the biotechnological potential of bacteria and archaea from karstic caves for industrial, environmental and medical applications. It provides an explanation of karstic cave habitats and their characteristics, followed by a demonstration of the microbial diversity and their valuable features for biotechnological purposes. Practical examples of biotechnological applications of these microbes in industry, environment and health sectors are also provided.

Chapter III is an original research paper on “Bioactivity of bacteria from under-explored environments against lung cancer and associated microbial infections”. This chapter presents the practical work developed, which goal was to assess the bioactivity of limestone cave microbes and their crude extracts against lung cancer (Lewis Lung Cancer cell line) and associated bacterial infections.

Chapter IV provides the concluding remarks and conclusions of this research study.

1.7. Scientific production under the Master thesis

Throughout this dissertation it was produced the following manuscripts, which will be meanwhile submitted to international peer-reviewed journals of the speciality:

- one review article, entitled “Light from the darkness: cave microbes as biotechnological tools for industry & health”. This review article approaches caves as microbe habitats and the biotechnological potential of karstic cave bacteria and archaea for different medical and industrial applications. To be submitted to*: Research in Microbiology*;

- one original research paper on “Cave bacteria as producers of antimicrobial and anticancer compounds”. It assesses the potential of limestone cave bacteria as producers of compounds with antimicrobial and anticancer activity. To be submitted to the journal: *Microbes and Infection*.

Chapter II - Light from the darkness: cave microbes as biotechnological tools for industry, environment & health

Light from the darkness: cave microbes as biotechnological tools for industry, environment & health

Abstract

Caves have singular ecological characteristics in what concerns aphotic conditions, low temperature, high levels of humidity, extreme oligotrophy, and pressure variations. Cave microbes capable of withstanding such conditions might have developed biological, genetic, metabolic and physiological adaptations to thrive in different cave ecological niches. Along this evolutionary run, cave Bacteria and Archaea evolved their secondary metabolism, meaning that they can synthesize valuable and exploited metabolites or natural products. This review intends to highlight karst caves as habitats harbouring a diverse microbial community of bacteria and archaea, as well as their potential for biotechnological applications for industrial, environmental and medical purposes. Karstic caves sustain a high diversity of Archaea and Bacteria, with a unique microbiome and parvome. Microbially-produced enzymes are very attractive for the industry arena given their high stability under low temperatures and wide panoply of properties. Cave bacteria also evolved resistance mechanisms to lead with contaminants, what may be explored towards environmental restoration. In clinics, cave bacteria and archaea have a yet undisclosed biosynthetic potential, which has been proven to be valuable for the discovery of novel antibiotic and anticancer drugs to cope with medical demands.

Keywords: karstic cave, microbial diversity, biotechnological potential, under-explored environments, biotechnological applications

2.1. Caves: hydrogeomorphological features and potential microbial habitats

Caves have unique ecological conditions, what makes them to be often considered as extreme environmental habitats[69,70]. This is mainly sustained by the extreme oligotrophy due to the low availability of organic matter and nutrients[71] to fuel microbes heterotrophic metabolism across the ecological niches found in caves (*e.g.,* soil, waterbodies)[72], the high levels of humidity[73], variable pressure, low temperature, and especially the absence of light, what prevents the establishment of photoautotrophs and inputs from photosynthetic activity[70,74]. Notwithstanding, these conditions may vary, depending on the range of light penetrance, energy input, depth of the cave, contact with surface, outside climate and chemoautotrophic activity[74–76].

Caves are classified according to their geomorphological origin and constitution, mainly presenting an horizontal or vertical configuration[77]. They can be karstic, sandstone, volcanic, marine and glacial caves[78]. Karst caves are cavities formed by dissolution of the sedimentary/carbonated/sulphated bedrock, such as limestone, gypsum or marble[71]. The dissolution of the mother-rock can occur by an upwards (epigenic system) or downwards (hypogenic system) water-flow[79]. The karstic landscape occupies almost 20% of the ice-free soil[80] and about 15% of the Earth[81]. Karst caves have rich groundwater sources that supply approximately 25% of the water used for worldwide consumption[82]. Their morphological structure can be quite zone-specific and often allows a separation into five large areas: entrance, twilight, transition, deep and stagnant air zone (not always present). The entrance area is where the surface and underground meet; the twilight zone encloses the area where outside light starts to disappear and the growing of vegetation ceases; in the transition zone begins the dark area, where abiotic changes are still happening; the deep zone in which the air-flow is absent and high levels of humidity can be recorded; the stagnant air zone is where almost no air exchanges occur and the environment can become stationary[77]. The terrestrial and aquatic compartments/habitats across these zones can hence elicit significant differences between each other[81]. The groundwater in these caves is divided in epikarst, unsaturated zone and saturated/phreatic zone. The water flows infiltrating from the sinkhole, forming compartments and cavities – epikarst –, and continues its descending way through the unsaturated zone until reaching the phreatic zone, thereby establishing large cavities. The groundwater usually discharges into a cave stream, which may drain into a spring or into the sea [81].

All these peculiar characteristics of available cave habitats constrain the organisms living in, which can withstand hostile environments due to biosynthetic, resistance, functional and

metabolic pathways they developed to adapt along an evolutionary trend. Some studies point out for the peculiar biodiversity of organisms and genetic pool harboured in caves worldwide, which has been deeply overlooked in nature conservation plans[78]. Microbes are particularly relevant inhabitants of karst caves as they are usually enrolled in nutrient (re)cycling that feed the basis of ecosystem trophic chains. The restricted energy input in karst cave systems, hence, tailors the microbial communities therein living. Karst caves can be distinguished in terms of their energy input. There are oligotrophic[83] karstic systems in which the sources of energy are mainly restricted to nutrients provided by streams, infiltrating water from sinkholes, wind debris, animals from cave inside/outside (*e.g.*, faecal material, dead bodies, nesting and hibernating sites) and roots[81,84]. But other karstic caves present quite poor soils, like arid soils, which have less organic carbon loads, causing a severe oligotrophic environment[85]. These conditions favour the proliferation of bacteria that can nurture themselves on matter deprived of nutrients, namely occurring in the saturated zone, where it is converted into minerals by epilithic bacteria[72]. Specialized populations of microbes, though, may be found in particular sources of energy, like bat guano[86].

As such, microbes can be found in several cave habitats, like waterbodies (*e.g.*, pits, ponds, streams, water drift from stalactites), sediments, soils, bedrock and moonmilk deposits, being demonstrated a great diversity of the microbial community structure between them[74]. Several studies have reported significant differences in the microbiome of close niches in the same cave, suchlike different pools[80] or moonmilk speleothems[71], showing a high heterogeneity. Leuko *et al.*[87] had estimated that there are *ca.* 10^6 microbial cells per gram of rock, demonstrating its high abundance. This is unexpected as there are limited nutrients in karst caves, as for Barton and Jurado[88] proposed an interesting model to explain mutualism between microbes in caves. First, there are microorganisms skilled in fixing organic carbon and their metabolites sustain other microbes, hence replacing competition by cooperation[88]. Thus, cave microbes thrive by cooperation[88,89], contrary to the known competitive-exclusion principle of ecology, meaning that two species will not coexist when they both need a limited resource[88,90].

2.1. Microbial diversity in karstic caves and ecological role

Archaea and Bacteria are the most predominant kingdoms in karstic caves[69,74], but since caves can provide very specialized niches[70,87], the abundance and diversity of representatives from each kingdom and phyla can vary, even between karstic caves with similar features. The genera most commonly reported in different cave habitats are *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Firmicutes*, *Planctomycetes*, *Nitrospirae*, *Verrucomicrobia* and *Chloroflexi*[90,91]. Still, a great variability has been observed in caves worldwide.

In western Europe, recently, there were quite a few studies. In the Italian gypsum and sulfidic caves[82,92,93] Frasassi[92] and Acquasanta Terme[93], the existent microbial biofilms were studied. Macalady *et al.*[92] had mainly found sulphur-cycling Proteobacteria in two distinct morphological biofilms of a stream in cave Frasassi, being *δ-*, *γ-* and *β-proteobacteria* the most dominant *taxa*. In the Acquasanta Terme, the occurrence of mixed sulphide and oxidant bacteria in groundwater[93] was observed, being an habitat of microbial lithoautotrophic primary productivity[92,93] for supplying food chains[93]. Hence , the most predominant bacteria in this latter system were *γ-proteobacteria* (*Thiobacillus baregensis*, *Thiofaba tepidiphila*) and *ε-proteobacteria* (*Sulfurovumales*), with scarce common species in regard to the Frasassi system, despite the habitat similarities[93]. The authors were not successful in amplifying archaeal 16S rRNA genes, though not necessarily meaning that they were not present[93]. Other study, that analysed the quality of water and microbiological composition, found that microbial counts, at niche level, were higher at habitats with a surplus of nitrate, what was not always connected with contamination events[82]. In Hundsalm cave (Austria), a fascinating study reported that the archaeal community was less diverse, but more abundant than the bacterial community in the microbiome of moonmilk deposits[94]. The species identified were mainly unknown lineages of the Euryarchaeota phylum, as well as representatives of the Thaumarchaeota phylum[94]. A similar research and trend was observed in the microbiome of moonmilk deposits of Austrian Alpine caves, since a higher abundance of archaeal *taxa* (*e.g.*, *Nitrosopumilus maritimus* and ammonia-oxidizing Archaea) were depicted, relatively to the bacterial ones, especially in the upper active layer[95]. As for the eastern part of Europe, some studies were developed in Slovenia[90] and Romania[96] caves. In a Slovenian cave, *Agrobacterium* and *Streptomyces* species were highly represented in microbial colonies of cave walls[90]. Epure and his colleagues[96] took a different approach, and analysed quaternary sediments, which keep a snapshot of the ancient microbial communities genetic pool that underwent evolutionary changes over time. The authors had

mainly identified unknown species from Proteobacteria, Chloroflexi, Firmicutes, Acidobacteria, Gemmatimonadetes and Nitrospirae phyla[96].

In the American continent, it was reported from an extreme Mexican oligotrophic karstic system, the isolation of two actinobacterial groups from cave soil and walls[97], being the bacteria identified as *Prauserella*, which closest relative is a marine actinobacteria[97]. It was the first time that this genus was identified in a cave[97], reinforcing the gap of knowledge on the microbial community structure and dynamics in such uncommon habitats.

As for Asia, Wu *et al.*[74] reported significant differences between cave niches (Jinjia Cave, China), as far as in the walls of the cave the most predominant *taxa* were γ-Proteobacteria and Actinobacteria, while in sediments and soil samples the Acidobacteria were more represented[74], enhancing the heterogeneity in close niches as mentioned above. A recent work in Heshang Cave, China[98], suggested that Archaea have a more crucial role in the formation of moonmilk deposits, though still fairly understood[94,95,99],

As already referred, Archaea and Bacteria, once being the most predominant kingdoms in karst caves, play an important role in cave ecosystems[69,74]. For instance, they are responsible for the non-photosynthetic primary production in caves[74,85,100], assuring the functioning of biogeochemical nutrient cycles of sulphur[100,101], nitrogen[87,98], phosphorus[102] and carbon[74]. These microbes possess several metabolic pathways of H_2S, carbon fixation, nitrogen and methane cycles[85,95,103]. Hence, their primary production occurs mainly through a chemolithotrophic metabolism that uses alternative sources of energy production, that sustain chemoorganotrophic organisms in the cave habitat[85,103–105]. It was also reported a symbiosis between a chemoautotrophic bacterium belonging to the *Thiothrix* genus and an amphipod, *Niphargus ictus*[106], showing how deeply enrolled microbes are in the cave biological interrelations of established food webs.

Karst cave bacteria also interact with minerals, inducing their precipitation and dissolution, shaping karst landscape[102,107–109]. More specifically, it could be pointed out the precipitation (via biomineralization processes) and dissolution (via release of acids) of cave minerals (*e.g.*, carbonates)[69,109,110], which processes also promote the mobilization or fixation of metals[111]. These include namely the formation of mineral deposits of phosphorous[102], ferromanganese[107] and calcium carbonate[108,109].

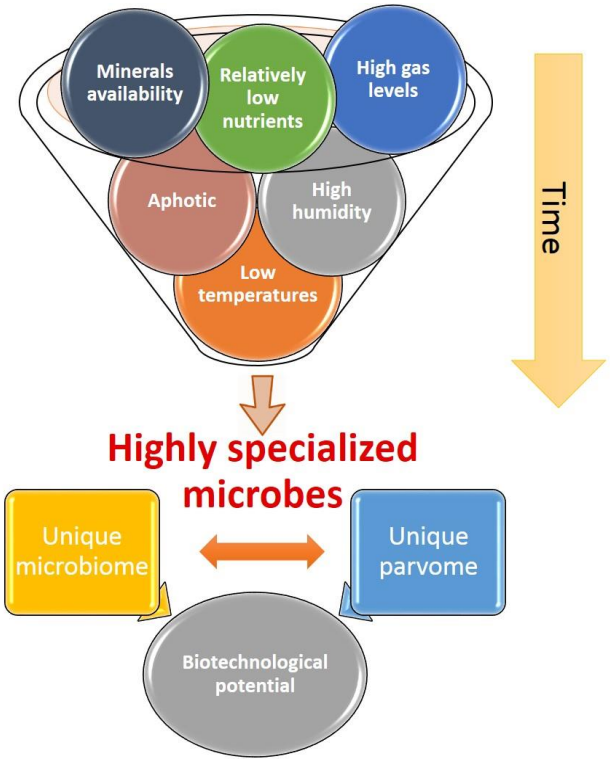

Fig. 1. Schematic explanation of karst cave microbes evolution. A stable habitat, with selective pressures, such as relativity low nutrients, high gas levels, aphotic habitat, high humidity, low temperatures and minerals availability, over time, leads to a specialization of cave microbes. These highly specialized microbes, with a unique microbiome and parvome presents high biotechnology potential.

2.2. Biotechnological applications of cave microbes

Cave microbes that live in unusual and practically untouched subterranean environments characterized by limited nutrient loads[87,89], low temperatures[81,112], varied pressure[77,85] and oxygen availability[77], are also able to adapt their metabolic pathways or physiological mechanism to survive[103], which may force the development of exceptional strategies for nutrient storage, tolerating low temperature and deprivation of oxygen, as well as high pressure environments[85] (Fig. 1). Consequently, the evolution of specific adaptations may have notably tailored their genetic pool and contributed for harbouring an extensively varied parvome[113–115](ensemble of secondary metabolites consisting of small molecules with diverse chemical structures). The parvome synthetized by cave bacteria function as biological strategies to cope with different ecological conditions or requirements, like interspecies competition and the need to fight against other pathogenic microorganisms, inter- or intra-species communication mediated by chemical cues (*i.e.*, quorum sensing), assist environmental adaptations (*e.g.*, biofilm formation) or even generate alternative nutrient sources[114,116]. Other remarkable characteristic of karstic cave

Bacteria and Archaea is the synthesis of enzymes that are stable under low temperatures, as cave cold-adapted (psychrophilic) microbes[117], opening a door to different applications requiring reduced energy in the production system[70,94] and applications in the industry at lower costs[70,114,118], while leveraging risks associated with the proliferation of potential pathogenic microbes growing at human body temperatures[117]. The *Streptomyces* genus often present in different cave systems has been particularly studied for their secondary metabolism, given the bioactivity of the synthesized metabolites, such as antimicrobial activity[113–115,119]. Other karst cave bacteria also have several antibiotic resistance genes in their genome[104], as deeply analysed in the Lechuguilla Cave, one of the most studied cave systems[104,120,121]. Furthermore, there are evidences that the more the exposure to metals, the more resistance genes the environmental bacteria acquire[122], which render them valuable resistance pathways to reduce metals toxicity and bioavailability through their metabolization/chemical transformation, adsorption and/or immobilization[107,112,123,124]. Thus, the secondary metabolites synthetized by cave bacteria can offer a huge biotechnological potential, as to fulfil different needs and supply broad applications[114114]. As for Archaea, only a fraction of their metabolism is known so far[95,99].

In summary, the ability of microorganisms to survive in such specialized environments, showing adaptive evolution, brands their microbiome and their secondary metabolism as unique features[113,114], meaning that cave microorganisms experienced a long and stable evolution, under selective pressures, that allowed a highly adaptability and specialization [87,98,114,119] (Fig. 1).

However, a comprehensive study on the morphological, biochemical, metabolic and physiological traits of environmental cave microorganisms cannot be thoroughly performed, as it is estimated that 99% are not culturable[113,125–127]. Several species are present in the environment in a viable but non-cultivable/growing state[113], if going through a dormant state in which cells are not dividing[125]. Bacterial cells can enter this state under stress conditions[125,127]. They could also be unculturable because their proper culture conditions are not known, and hardly can the environmental properties of their habitat matrices be thoroughly mimicked under the laboratory[128]. As for archaea cells, the culture problems also remains[94]. Some features of cave microbes can make difficult their laboratorial culture and biotechnological exploitation: they are usually slow-growers and may enter temporary stages of reduced metabolic activity, as well as they may depend on metabolically-cooperating microbial consortia[127]. Since they are used to extreme oligotrophic and mineral-rich environments[127], an unfavourable growth is observed under nutrient-rich media[70,125]. To overcome the non-cultivability of bacterial and archaeal cells, an improvement in the cultivation techniques has been applied[113,129]. This includes modification of

growth media[111] and/or incubation conditions (*e.g.*, growing time[70,130], addition of stimulatory compounds[131]) [90,97,114], as well as the establishment of community/co-cultures (cultivation with helper bacteria)[132] and *in situ* culture[133]. One recent system, the iChip, had demonstrated promising results towards the isolation of compounds from environmental unculturable microorganisms[134,135], an *in situ* culture system. Nevertheless, the cultivation of archaea mixed cultures with bacteria[136] and selected cultivation tactics, resembling habitat features[95] had already been successfully implemented. Yet, if biotechnological applications are intended to be developed from cultured microbes, it should be kept in mind that their (bio)activity under laboratorial culture conditions may not resemble that occurring in the natural habitat of the microbes[137,138].

As to overcome microbial cultivation challenges for biotechnological purposes[127], culture-independent methods have been also implemented to enable the study of non-cultivable microbes[126,137]. These methods allow for the search of characteristics of interest more rapidly. Culture-independent methods can be divided in whole-community analysis or partial community analysis[127]. In PCR-based methods, a usual approach involves 16S rRNA gene sequencing[90,96,119,126,139]. Besides 16rRNA-PCR techniques, denaturing gradient gel electrophoresis[82,96], restriction fragment length polymorphisms[126] and quantitative-PCR[74,138] have been applied. Other molecular techniques applied to the DNA were as well implemented, such as fluorescence in situ hybridization[93,140] and next-generation sequencing[86,126,137]. Nowadays, the onset of omics techniques[85,126], has been allowing the study of the whole-community diversity and functions of environment microbes[126].

The ideal scenario is to implement both techniques, independent- and dependent-cultivation strategies, as a complement to each other[127]. For example, phenotype characteristics have been identified using bioinformatic tools[103], but only cultivation techniques can confirm the expression of this phenotype, such as the identification of metabolic and biochemical properties[125,127].

2.2.1. Industrial and environmental applications of cave bacteria

The main use of cave bacteria and archaea for industry is based on the enzymes they synthesize, since they are more stable, may provide a wide range of properties or catalytic activities and, hence, of applications[118]. Psychrophilic microorganisms have enzymes with a flexible structure, capable of functioning at a low temperature[141] , thus demanding lower energy consumption in industrial processes[142]. One of these enzymes industrially-exploited, which is synthesized by cave bacteria and archaea, is urease[143]. It catalyses calcite precipitation[94,111,138,143]

and can therefore be used in the construction industry, as far as it can improve the toughness of construction matrix[143], or function as a protective coat in cement-based materials[142]. *Sporosarcina pasteurii*, which was isolated from the alkaline and calcium-enriched Sarawak cave, was proposed for biocementation processes given its ability to produce urease as well[143]. In Magura Cave, Bulgaria, the screening of bacteria capable of synthesizing other biotechnologically-relevant enzymes were done[70]. This study allowed the isolation of various hydrolytic enzymes, like phytase (capable of hydrolysing phytic acid into inorganic phosphorous; used in animal feeds[144]), xanthan lyase (xanthan lyase are enzymes that depolymerize xanthan[145]; xanthan itself is used commercially as emulsion stabilizer and thickener in food industries[146]), polygalacturonase (involved in the ripening of fruit; used in agriculture[147]), and β-glucosidase (able of cellulose degradation[148]; used in industrial recycling of biomass to obtain energy[149]). Hence, these studies highlight the potential of caves as reservoirs of industrially-exploited enzymes of bacterial/archaeal origin.

Food and textile industries have indeed searched for biotechnological applications of cave bacteria, namely regarding the production of pigments like carotenoids, quinones, flavonoids and rubramines[150]. In the caves of Miroc mountain (Serbia), Stankovic *et al.* [150] isolated and identified a gram-positive and red-pigment producer, *Streptomyces sp*. JS520. This strain showed potential for the large-scale production of the pigment undecylprodigiosin, which also presented antioxidant and UV-protective properties that can be explored towards different industrial sectors like textile, food, pharmaceutical[150] or cosmetics manufacturing.

The capacity of cave bacteria to cope with contaminants (*e.g.*, methane, metals, aromatic compounds), make them promising candidates for being explored as bioremediation agents[112,151,152], thereby reinforcing their potential for environmental purposes regarding the restoration of contaminated compartments. One of bioremediation techniques that has been proposed involves the use of acidophilic microorganisms, which produce enzymes that catalyse metal solubilization - bioleaching[112,153], and could be applied to clean up contaminated coastal sediments[112]. Acidophiles in sulphide-rich caves with low temperatures have hence great biotechnological potential for metals bioleaching, and could be more efficient than mesophilic or thermophilic acidophile strains, because of their lower temperature optimum for metabolic activity[112]. In Frasassi cave system, were isolated *Acidithiobacillus thiooxidans* strains from biofilms, which revealed great potential for arsenic bioleaching[112]. Cave bacteria also have the ability to oxidize/reduce other metals, like iron[107,123,124]. Bacteria in limestone karst cave rocks were further pointed out as successful oxidizers of methane in mesocosms, thereby contributing

for the scavange and sink of atmospheric methane, through methane monooxygenase catalysis[151], preventing the greenhouse impacts of this powerful greenhouse gas[151,152].

The potential of cave bacteria for biocontrol purposes was also already assessed[117]. In the context of space missions, plants have been essential living elements to clean the air, as well as a source of food for crews[117]. But this "space plants" are susceptible to fungal infections[117]. As such, the antifungal activity of the cave soil bacterium *Pseudochrobactrum kiredjianiae* was evaluated, being highlighted its considerable biocontrol potential against fungal infections and strong plant growth-promotion[117].

2.2.2. Applications of cave bacteria microbes for medical purposes

Antibiotic resistance genes are present and common in environmental bacteria since ever, being indeed similar to the ones found in pathogens[104]. The resistome constituted by the collection of the genetically varied and extensively distributed resistance elements, has been subjected to evolutionary pressures. With the medical use of antibiotics, it is believed that these mobile resistance genes elements are positively selected[104,121], what confers microorganisms improved drug resistance abilities namely due to the cumulative acquisition of multiple resistance genes[104,121]. This brings a major problem regarding the containment and control of microbial infectious diseases. Therefore, there has been made an effort to find new bioactive compounds, such as antibiotics, from different biological sources and environments[154].

As previously mentioned, the most studied cave microbes as producers of bioactive compounds and secondary metabolites, are Actinobacteria (70%)[113,115,119,154,155] and, especially, the *Streptomyces* (55%) genus[113,114,150,156,157]. They are particularly known for their antimicrobial activity, but only few studies proceeded to identify the inherent bioactive compounds[113,115,154,155]. In a brief description of recent studies developed under this scope, Nakaew *et al.* [158], isolated a rare *Actinomycete* from soil samples of the Phanangkoi cave, Thailand, which crude extract presented antimicrobial activity against different bacteria (*Bacillus cereus*, a methicillin-resistant *Staphylococcus aureus* and *Paenibacillus larvae*), but also anticancer activity against a human small lung cancer cell line[158]. Axenov-Gibanov *et al*. [119] isolated and confirmed the antifungal and antibacterial activity of compounds synthesized by new strains of Actinobacteria, like Cyclodysidin D and Chaxalactin B, from an underground lake and moonmilk speleothem in Bolshaya Oreshnaya cave (Siberia). Belyagoubi *et al.* [154], isolated Actinobacteria from sediments of a karstic cave (Chaabe Cave) in Algeria and verified that their antimicrobial activity was more pronounced and

frequent against Gram-positive pathogens (*e.g.*, *Staphylococcus aureus, Micrococcus luteus*, *Listeria monocytogenes*, *Bacillus subtilis*)[154]. The authors ascertained that the isolates co-produced polyene and non-polyene metabolites, although they were not identified[154]. Maciejewska *et al.*[114], reported similar results, with a *Streptomyces* isolated from moonmilk. The isolated strains showed greater inhibition of growth against Gram-positive bacteria than against Gram-negative pathogens, having a more significative antifungal activity[114]. The majority of the isolates presented activity against *B. subtilis* and *M. luteus*, whereas the most resistant to growth inhibition was *P. aeruginosa*[114]. In particular, most of the *Streptomyces* isolated showed antifungal activity against *R. argillacea*, a fungi responsible for infections in humans, being this outcome corroborated by the genome mining of non-ribosomal peptide synthetases (NRPS) polyketide synthase (PKS) gene clusters[114]. Adam and colleagues[113] isolated rare moonmilk Actinobacteria strains and observed that their antimicrobial activity was particularly evidenced against Gram-positive bacteria, being detected the presence of biosynthetic clusters of putative secondary metabolites, *i.e.*, PKS and NRPS gene clusters. Rajput *et al.*[157], screened the antimicrobial activity and antibiotic susceptibility of *Streptomyces* strains isolated from Kotumsar cave (India). The bacteria from deeper zones presented enhanced antimicrobial activity, especially against *E. coli*[157]. An already analysed red-pigment (undecylprodigiosin) producer in another section of this review will be sectioned here[150]. The crude extract of this *Streptomyces* sp. JS520 strain showed antimicrobial activity against the human pathogens *M. luteus*, *B. subtilis* and *C. albicans*, but extracts of nonpigmented cultures had no antimicrobial activity[150].

Cervimycins A–D are four polyketide glycosides extracted from the cave bacterium *Streptomyces tendae* (Grotta dei Cervi, Italy), which inhibited the multidrug-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*[159]. The cervimycin class of antibiotics is yet to be thoroughly explored, and even cervimycins A and B are novel antibiotics[159]. Cervimycins A-D can bring a valuable opportunity to cope with the rising resistance of vancomycin[159]. More recently, the Xiakemycin A, a novel pyranonaphthoquinone antibiotic, was identified from *Streptomyces* sp. CC8-201, a cave soil bacterium[156]. The substance presented strong inhibitory activities against Gram-positive bacteria, like *Staphylococcus aureus* and *Enterococcus faecalis*[156]. The antibiotic also showed antitumoral activity against several human tumour cell lines, such as human lung cancer cells and breast cancer cells [156].

Firmicutes from cave soil, walls, sediment, speleothems and water samples had also evidenced antimicrobial activity, being demonstrated that it increased with cave depth[89]. Furthermore, bacteria activity is often associated with a mixture of bioactive compounds or

metabolites, being some of them volatile and mediators of inter/intraspecific interactions between cave microbes[89]. Other alternatives of interest in cave microbes are isolation of antibiofilm compounds, that could inhibit biofilm formation by human pathogens such like *Staphylococcus aureus*[160].

2.2. Final remarks

Caves are singular habitats, still poorly explored, with great probability of finding novel microorganisms and therefore novel enzymes and compounds[114,119]. Although in these last decades the scientific interest and research in caves has increased[161], the limited number of reported studies expressed a major potential in cave microbes for biotechnological applications in industry, showing how they are still undervalued. There is a rising need to fight resistance to substances used as antivirals, antimicrobials, herbicides and antitumoral agents[113]. The World Health Organization asserted the deficiency of antibiotics capable of fighting the escalating resistance of pathogens[154]. For example, vancomycin, which was considered the last option against resistant infections, has been failing against the growing antibiotic-resistant microbes[159]. Despite the numerous studies focusing on caves microbiome and their natural bioactivity, only a few compounds were chemically identified and characterized, being essentially accomplished the screening of their antimicrobial activity against human pathogens[14,150,156,157,162] or anticancer abilities[156]. Likewise, the results of these latest studies analysed in this review confirms the yet untapped health applications of caves microbes.

Chapter III – Bioactivity of bacteria from under-explored environments against lung cancer and associated microbial infections

Bioactivity of bacteria from under-explored environments against lung cancer and associated microbial infections

Abstract

Respiratory tract infections are common in immunocompromised lung cancer patients, partly due to chemotherapeutic treatment. In turn, the limits in bringing new synthetic products, more effective and with lower toxicity to treat lung cancer is leading the pursuit to find novel natural compounds. In this study it was aimed to evaluate the bioactivity of bacteria from limestone caves against bacteria inducing respiratory tract infections (BRTI) in lung cancer patients. Plus, the anticancer activity of cave bacteria crude extracts was tested on murine Lewis lung cancer (LLC) cell line. The targeted BRTI were *Escherichia coli*, *Klebsiella pneumoniae* 1, *K. pneumoniae* 700603, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Micrococcus luteus*. Antimicrobial activity against BRTI was confirmed for 14% of the tested cave isolates (mainly *Pseudomonas* species), being *M. luteus* the most susceptible pathogen (inhibited by 92.5% of the bioactive bacteria) and *P. aeruginosa* the most resistant. Several cell fraction and cell-free fraction crude extracts of the selected cave bacteria were capable of inhibiting the activity of LLC cells by the MTT assay, thereby highlighting their anticancer activity. The outcomes achieved reinforce the potential of these cave bacteria for future biomedical applications towards lung cancer treatment, particularly squamous cell carcinoma, though further studies are yet to be performed.

Keywords: cave bacteria, crude extracts, antimicrobial activity, anticancer activity, MTT assay

3. Introduction

Lung cancer leads the mortality rate in cancer patients worldwide[163]. It is an extremely invasive and metastasizing cancer[7]. Currently, the major procedures of treatment for lung cancer are surgery, chemotherapy, radiation therapy and targeted therapy[164]. Among the patients at the early-stage of lung cancer that are subjected to surgical resection, only 50% can survive and do not show relapse in the first 5 years[165]. Chemotherapy drugs have several side effects, like immune dysfunction[166,167], toxicity[6,7,25] and drug resistance[9,24]. In contrast, it has been achieved attractive outcomes and efficacy improvements with the use of targeted therapies[168]. Yet, the development of drug resistance tend to reduce the effectiveness of these therapies[169]. Another issue that complicates lung cancer patients' recovery and/or survival is the occurrence of respiratory infections, which have been reported in up to 84% of patients[31]. Chemotherapy treatments can have highly deleterious effects on the normal functioning of patients' immune response. For example, the chemotherapeutic drugs used to treat lung cancer incites febrile neutropenia in 10 to 40% of patients, what can be a result of bacterial infection[170]. Febrile neutropenia can cause treatment interruptions that may in turn compromise clinical results[167]. Currently, it is already evaluated what is the risk of infection in febrile neutropenia patients, and there are several approaches of treatment to prevent serious complications derived from bacterial infections that could incur[171–173]. However, other factors besides chemotherapy side-effects can be as well linked to the occurrence of infections, like disease progression itself[31,166], prior lung diseases[166], continuous contact with hospital environments[174] and general frailty of patients[166].

Thereby, there is a need to find new drugs and strategies able to turn the odds in favour of health requirements, namely regarding lung cancer and associated-infections in lung cancer patients. The discovery of unknown natural compounds might help counteract these issues[27]. In particular, natural compounds of microbial origin have been showing relevant bioactivities towards anticancer[27] and antimicrobial[175] medical applications. The greater probability of finding them is in sub-explored habitats[113], such as caves. Recently, it has been identified novel antibiotics synthetized by cave bacteria, like Cervimycins A–D[159] and Xiakemycin A[156], the latter proved to be cytotoxic against the human lung cancer A549 cell line.

Therefore, the need for searching new natural compounds, that could present lesser side-effects and resistance, and in turn greater efficacy is greatly claimed and has been lately pursued. This way, the present work aims the screening and identification of clones with antimicrobial and anticancer activity in the context of lung cancer. In order to accomplish that, it was evaluated the

antimicrobial activity of bacteria isolated from limestone caves against bacteria responsible for respiratory tract infections in lung cancer patients (BRTI). Besides, the anticancer potential of extracts of these bacteria was tested against a murine Lewis lung cancer cell line.

3.2. Materials and methods

3.2.1. Bacteria

Cave bacterial strains targeted in this study were originally extracted from water (shallow pools, dripwater from stalactites), sediment and soil samples collected in limestone caves. Serial dilutions of water samples and sediment/soil extracts were incubated in R2A agar medium plates at room temperature. Grown colonies with different morphological aspects were isolated into fresh agar plates, being re-inoculated until obtaining isolated bacterial clones. These clones were stored at -80ºC in 15% glycerol. The pathogenic bacteria or BRTI *Escherichia coli*, *Micrococcus luteus*, *Aeromonas hydrophila*, *Klebsiella pneumoniae* strain 1, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* were grown in TSA or TSB medium at 37ºC.

3.2.2. Screening of cave bacteria antimicrobial activity

The antimicrobial activity of the isolated strains was firstly screened against BRTI, especially under lung cancer pathological scenarios: *Escherichia coli*, *Micrococcus luteus*, *Aeromonas hydrophila*, *Klebsiella pneumoniae* strain 1, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa*. This assay was done by the spot-on-lawn method[176] on Mueller Hinton Agar plates. The thin lawn was prepared with the overnight culture of each BRTI species, over which a colony of each cave bacteria isolates under screening were inoculated (spotted) (Fig. 2). Three replicates per cave bacteria were considered. The plates were incubated at room temperature for 72h. At the end of incubation, the radius (mm) of the inhibition zone was recorded and interpreted as a measure of the antimicrobial activity against the BRTI species. Average and standard deviation of the measured radius were computed for each three replicates tested in each treatment.

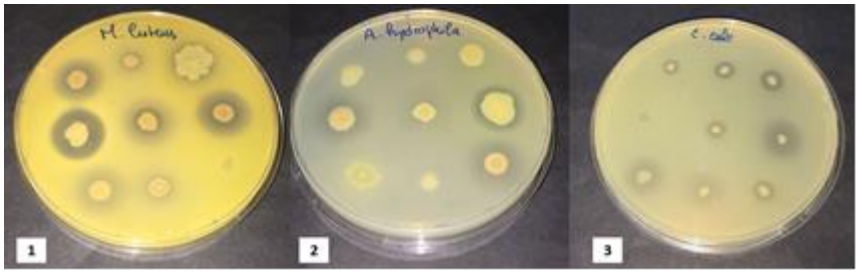

Fig. 2. Spot-on-lawn assay to test the antimicrobial activity of cave bacterial strains against 1- *Micrococcus luteus*, 2- *Aeromonas hydrophila*, 3- *Escherichia coli*

3.2.3. 16S rRNA gene sequencing and bacteria identification

Amplification of the 16S rRNA gene was done by PCR, with universal primers 27F (5'- AGA GTT TGA TCM TGG CTC AG - 3') and 1522R (5'- AAG GAG GTG ATC CAN CCR CA - 3')[177,178]. The 25 µL of PCR reaction mixture contained 12.5 µL of DreamTaq Master Mix, 0.3 µM of each primer and 1µL of (genomic, gDNA) DNA template, obtained upon heat lysis of a bacterial colony suspension. For spore-forming bacteria the DNA was extracted with JetFlex™ Genomic DNA Purification Kit (Invitrogen) before amplifying the 16S rRNA gene with PCR reaction as described above. PCR conditions were as follows: preheating at 94^{o}C for 9 minutes, 28 cycles of PCR amplification at 94^{o}C per 30 seconds of denaturation, 57^{o}C per 30 seconds of annealing, and 72^{o}C per 1.30 min of extension, and a final extension at 72^{o}C for 10 minutes. When needed, PCR products or agarose gel bands were purified with a DNA purification kit. Reverse strands of the amplified DNA fragments were sequenced with Sanger technique. The generated sequences were compared with all accessible entries in NCBI database using BLAST® software. Multiple sequence alignment was performed using CLC Sequence Viewer.

3.2.4. Preparation of crude extracts

The cave bacteria isolates with antimicrobial activity were incubated in liquid medium and grown for three days at room temperature with orbital shaking (160 rpm). The grown cultures were centrifuged to separate cells from the supernatant, thereby obtaining the cell (CF) and cell-free (CFF) fractions, respectively. The supernatant was treated with ethyl acetate:water (1:1), and the pellet/cells with acetone:ethyl acetate (1:1)[179]. Both fractions were shaken for 1h. The solvent

phase of each fraction was obtained in a separation funnel (Fig. 3A), and then the solvent was evaporated in a rotatory evaporator (Büchi, Switzerland) (Fig. 3B). The dried crude extracts (Fig. 3C) were resuspended in dimethyl sulfoxide (DMSO) (Fig. 3D) and stored at -20^{o}C until further tests. The mass of the crude extracts was weighted, and their concentration was determined relatively to the volume of DMSO used. For CFF of DI1, DI3 and DI27, and the CF of DI5, only one replicate was tested because of the limiting volume of extract (the preparation and testing of these extracts will be repeated in the future). The preparation of crude extracts was carried out for bioactive bacteria for which an average radius of inhibition halo was higher than 0.5 mm in the spot-on-lawn assay. However, extracts of bioactive cave isolates with identical molecular identification (homology > 91%) and equal spot-on-lawn results were not performed.

Fig. 3. Preparation of crude extracts. A- solvent phase separation. B- Evaporation of solvent phase. C- Dried extract. D- Resuspension in DMSO.

3.2.5. Activity of crude extracts

a. Inhibition of BRTI

The activity of the CFF and CF extracts was confirmed by the Kirby-Bauer disc diffusion method[180]. Paper discs were individually impregnated with 15 µL of each concentrated extract and tested against the respective pathogens/BRTI, which growth was inhibited in the spot-on-

lawn assay previously performed. Two replicates were tested per BRTI and extract, being also included a DMSO control in order to test if it interfered or not with BRTI growth. Regarding positive controls, one antibiotic was chosen for each BRTI. More specifically, penicillin (10U) for *M. luteus,* chloramphenicol (30 µg) for *A. hydrophila*, streptomycin (300 µg) for *E. coli*, cephalothin (30 µg) for *K. pneumoniae* strain 1, and gentamicin (30 µg) for *K. pneumoniae* ATCC 700603. A thin layer of agar medium individually inoculated with each BRTI was poured onto Mueller Hinton Agar plates. The impregnated paper discs were transferred to the test plates and incubated for 48h at 37^{o}C. The radius of inhibition zone (mm) was measured for evaluating the bioactivity of CFF and CF extracts against the BRTI. Average and standard deviation were computed for the two replicates considered per BRTI and extract.

b. Cytotoxicity MTT assay

The cytotoxicity assays were performed with murine Lewis lung cancer (LLC) cells. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) 1x media (Gibco), supplemented with 10% (v/v) of foetal bovine serum (FBS) and 1% (v/v) of antibiotic Penicillin-Streptomycin at 37ºC and 5% CO_2.

The cell viability was assessed by the colorimetric (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) - MTT assay[181,182] after a 24h exposure to the extracts. This viability assay allows evaluating the impact of the extracts on the activity of mitochondrial enzymes, which catalyse the reduction of MTT to formazan, forming violet crystals that can be spectrophotometrically measured at 550-590 nm[183,184]. The exposure and MTT assay conditions were first optimized in a preliminary test. More specifically, the influence of several variables was evaluated: i) initial cell density - 1 000, 5 000, 10 000, 50 000 cells mL^{-1}; ii) DMSO concentration since extracts were dissolved in this solvent – 0, 0.5, 0.8, 1 and 2% (v/v); iii) measuring times after dissolution of formazan crystals - 5min, 10min, and 15min; (iv) measured wavelengths - 500, 540, 570, 590, and 630 nm. From a 70% confluent cell culture was prepared a cell suspension, which cell density was estimated in a Neubauer chamber. The different cell densities were prepared in DMEM and dispensed in the respective wells of a 96-well cell culture microplate, being left one empty column to be used as blank of spectrophotometric readings. Cells were let to attach for approximately 24h. The culture medium was then removed and replaced by the different DMSO test solutions prepared in DMEM. Four technical-replicates were considered per cell density and DMSO concentration, being empty wells used as a blank. After 24h exposure under the same culture conditions, the DMSO test solutions were removed and the MTT assay was initiated for

about 2-3h. At the end of this exposure time, the MTT solution was discarded and the formazan crystals were dissolved with concentrated DMSO. After 5, 10 and 15 min was measured the optical density at 500, 540, 570, 590 and 630 nm, being the last used to correct the absorbance readings. Based on the results of the optimization assay, an initial cell density of 25 000 cells mL^{-1}, absorbance readings at 570 nm after 10min incubation, and 1% (v/v) DMSO were established for the crude extracts assays. Although this cell density was not included in the optimization trial, a 1% DMSO control was included in the assays of extracts in order to confirm its effect.

The cytotoxicity of crude extracts was tested against LLC cells, which were exposed to a test concentration of 1% (v/v). In this assay were also included: a blank, a negative control (cells + culture medium) and a solvent control (cells + culture medium with 1% DMSO). For each treatment (*i.e.*, controls and extracts) were tested four technical-replicates (*i.e.*, 4 wells) and two experimental replicates (*i.e.,* 2 microplates). The spectrophotometric measurements were made after 10 min dissolution at 570 nm and 630 nm. The average and standard deviation of the replicated absorbance readings were computed and plotted. A one-way analysis of variance (ANOVA) followed by the Dunnett's test multicomparison test was performed on Sigmaplot® v14 software to test if the DMSO concentrations or bacterial extracts induced a significant effect on lung cancer cells activity, in comparison to that of the control.

3.3. Results and discussion

3.3.1. Screening for antimicrobial acitivity

Among the 264 cave bacteria isolates tested against BRTI, 14% (37 isolates) presented antimicrobial activity, according to the spot-on-lawn assays. A low percentage of cave bacterial isolates with antimicrobial activity were reported in other similar studies, like Krubera-Voronja Cave[89]. This low percentage of antimicrobial activity may not necessarily mean an absence of antimicrobial properties of the apparently non-active bacteria, but instead the assay or culture condtions may not trigger their secondary metabolism towards the production of bioactive compounds[113,139]. None of the isolates demonstrated antimicrobial acitivity againt *P. aeruginosa* (Fig. 4), being this antimicrobial resistance of *P. aeruginosa* already previously observed[139,154]. On the contrary, it was recorded a predominance of antimicrobial activity against *M. luteus* (92%), followed by 51% (19 isolates) against *A. hydrophila*, 27% (10 isolates) against *E. coli*, and 11% (4 isolates) against *K. pneumoniae* 1, and *K. pneumoniae* 700603 (Fig 4). The greater antimicrobial

susceptibility of Gram-positive BRTI comparatively to that of Gram-negative BRTI when co-incubated with cave microbes has been also documented in other studies[113,154], most likely because Gram-negative bacteria have an outer membrane that offers extra-protection against antibiotic action of bioactive compounds synthesized by other bacteria[185].

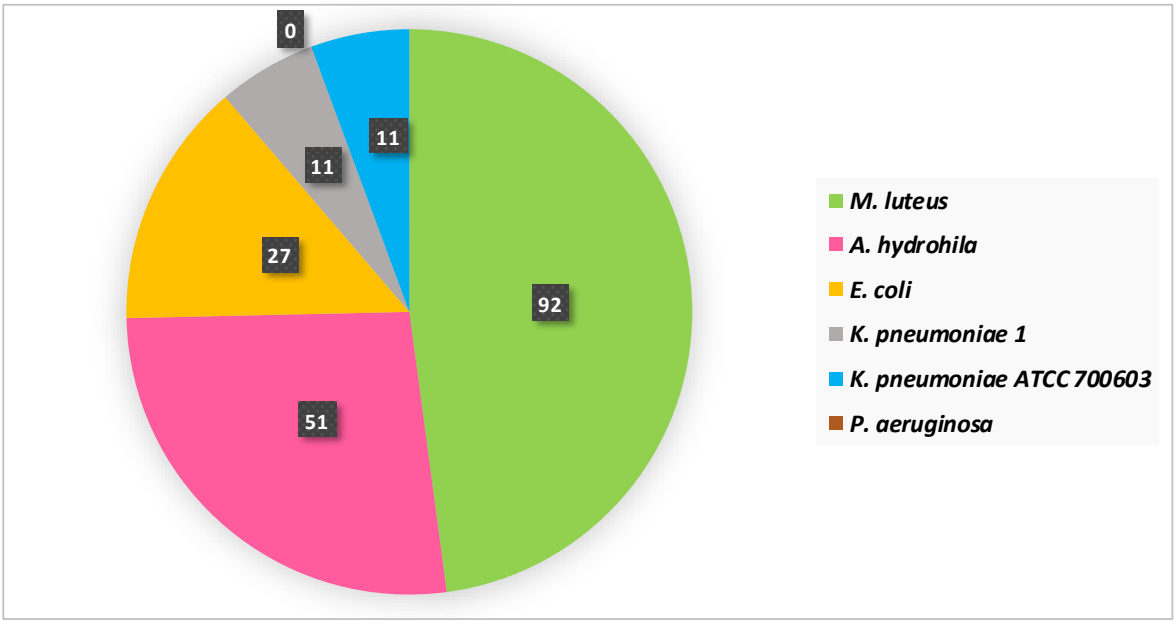

Fig. 4. Percentage (%) of cave bacteria isolates showing antimicrobial activity against BRTI pathogens in the spot-on-lawn assay. Percentages calculated relatively to the total number of bioactive cave bacteria.

The larger growth inhibition zones were also determined to be against *M. luteus*, and several cave bacteria showed antimicrobial activity against two otgr more BRTI (Fig. 5). Amongst these cave bacteria, DI25 and DI37 inhbited the growth of five BRTI, meaning, all except *P. aeruginosa* (Fig. 5), hence marking them as isolates of interest. Regarding the intensity of the inhibition, the cave bacteria DI3, DI4, DI5, DI14 and DI27 induced wider inhibition zones than other cave bacteria, particularly against *M. luteus* (Fig. 5).

3.3.2. Taxonomic identification of cave bacteria

The 37 cave bacteria isolates that presented antimicrobial activity against BRTI in the spot-on-lawn assay were sequenced and, except for 4 to which no sequence could be yet amplified, the matching performed in BLASTN retrieved matches with an homology ≥98% for 8 isolates, and <98% for 25 bacteria. Above 98% homology it is likely to be the same species though it could be a different strain[186].

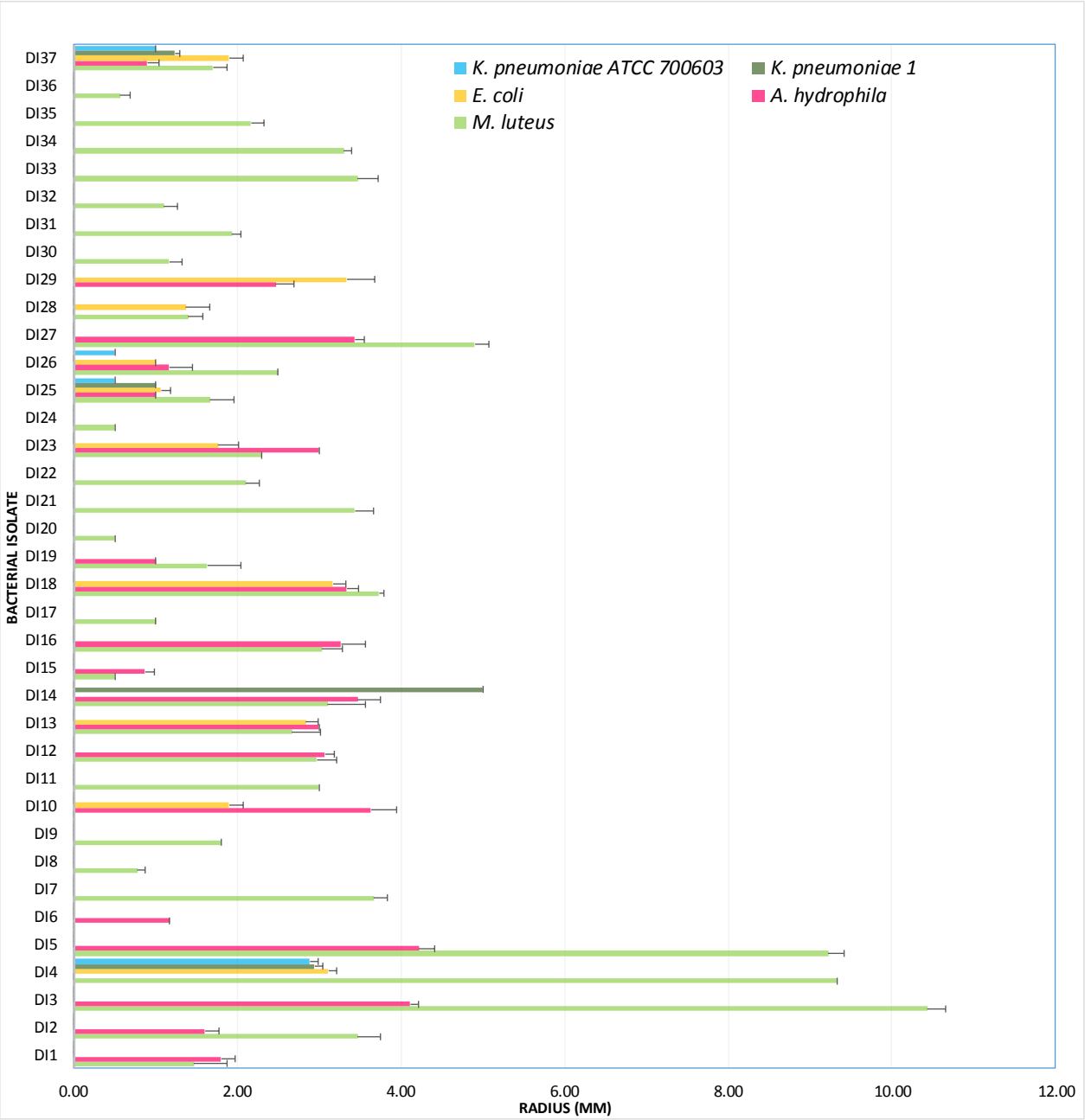

Fig. 5. Average radius (mm) of growth inhibition halo against BRTI pathogens, by the spot-on-lawn method. Error bars represent average standard deviation.

However, an homology below 98% require the taxonomic confirmation through the amplification of other genes capable of providing molecular phylogeny of bacteria or through other molecular, biochemical and physiological testing. But if lower than 95% it may correspond to new bacteria species. Nevertheless, even at >99% homologies, some studies found that bacteria under phylogenetic identification were new species (*e.g.*, Rajendhran[187]). This would not

be surprising in the present study, considering that cave habitats are extremelly under-explored, especially in Portugal. Hence, there is great chance that most of the cave bacteria isolates are actually new species, what will be fine-tuned in the near future following a polyphasic approach. The size of amplified sequences ranged between 364 and 1644 bp (Table 1). The size of the fragment we aimed was 1500 bp, but with the purification of PCR products or gel bands, some DNA might have been lost, what can partly explain that some sequenced 16S rRNA gene fragments of cave bacterial isolates presented small sequences, particularly for *Streptomyces* species.

Overall, the closest relatives for most 16S rDNA sequences of cave bacteria were *Pseudomonas* species (*ca.* 68%) (Table 1). Fifteen cave bacteria isolates were identified as *Pseudomonas wadenswilerensis* (60% of the Pseudomonads) with an homology higher than 91%. Isolates DI-14 and DI-27, which induced a high of BRTI growth in the spot-on-lawn assay, were idientified as *Pseudomonas donghuensis* (98%) and *Pseudomonas tolaasii* (96%), respectively. The high proportion of Gammaproteobacteria in rock deposits and other limestone cave habitats was reported in previous studies[70,74]. Isolates DI7, DI8, DI9, DI24 and DI31 were identified as *Streptomyces*, which belongs to the Actinobacteria phylum and is also a frequently detected *taxon* in limestone caves[114]. *Streptomyces* are broadly known for their wide secondary metabolism[114,139], specially regarding the synthesis of antimicrobial compounds[150,156]. Nevertheless, the Actinobacteria phylum is not significantly represented among the isolated and bioactive cave bacteria selected in this study, what could be due to the culture medium used, as several authors use specific media for the isolation and cultivation of Actinobacteria[89,139]. It was indeed noticed that almost all of the cave isolates are Gram-negative bacteria, except for the *Streptomyces* species. The prevalence of Gram-negative isolates in this type of caves has been reported also in Magura Cave (Bulgaria), what suggests that the dominance of Proteobacteria could be associated with higher energy inputs[70]. Indeed, Gammproteobacteria (*e.g.*, *Pseudomonas* spp.) are copiotrophs and have been described as oppottunistic and fast-growing bacteria[188]. They are involved in nitrogen[189] and carbon[190] cycling as well, what could further elucidate the abundance of this class in habitats with more energy input.

Table 1 Identification of isolated cave bacteria based on 16S rRNA gene sequencing. ni – not identified.

Bacterial clone	Phylotype	Closest relative	Phylum	Homology (%)	Cover (%)	bp
DI1	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	95	92	999
DI2	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	98	79	1365
DI3	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	96	86	1115
DI4	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	91	92	949
DI5	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	96	72	1183
DI6	ni	ni	ni	ni	ni	ni
DI7	*Streptomyces purpureus*	*Streptomyces purpureus* NRRL B-5403	Actinobacteria	96	83	395
DI8	*Streptomyces avidinii*	*Streptomyces avidinii* NBRC 13429	Actinobacteria	99	100	671
DI9	*Streptomyces subrutilus*	*Streptomyces subrutilus* NBRC 13388	Actinobacteria	97	94	364
DI10	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	98	42	680
DI11	*Pseudomonas chlororaphis spp. aurantiaca*	*Pseudomonas chlororaphis spp. aurantiaca* NCIB 10068	Proteobacteria	97	89	1397
DI12	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	96	67	1644
DI13	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	97	93	1170
DI14	*Pseudomonas donghuensis*	*Pseudomonas donghuensis* HYS	Proteobacteria	98	89	1509
DI15	ni	ni	ni	ni	ni	ni
DI16	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	96	81	1246
DI17	*Variovorax boronicumulans*	*Variovorax boronicumulans* BAM-48	Proteobacteria	98	94	898
DI18	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	96	87	1030
DI19	*Pseudomonas donghuensis*	*Pseudomonas donghuensis* HYS	Proteobacteria	95	96	1394
DI20	*Pseudomonas kilonensis*	*Pseudomonas kilonensis* 520-20	Proteobacteria	97	74	1556
DI21	ni	ni	ni	ni	ni	ni
DI22	*Pseudomonas baetica*	*Pseudomonas baetica* a390	Proteobacteria	97	85	1469
DI23	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	98	87	1414
DI24	*Streptomyces subrutilus*	*Streptomyces subrutilus* NBRC 13388	Actinobacteria	96	82	433
DI25	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	98	98	1033
DI26	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	97	90	1216
DI27	*Pseudomonas tolaasii*	Pseudomonas tolaasii LMG 2342	Proteobacteria	96	93	1480
DI28	*Pseudomonas donghuensis*	*Pseudomonas donghuensis* HYS	Proteobacteria	98	60	1532
DI29	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	96	75	1566
DI30	*Pseudomonas lurida*	*Pseudomonas lurida* P 513/18	Proteobacteria	96	100	955
DI31	*Streptomyces subrutilus*	*Streptomyces subrutilus* NBRC 13388	Actinobacteria	97	70	1594
DI32	*Pseudomonas migulae*	*Pseudomonas migulae* CIP 105470	Proteobacteria	96	79	1439
DI33	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* DSM 50071	Proteobacteria	97	89	1302
DI34	*Pseudomonas baetica*	*Pseudomonas baetica* a390	Proteobacteria	97	85	1528
DI35	ni	ni	ni	ni	ni	ni
DI36	*Pseudomonas alcaligenes*	*Pseudomonas alcaligenes*	Proteobacteria	96	81	1510
DI37	*Pseudomonas wadenswilerensis*	*Pseudomonas wadenswilerensis* ID2	Proteobacteria	95	48	547

3.3.3. Crude extracts activity

a. Inhibition of BRTI

In the disc-diffusion assay, the DMSO control did not inhibit BRTI growth, what validated its use as solvent of crude extracts. Moreover, the BRTI were susceptible to the antibiotics chosen as positive controls, showing similar growth inhibitions as reported in the literature. The average±standard deviation of the halo radius for penicillin-exposed *M. luteus* was 19.0±0.00 mm, for chloramphenicol-exposed *A. hydrophila* was 8.67±0.00 mm, for streptomycin-exposed *E. coli* was 5.0±0.00 mm, for cephalothin-exposed *K. pneumoniae* 1 was 2.9±0.12 mm, and for gentamicin-exposed *K. pneumoniae* ATCC 700603 was 5.0±0.00 mm. In what concerns the exposure of BRTI to the cave bacteria crude extracts, it was noticed a slight decrease in the average radius of the inhibition zone in the disc-diffusion assay, in comparison to that induced by living colonies on the spot-on-lawn assay (Fig. 6). Besides, some of the bacterial extracts did not present activity against BRTI, though the latter were previously susceptible to the respective cave bacteria when co-incubated alive in the spot-on-lawn assay (*cf.* section 3.2.1.). This could be explained by our crude extracts being a mixture of compounds and not yet a refined product[119]. On the other hand, it can be indicative of the need to depart from higher volumes of bacterial culture, in order to extract more quantities of the bioactive compound. Plus, the cultures conditions (*e.g.*, medium, incubation time and temperature, pH) can indeed constrain the biosynthesis of natural products by cave bacteria. As such, future attempts should be done towards the optimization of extracts production.

In a general view, it was also possible to verify differences in the average radius of the CFF *versus* CF extracts. The synthesized compounds can be kept intracellularly (in cell cytoplasm or membrane) or released to the extracellular environment, thereby rendering different activities of bacterial fractions[119].

Despite the apparently similar phylogenetic identification of the cave bacteria isolates, the different antimicrobial activities induced by each one, strongly highlights they might actually be different strains or species. The crude extracts that showed the most powerful activity against *M. luteus* were DI27-CF, and CFF of DI26 and DI37 (Fig.6a). For some extracts, both fractions presented antimicrobial activity, namely those from DI2, DI3, DI5, DI16 (Fig.6a). DI27 had similar antimicrobial results for *M. luteus* in the spot-on-lawn assay and it was identified as *P. tolaasii*, which is known for the production of tolaasiin, a phytotoxin[191] (Tables 1 and 2). For the fifteen

isolates identified as *Pseudomonas wadenswilerensis*, some evidenced similar activities against *M. luteus* when tested as colony (spot-on-lawn assay) or extract (disc-diffusion assay) (*e.g.,* DI2, DI3, DI4, DI5, DI16); whilst for others (DI26 and DI37) the outcome obtained in each assay was not coherent (Figs. 5, 6).

As for *A. hydrophila*, the extracts that presented strongest inhibitory activity were the cell-free fraction of DI5 and DI37, as well as the cell fraction of DI-1 and DI-3 (Fig. 6b). The cell-free fraction extraction of DI37, in particular, demonstrated to have a broad antimicrobial activity against five BRTI (*M. luteus, A. hydrophila, E. coli, K. pneumoniae* 1 and *K. pneumoniae* ATCC 700603), what highlights the biosynthetic potential and biotechnological value of this cave bacteria for medical purposes (Fig. 6c). The extracts prepared from both fractions of DI1, DI5, DI12, DI16, and DI23 could inhibit *A. hydrophila* growth. The isolates DI23 and DI14, which inhibited the growth of *M. luteus, A. hydrophila* and *E. coli* when tested as live colonies in the spot-on-lawn assay (Fig. 5), in the disc-diffusion assay only presented antimicrobial activity against *M. luteus* (CF: DI14, DI23) and *A. hydrophila* (CF: DI14, DI23; CFF: DI23) (Figs. 6a, 6b), while DI1, DI3, DI5, DI12 and DI16 presented similar results in both tests. DI14 was identified as *Pseudomonas donghuensis*, which was firstly isolated from a lake in China[192]. To this bacteria it was attributed antifungal[193,194] and antibacterial[194] activities (Table 2).

The 15 isolates identified as *P. wadenswilerensis*, though, induced different antimicrobial activities. Hence, they could not only represent different strains, but also different bacteria, as the 16S rRNA gene has several intra-genomic copies that could diverge in the sequences within the bacterium, thereby misleading the identification[187,195]. *P. wadenswilerensis* was first isolated from a forest soil, and initially assigned to the *Pseudomonas putida* group based on 16S rRNA sequencing, but upon a deeper analysis of the molecular, biochemical and physiological traits, it was classified as a new species[196,197]. Therefore, a future approach in which a combination of multilocus sequence typing analysis (MLSA), together with other complementary approaches, should be undertaken as to accomplish a more reliable identification of the 15 cave Pseudomonads[114,187]. Anyway, this group is recognized by its biotechnological potential in both industry and medicine[196,197], but to the best of our knowledge, no bioactive compound specifically produced by *P. wadenswilerensis* or bioactivity was so far identified and characterized (Table 2).

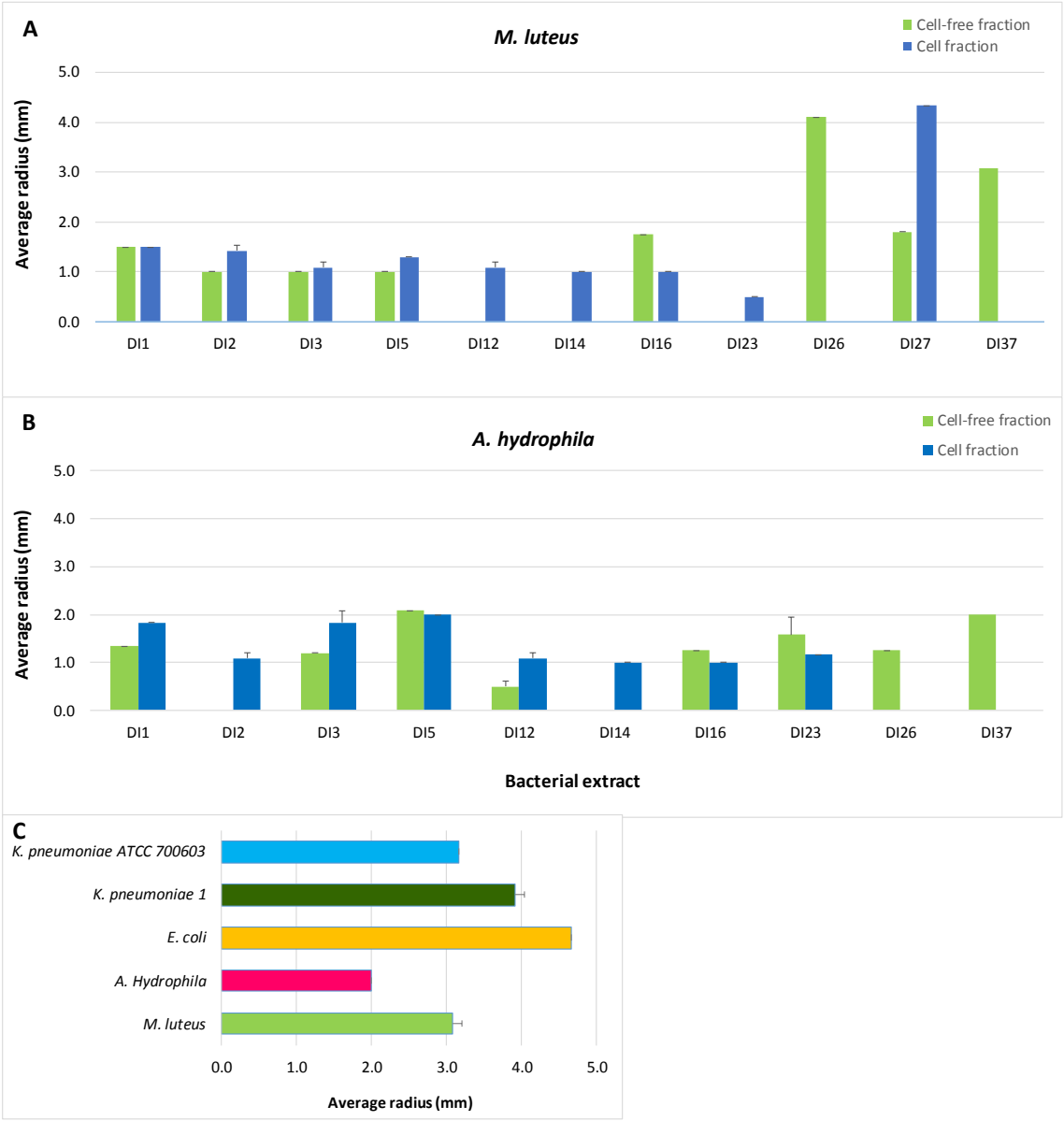

Fig. 6. Average radius (mm) of the inhibition halo induced by cave bacteria extracts (cell-free and/or cell fractions) against **(a)** *Micrococcus luteus*; (**b)** *Aeromonas hydrophila*, through the disc diffusion assay. **(c)** Antimicrobial activity of one particular cave isolate (DI37), which cell-free fraction extract evidenced a broad-spectrum action against five BRTI (the outcome obtained for DI37 against *M. luteus* and *A. hydrophila* in figures 6A and 6B was represented again in 6C for comparison means). Error bars indicate standard deviation.

Table 2. Compounds synthesized by the phylotypes assigned for the cave bacterial isolates, according to the literature. ni – not identified.

Species	Identified compound	Activity/pathogenicity/ application	References
Pseudomonas tolaasii	Tolaasiin	Phytotoxin	191
Pseudomonas wadenswilerensis	ni	-	196,197
Pseudomonas donghuensis	ni	Antifungal, antibacterial	193,194
Pseudomonas migulae	ni	Antibacterial	198
Pseudomonas baetica	ni	Fish pathogen	199,200
Pseudomonas aeruginosa	ni	Human pathogen	201
Pseudomonas chlororaphis spp. *aurantiaca*	Phenazines	Antifungal	202,203
Pseudomonas kilonensis	ni	Antifungal	204
Variovorax boronicumulans	ni	Bioremediation	205
Pseudomonas alcaligenes	Rhamnolipids	Fish-pathogen, bioremediation	206–208
Pseudomonas lurida	Rhamnolipids	Antifungal, plant growth promotion	209–211
Streptomyces subrutilus	ni	Bioremediation agent, antifungal	212,213
Streptomyces avidinii	ni	Anticancer	214
Streptomyces purpureus	ni	Antibacterial	215

b. Cytotoxicity assessment

As to optimize the exposure and MTT assay conditions for the LLC cell line, a preliminary trial was performed for evaluating the effect of initial cell density, measuring time, wavelength and DMSO concentration. Regarding the wavelength and time of measurement, it was verified that the absorbance of the dissolved formazan crystals was higher at 570 nm and 10 minutes, respectively (Fig. 7). Concerning the initial cell density, the one at which a linear relationship could be obtained in regard to absorbance was between 10,000 and 50,000 cell mL^{-1}. For that reason, it is proposed an initial cell density of 25,000 cell mL^{-1} for the exposure assays with extracts (Fig. 7).

Since the bacterial extracts were dissolved in DMSO, it must be analysed up to which concentrations DMSO can be safely used without inducing an impairment on LLC cells activity *per se*. Based on the outcome of the optimization trial described above, the absorbance measurement was performed after 10 minutes of dissolution of formazan crystals. According to

the results obtained, it was possible to verify that for 1000 cells mL^{-1} DMSO was already toxic at 0.5% (500 nm) (Fig. 8). As for 5,000 cell mL^{-1}, DMSO was toxic for concentrations above 1% (v/v) for all wavelengths (Fig. 10). Regarding 10,000 and 50,000 cells mL^{-1}, only 2% (v/v) of DMSO induced a statistically significant reduction on LLC cells activity (Fig. 10). These results show that DMSO can affect LLC cells viability, depending on their initial densities, being particularly toxic for lower cell densities. In the light of this, it was decided to perform the cytotoxicity assay with the extracts at a concentration of 1% (v/v).

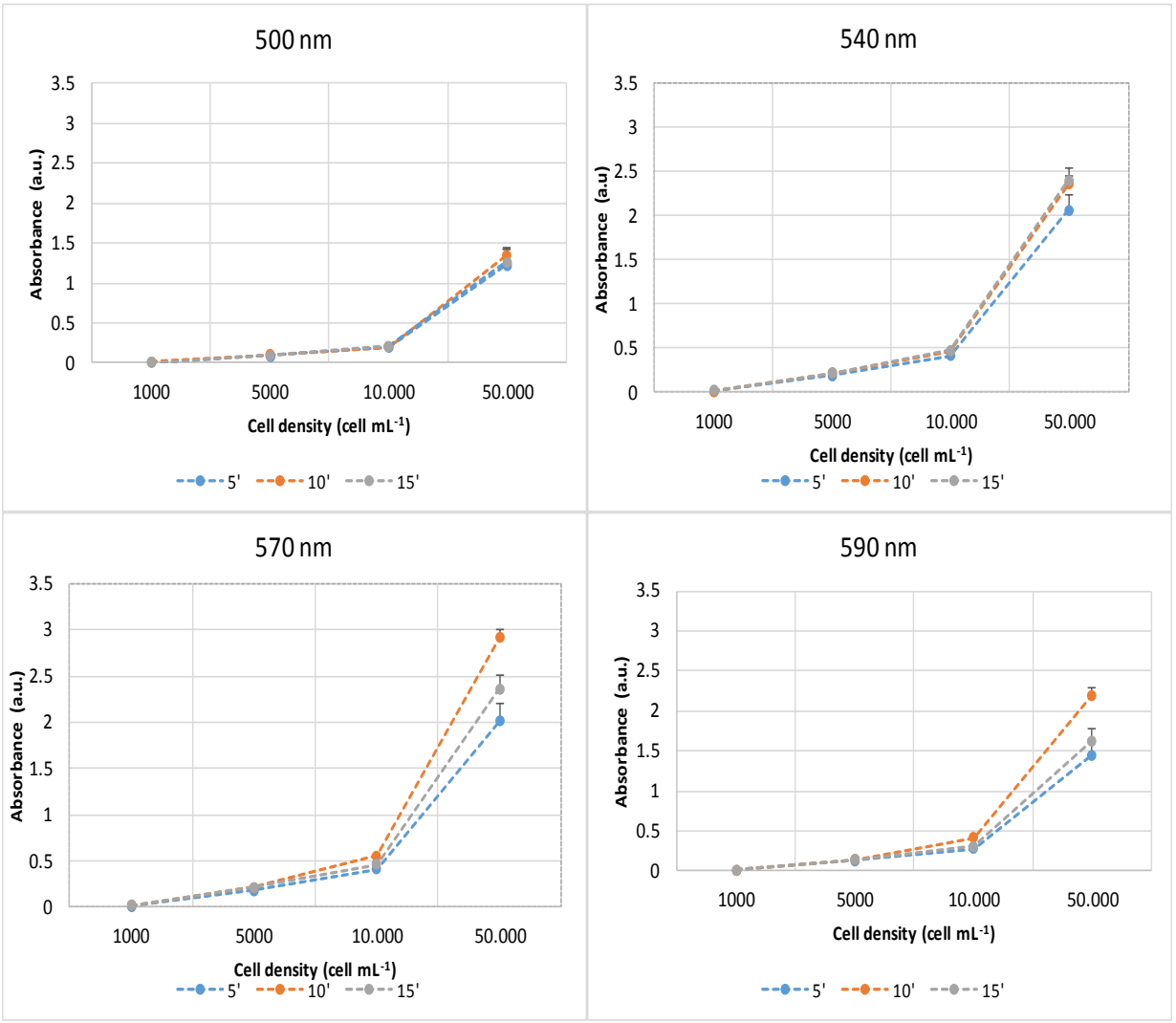

Fig. 7. Average absorbance readings resulting from the MTT assay performed to test the influence of initial cell density (1000, 5000, 10.000 and 50.000 cells mL^{-1}), time to absorbance measurement (5, 10 and 15 min), and wavelength (500, 540, 570 and 590 nm) on Lewis lung cancer cell line activity. Error bars represent standard deviation.

The bacterial extracts were prepared from the CF and CFF of grown bacterial cultures, being obtained stock concentrations ranging between 9 and 20 mg mL^{-1} for CF, and between 8 and 20 mg mL^{-1} for CFF (Table 4). Therefore, the tested concentrations of CF or CFF crude extracts varied between 80 and 200 µg mL^{-1}.

The evaluation of the cytotoxicity of the bacterial crude extracts allowed to observe that there were several of them capable of affecting murine LLC cells. More precisely, the biological response induced by 73% of the extracts was significantly different from that of the negative control (Fig. 9). Besides, it was confirmed that the DMSO control did not inhibit LLC cells activity as far as no statistically significant outcome was not achieved in regard to the negative control. Overall, a higher percentage of CF (80%) crude extracts showed to be more cytotoxic to LLC cells activity than that of CFF extracts (61%), according to the MTT assay outcome (Fig. 9). The most cytotoxic CF extracts were obtained from DI1, DI5, DI27, whilst the most cytotoxic CFF extracts were obtained from DI4, DI9, DI26, and DI27 (Fig. 9). The CF of DI7 and DI35 induced a significant reduction on cells activity even at a concentration as low as 9 µg mL^{-1} (Table 4).

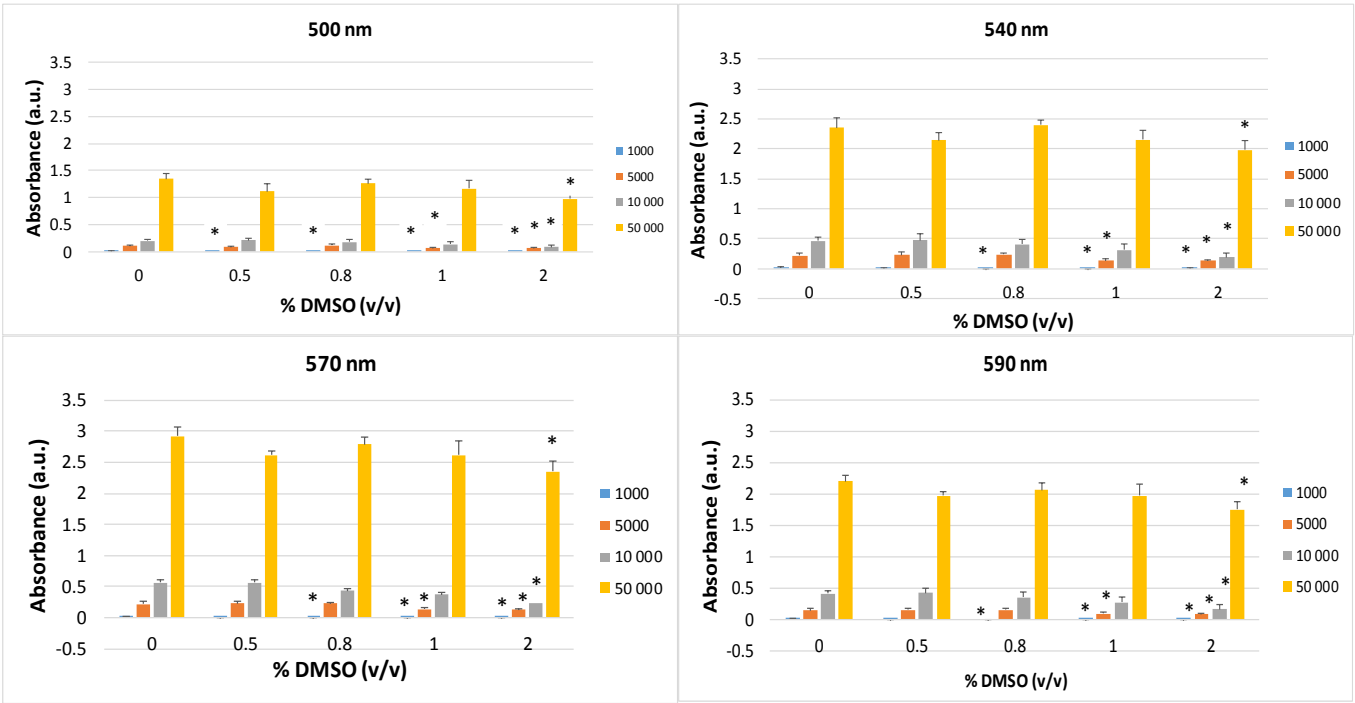

Fig. 8. Average absorbance readings resulting from the MTT assay performed to test the effect of DMSO concentration in Lewis lung cancer cell viability, upon different initial cell densities (1000, 5000, 10.000 and 50.000 cell mL^{-1}) and wavelengths. Error bars represent average standard deviation. * - result significantly different from the negative control ($p<0.05$).

Similar results were obtained with the crude extracts of a *Nonomuraea* sp. strain that was isolated from cave soil[216]. The CF extracts of this bacterium were analysed and showed anticancer activity against a human small cell lung cancer cell line, with 6.96 µg mL^{-1} of crude extract necessary for the cytotoxic effect. However, in a breast cancer cell line it did not present cytotoxicity, even at 50 µg mL^{-1} [216]. It must be retained, however, that the extracts herein prepared and tested are a mixture of compounds, so the concentration of the active compound is unknown yet. Still, this study provided enough evidences to demonstrate that cave bacteria are an unexploited source of anticancer compounds. The LLC cell line was isolated from a squamous cell carcinoma in the lung of the mouse by Dr. Margaret Lewis[217]. This type of NSCLC has not many therapeutic choices[218,219], what greatly boosts the interest on the promising results herein achieved.

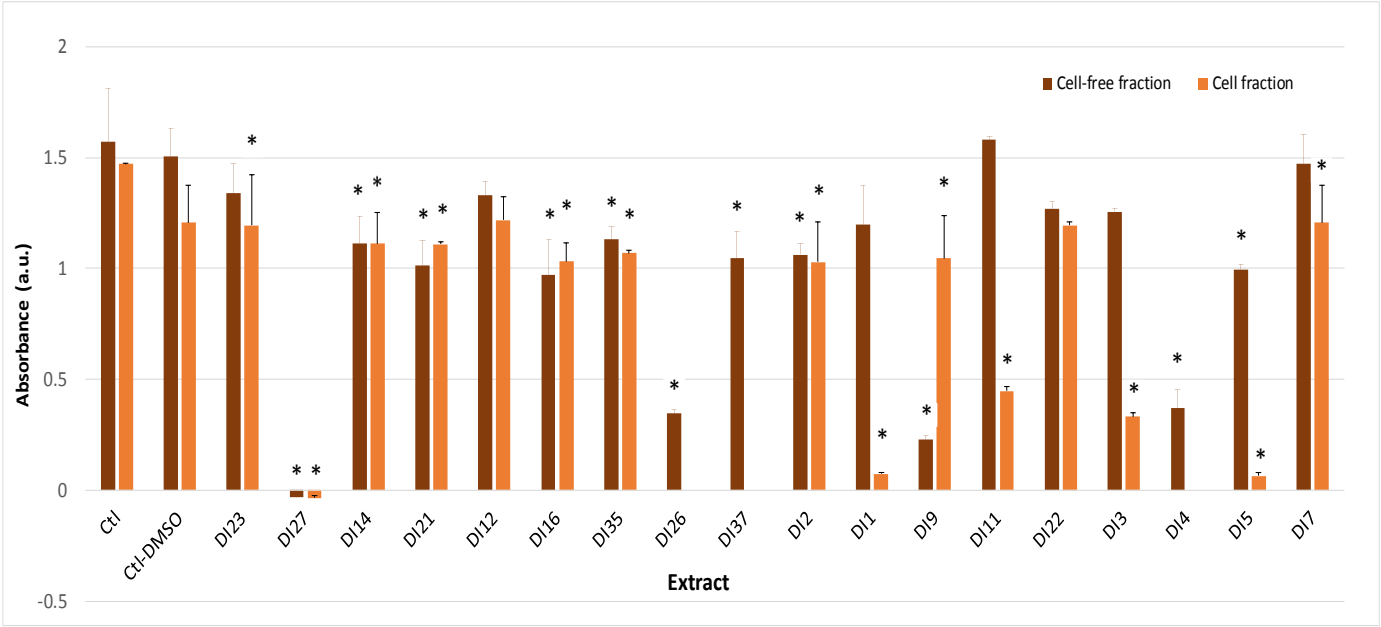

Fig. 9. Cytotoxicity (measured as a reduction on absorbance values) of extracts against murine Lewis lung cancer cells, according to the MTT assay. Negative control was composed by culture medium (Ctl) and the DMSO control contained medium (Ctl-DMSO), cells and 1% (v/v) of DMSO. Initial cell density of 25 000 cells mL^{-1}. Error bars represent average standard deviation. * - statistically significant different from negative control ($p<0.05$).

Nevertheless, further tests are needed, such as confirmation of these results with other cytotoxicity assays, like the Trypan Blue exclusion test[220]. Trypan Blue exclusion assay is based on the principle that live cells have intact cell membranes that do not allow the entering of the dye[220], or the contrary if cell membranes were damaged, hence indicating a cytotoxic effect. It is also needed the cytotoxicity evaluation on a non-tumoral cell line. In the possibility of cytotoxicity

against non-tumoral cells, this does not invalidate our results, as the future refined compounds could be administered more locally to tumoral cells. This could be accomplished by drug-delivery systems, such as nanomaterials[221]. The optimization of the fermentation conditions for inducing the production of the compounds is likewise necessary[89,222], as it could increase the number of extracts with anticancer activity . The minimum inhibitory concentration of extracts should also be assessed[222], as well as the isolation and purification of the bioactive compounds coupled with chemical methods such as HPLC[216,223] or identification by methods like Ultra-Performance Liquid Chromatography associated with Mass Spectrometry[114], could help to fine-tune the identification and characterization of the chemically-active structures and their stability[216,223–225].

Table 3. Stock concentration of extracts (mg mL^{-1}). nt=not tested.

Extract	Concentration (mg mL^{-1})	
	CFF	**CF**
DI23	20	20
DI27	20	20
DI14	19	10
DI21	8	19
DI12	8	10
DI16	10	10
DI35	10	9
DI26	11	nt
DI37	10	nt
DI2	20	20
DI1	20	20
DI9	20	20
DI11	20	10
DI22	20	20
DI3	20	19
DI4	20	nt
DI5	20	19
DI7	10	9

4. Conclusion

Facing the current problems related with antimicrobial and drug resistance, it is time to turn into the discovery of novel and bioactive natural compounds. With this work was shown that live bacteria from under-explored caves, as well as their crude extracts, may offer a great potential to be a source of novel antimicrobial and anti-lung cancer compounds, particularly squamous cell

carcinoma. In general, the extracts were more active against the LLC than BRTI. Most of the phylotypes relative to the cave bacterial isolates do not have any identified compound so far, thereby reinforcing the relevance for their future bioprospection and study as to find out which compounds are mediating the screened bioactivities.

Chapter IV – Final considerations

5. Final considerations

Our study provides a window to the sub-explored potential of cave bacteria. Overall, we tested 264 cave bacteria isolates and identified 37 with antimicrobial activity. This antimicrobial activity was higher against Gram-positive pathogens that are responsible for several nosocomial infections. The crude extracts of the isolates also showed antimicrobial activity and could be promising towards the isolation of novel compounds with clinical importance for the treatment of infections, specifically in lung cancer patients-associated infections. The anticancer activity presented by the crude extracts of cave bacterial isolates was also high, hence suggesting a great biomedical potential in what concerns lung cancer therapeutics. As LLC is a squamous cell lung carcinoma cell line, which cancer has little therapeutic options, it enhances the potential for future medical applications.

It was reached the expected outcome, that is, to provide preliminary data that points out caves as a rich source for the isolation of bacteria producing bioactive compounds. The literature also supports this hypothesis, *i.e.*, the need to find new compounds for health applications and the higher chance of finding them in under-explored environments. It has thereby been highlighted the attractive characteristics of cave bacteria for biotechnological purposes.

To the best of our knowledge there is a great gap concerning the availability of studies directed to the bacteria inhabiting Portuguese limestone caves and their biotechnological abilities. There are some studies in Azores islands, but they were developed in lava cave bacteria[226–228]. Hence, the present work confirms the high relevance that cave bacteria can assume in the future to cope with clinical applications, besides suggesting that the Portuguese underground microbial world is still a deeply under-explored resource.

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