Universidade de Aveiro Departamento de Biologia
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Avaliação da atividade antimicrobiana de bactérias da Gruta da Cerâmica (Portugal): O caso de estudo de *Cupriavidus* sp.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Sérgio Miguel Marques, Investigador em Pós-Doutoramento do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro e do Doutor Fernando Gonçalves, Professor Associado com Agregação, do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

Por um futuro e para um futuro melhor.
A todos um Obrigado!

"The extensive variability and the environmental constraints found in caves, makes one wonder how living things "make ends meet""

Ghosh *et al.* (2017)

o júri

presidente

Doutor Artur Jorge da Costa Peixoto Alves
Investigador Principal do Departamento de Biologia & CESAM, da Universidade de Aveiro

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palavras-chave

Sistemas cavernícolas, Gruta da Cerâmica, bactéria, atividade antimicrobiana, triagem *in vitro*, *Cupriavidus* sp., pigmentos, meios de cultura.

resumo

Atualmente, o contínuo aumento de organismos patogénicos multirresistentes é uma das maiores problemáticas que a comunidade científica enfrenta. Isto é acentuado pela incapacidade de encontrar antibióticos com novas estruturas químicas e/ou modos de ação. Por este motivo, a procura por novos metabolitos tem vindo a expandir-se para a prospeção de novas estirpes bacterianas produtoras de antibióticos em ambientes extremos, tais como grutas. Os ambientes cavernícolas diferem dos de superfície em alguns aspetos chave, sendo responsáveis por criar um ambiente extremo e menos favorável para os microrganismos. A ausência de luz, a elevada humidade e a típica falta de nutrientes gera nichos oligotróficos, ao longo da gruta, com elevado potencial para possuir populações microbianas únicas e não exploradas. Por este motivo, estes ambientes representam um reservatório desconhecido de diversidade bacteriana, potencialmente com organismos produtores de antibióticos. Adicionalmente, poucas grutas foram exploradas com o foco específico de encontrar estes microrganismos. Por este motivo, este trabalho focou-se na atividade antimicrobiana de estipes bacterianas isoladas a partir de sistemas cavernícolas. Os objetivos específicos deste trabalho foram: i) a revisão dos métodos usados na obtenção de estirpes com atividade bacteriana, assim como nos métodos usados na avaliação das atividades antibacterianas; ii) a identificação e avaliação de atividade antimicrobiana de estipes bacterianas isoladas a partir da Gruta da Cerâmica, Portugal; iii) modular a produção de compostos antimicrobianos e pigmentos pelo isolado CC166 (*Cupriavidus* sp.) assim como avaliar as suas bioatividades.

Foi realizada uma extensa revisão bibliográfica, que resultou na conclusão de que os ambientes cavernícolas podem ser um dos próximos nichos mais promissores para a descoberta de estirpes bacterianas produtoras de novos metabolitos. Entre os métodos de isolamento empregues, o uso de suplementos, de uma grande variedade de meios de cultura e de pré-tratamentos, têm sido aplicados com sucesso no isolamento de estirpes produtoras de antibióticos. Com base na literatura, os métodos mais aplicados na avaliação da atividade antimicrobiana das estirpes bacterianas incluem o método disco-difusão e o método de difusão em ágar por poço.

Através da realização do segundo objetivo, várias bactérias produtoras de antibióticos pertencentes a diversos géneros foram reveladas. Estes incluíram os géneros *Streptomyces*, *Cupriavidus*, *Bacillus* e *Paenibacillus*. Relativamente ao terceiro objetivo, o uso de vários meios de cultura revelou padrões de atividade antimicrobiana notáveis, assim como de modulação de pigmentação por *Cupriavidus s*p. CC166. Notavelmente, *Cupriavidus* sp. CC166 demonstrou a capacidade de inibir todas as bactérias patogénicas testadas neste trabalho.

keywords

Cave systems, Cerâmica Cave, bacteria, antimicrobial activity, *in vitro* screening, *Cupriavidus* sp., pigments, culture media.

abstract

Presently, the increasing number of multidrug resistant pathogens continues to be one of the major issues to be tackled by the scientific community. This is aggravated by the inability to find antibiotics with new chemical structures and/or modes of action. Therefore, the search for novel metabolites has expanded to the prospection of bacterial drug producer's strains in extreme environments such as caves. Caves differ from surface environments in some of their core aspects, creating an extreme and unfavourable environment for microorganisms. The absence of light, high humidity and typical lack of nutrient input creates oligotrophic niches throughout the cave, with high potential for harbouring unique and unexplored microbial populations. Caves represent, thereby, a reservoir of unknown bacterial diversity potentially with antibiotic producing organisms. Additionally, few caves have been explored with the specific focus of finding these microorganisms. Taking this into consideration, this work focused on the antimicrobial activity of bacterial strains isolated from cave systems. The specific objectives of this work were: i) to review the methods used in the isolation of drug producer strains as well as in the evaluation of *in vitro* antibacterial activity ii) to identify and screen for antimicrobial activity bacterial strains isolated from Cerâmica Cave, Portugal; iii) to modulate the production of antimicrobial compounds and pigments by isolate CC166 (*Cupriavidus* sp.) and assess their bioactivity.

An extensive review was made leading to the conclusion that caves may be one of the next most promising niches for novel drug producer bacterial strains. Among the isolation methods employed, the use of supplements, a wide variety of culture media and pre-treatments has been successfully applied in the isolation of drug producer strains. Based on the literature, the disk diffusion method and the agar well diffusion assay have been successfully employed on the screening for drug producer strains.

Through the tackling of the second objective, antibiotic producer bacteria belonging to several genera were revealed. These included *Streptomyces*, *Cupriavidus*, *Bacillus* and *Paenibacillus*. Regarding the third objective, the employment of several culture media revealed remarkable patterns in the antimicrobial activity as well as in the modulation of pigments by *Cupriavidus* sp. CC166. Notably, *Cupriavidus* sp. CC166 was able to inhibit all pathogenic bacteria used as test agents in this work.

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Chapter 1: General introduction and objectives

1. General introduction and objectives

1.1.The antibiotic crisis

The discovery of antibiotics is considered one of the greatest turning points in medicine leading to enormous benefits to humankind with the treatment of previously untreatable infectious diseases (Bérdy, 2012). Most of the antibiotic classes were discovered between the 1950s and 1970s and effectively used in the treatment of infections, ultimately leading to an increase in the average life expectancy of the population (**Figure 1**) (Bérdy, 2012). Nevertheless, an evident decline on the discovery of new antibiotic classes allied with the misuse of antibiotics over the following decades resulted in the rise of resistance mechanisms observed against commercially available therapeutic agents in several pathogenic and non-pathogenic organisms (Ahmad et al., 2017; Alanis, 2005; Bérdy, 2012; Davies and Davies, 2010; Dias et al., 2012; Hong, 2012; Sass, 2017; Walsh, 2013; Walsh and Duffy, 2013). In fact, any therapeutic agent that enters in contact with bacterial populations has the potential to pressure the population and therefore the ones with tolerance and/or resistance to the active compound will survive, compromising future treatments as observed in bacterial and fungal pathogens (Davies and Davies, 2010). Moreover, there is a wide variability of factors contributing to the emergence as well as to the dissemination of resistance mechanisms among clinical and non-clinical strains and few studies have yet grasped the complexity associated in specific strains (Alanis, 2005; Davies and Davies, 2010). Therefore, the need for new pharmacological agents capable of treating bacterial infectious diseases is not surprising (Sass, 2017). In this context, natural product sources are a reliable reservoir with several niches yet to be explored (Rossiter et al., 2017). As it is known, the microbial diversity of each environment is driven and shaped by several constrains linked to their surroundings leading to a wide diversity of adaptations (Fierer, 2008). Taking this into consideration, several extreme ecosystems such as mines, hyper-arid deserts and caves, among others, may house potential drug producer strains with molecules worth exploring (Bérdy, 2012; Ghosh et al., 2017; Mohammadipanah and Wink, 2016).

Cave systems are one of these extreme environments, with multifactorial pressures (e.g. deep depletion of nutrients, absence of light among others) that have driven microbial populations and new and unknown mechanism have been observe in order to survive these habitats (Barton, 2015; Barton and Jurado, 2007; Engel, 2010; Gabriel and Northup, 2013;

Ghosh et al., 2017). Several studies have already demonstrated the vast diversity of microbial communities in these highly oligotrophic systems while novel species have been found on several caves around the world such as *Beutenbergia cavernae* gen. nov., sp. nov. (Groth et al., 1999), *Rhodococcus antrifimi* sp. nov. (Ko et al., 2015), *Paenibacillus cavernae* sp. nov. (Dong Lee, 2016) and *Streptomyces lunaelactis* sp. nov. (Maciejewska et al., 2015), among others (Chen et al., 2016; Jurado et al., 2008; Tomczyk-Żak and Zielenkiewicz, 2016). Nonetheless, few studies have been performed regarding drug potential from cave strains and most caves are yet to be explored regarding their microbial diversity. Therefore, the exploration of extreme habitats such as caves may indeed be one way to find bioactive agents able to cope with infectious diseases (Adam et al., 2018; Barton, 2015; Cheeptham et al., 2013; Ghosh et al., 2017; Tomczyk-Żak and Zielenkiewicz, 2016).

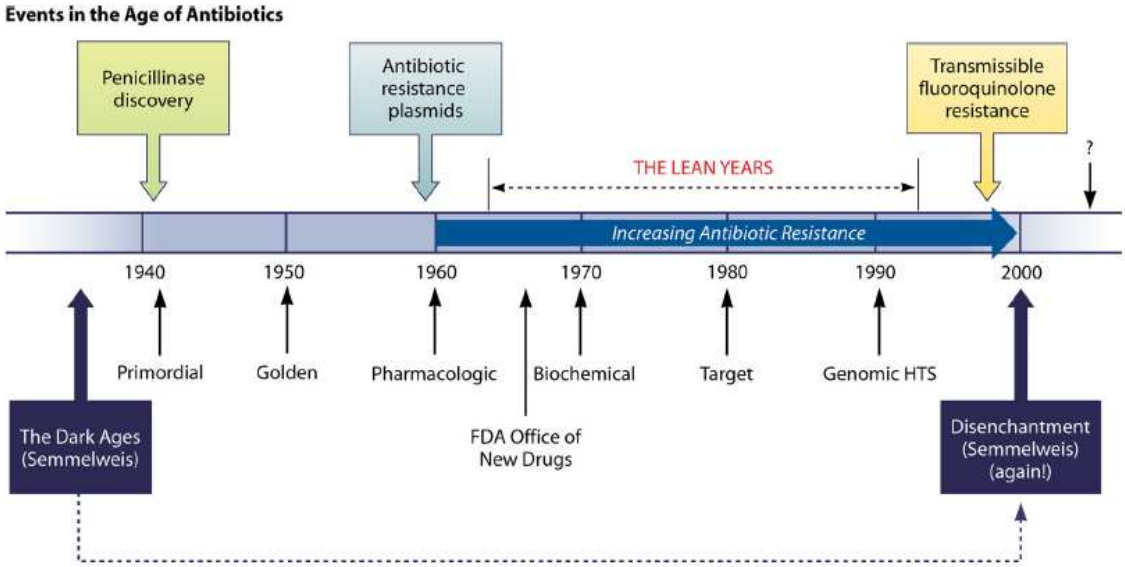

Figure 1. Schematic representation of the history of antibiotic discovery and the concomitant development of antibiotic resistance, (Adapted from Davies and Davies, 2010).

1.2.The particular environment of caves

1.2.1. Formation of natural caves

Caves are geological formations, simply defined as natural cavities with some part of it in total darkness (Lee et al., 2012). Depending on the method of speleogenesis (how a cave is geologically formed and developed), caves can be classified upon various factors

including: (1) the types of minerals and bedrock (e.g., limestone or lava tube); (2) the proximity to the groundwater table (e.g., above, at, or below it); (3) their geometric structure and morphology (e.g., horizontal or vertical development); (4) the time in which they were created in relation to the rock in which they are found (e.g., primary, secondary, or tertiary) and (5) the mechanism by which they were formed (e.g., solutional or non-solutional) (Engel, 2011; Ghosh et al., 2017). Overall, intrinsically dependent on the rock type and geochemical/geophysical conditions, several speleogenetic processes may take place. These may include solubilization of the host rock and precipitation of minerals and sulfuric acid-driven speleogenesis where solubilization of the host rock (such as calcareous rocks) may generate large caves (Lee et al., 2012). Among other examples of caves are the lava tubes originated from lava flows upon volcanism as well as the formation of anchialine caves freshwater-saline water interface systems (Lee et al., 2012).

Karst landscapes encompass a great number of caves worldwide and constitute a total of ~15–20% of the Earth's ice-free land surface (Engel, 2010). Most karst caves are formed due to the dissolution of carbonate rock by the action of the water flowing on and through the rocks from epigenic or hypogenic fluids (Engel, 2010). Overall, the main sedimentary carbonate rock in karst caves is usually limestone and dolomite (Mulec, 2015). Depending on the rock type, several redox gradients for microbial colonization are observed, since the rock itself may contain several reduced molecules. These molecules are responsible for the origin of a diverse array of redox gradients within short distance, crucial for the expansion of complex microbial communities (Barton, 2015; Barton and Jurado, 2007; Engel, 2015; Lee et al., 2012; Northup and Lavoie, 2001). Interfaces between the host rock and cave passage atmosphere, or rock and water, create additional redox gradients and nutritional niches for the development of a complex structural microbial community (Barton and Jurado, 2007; Lee et al., 2012). Furthermore, some of the described cave minerals are new to Science (Ghosh et al., 2017) which may represent unknown niches for microbial specialization and/or development (Rooney et al., 2010). This, allied with the lack of exploitation regarding the microbial community of cave systems, makes these ecosystems an emergent field of investigation (Rooney et al., 2010).

Studies in cave microbiology focusing in microbial diversity have been attempted at least since the 1940s with unrealistic representation due to the limitation of the methods used (Cheeptham, 2013). For several decades, the methods applied relied heavily on microscopy

and culture-based approaches developed for medical microbiology (Cheeptham, 2013). Recently, advances in molecular methods have unlocked a deeper and more complete perspective of caves microbial diversity and nowadays it is known that cave habitats harbor several groups of extremophilic microbes which belong to acidophilic, oligotrophic, sulfidophilic and thermophilic bacterial populations in its microbiomes (Engel, 2010).

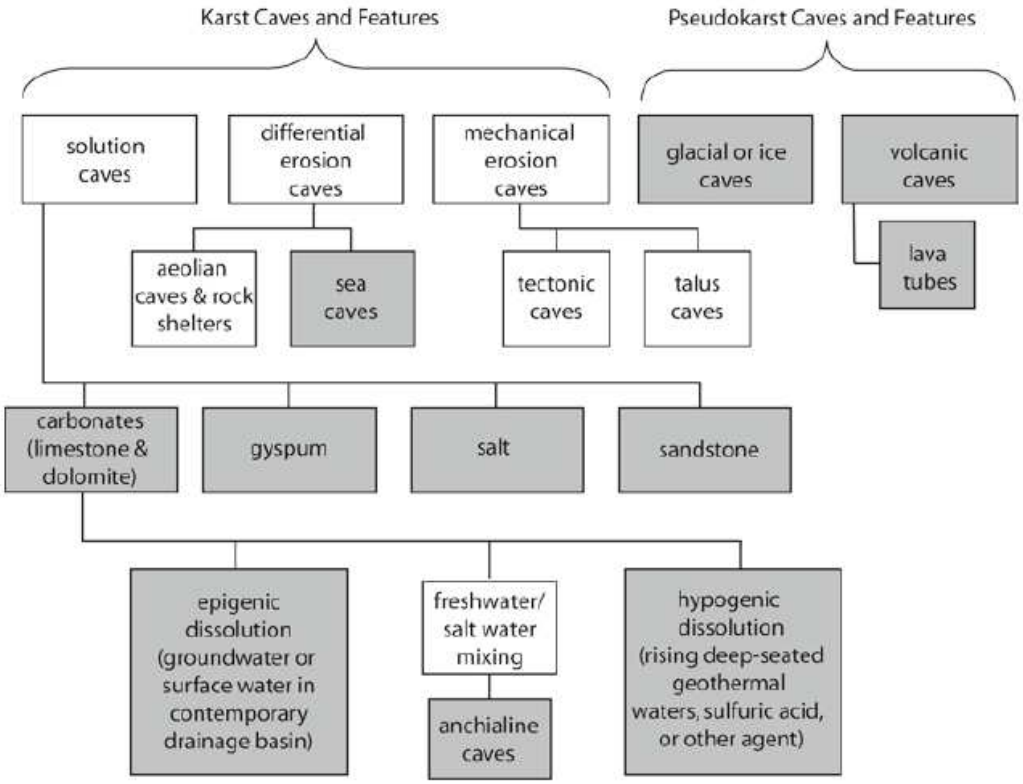

Figure 2. Common types of caves and the processes responsible for their formation, including dissolution and weathering, (Adapted from Engel, 2010).

1.2.2. Cave zonation and oligotrophy

Caves differ from surface environments in some of their core aspects, including in the amount of light, energy, and nutrients available, as well as in the stability of several abiotic factors which are responsible for the creating of an extreme and unfavorable environment (Gabriel and Northup, 2013). Overall, caves constitute an oligotrophic environment [defined by less than 2 mg of total organic carbon (TOC) per soil], with low levels of light, stable

temperatures and high humidity conditions (Tomczyk-Żak and Zielenkiewicz, 2016). Cave's interior can be divided into four main zones, depending on the amount of light that enters in the cavern and on the fluctuations between surface and subsurface (e.g. air currents). The first area is the entrance zone (interface between surface and underground environments), followed by twilight zone (from low amount of light to complete darkness) and transition zone (no light is detectable but slight environmental fluxes such as temperature and moisture from surface are still observed). The last area constitutes the deep zone, characterized by complete darkness, high humidity and constant temperature (Cheeptham, 2013; Gabriel and Northup, 2013; Lee et al., 2012). In the Entrance zone, size and shape constitute one of the major limitations to light penetration, affecting the distribution of mixed population from surface and subsurface microbial communities due to the degree of phototropism observed where primary energy sources are still present. Moving beyond from the twilight zone, the influence of phototropism is absent, leading to an increase in microbiomes adapted to low nutrient system. In the Deep zone, the air remains unchanged and saturated with high humidity, where the potential evaporation rate is negligible over time, creating a stable environment for oligotrophic communities (Cheeptham, 2013; Gabriel and Northup, 2013; Lee et al., 2012).

In caves, most microbiomes are not established in a conventional energetically favorable system (due to oligotrophism), nonetheless, microorganisms growing in these ecosystems are routinely found at amounts of 10^6 cells.g^{-1} of rock (Barton and Jurado, 2007). In spite of the oligotrophic enviounment, the extensive variability of energy and nutrients that may enter the cave as well as the origin of the nutrients are yet to be determined (Ghosh et al., 2017). For instance, organic carbon, that fuels heterotrophic communities, enters into caves in several forms such as plant debris that are transported by water into the cave (Gabriel and Northup, 2013). Air currents, especially in caves with more than one entrance, bring various reduced carbon energy sources, as well as particulate matter. Feces from a variety of animals that visit or live in caves provide microbial habitats rich in nutrients, especially nitrogen, carbon, and phosphorus. In fact, cave roosting bats provide deposits of bat guano, which are inhabited by many microorganisms (e.g. *Streptomyces* genus) (De Leon et al., 2018; Leon et al., 2018; Newman et al., 2018; Tomova et al., 2013). Human impact by tourism introduces a variety of foreign materials such as hair, skin cells, sweat, skin oils, fibres from clothing,

and dust particles that could potentially provide nutrients to microbes (Gabriel and Northup, 2013).

Alternative methods for carbon assimilation can be also associated with chemoautotrophy. In such conditions, primary organic matter is produced by chemolithoautotrophic microorganisms, which derive energy from binding not only hydrogen, nitrogen, or volatile organic compounds, but also from oxidation of reduced ions (from different metals e.g., manganese, iron) present in the rocks (Kumaresan et al., 2017; Porter et al., 2009; Tomczyk-Żak and Zielenkiewicz, 2016).

In such complex deprived environment, caves harbor an outstanding diversity of microorganisms. On one hand, their existence under oligotrophy conditions results in new metabolic versatility allowing organisms to acquire energy by several pathways, such as (1) by fixing volatile organics, carbon dioxide, and nitrogen from the atmosphere, (2) by oxidizing reduced metals within the cave rocks and (3) by breaking down surface-derived complex aromatic compounds (Barton et al., 2007; Barton and Jurado, 2007). On the other hand, the microbial communities have accumulated biological adaptations to a habitat with low alterations, being well established for thousands or millions of years, with a unique dynamic and cooperation between different species. In fact, in this limited complex nutrient environment, only few microbial species can uptake and catabolize the reactions necessary for growth (Barton et al., 2007; Barton and Jurado, 2007). However, by cooperative and mutualistic associations, such as the ones observed in biofilm communities, it is possible to achieve a higher diversity (Barton et al., 2007; Barton and Jurado, 2007; Borsodi et al., 2012; Dapkevicius, 2013; Macalady et al., 2007).

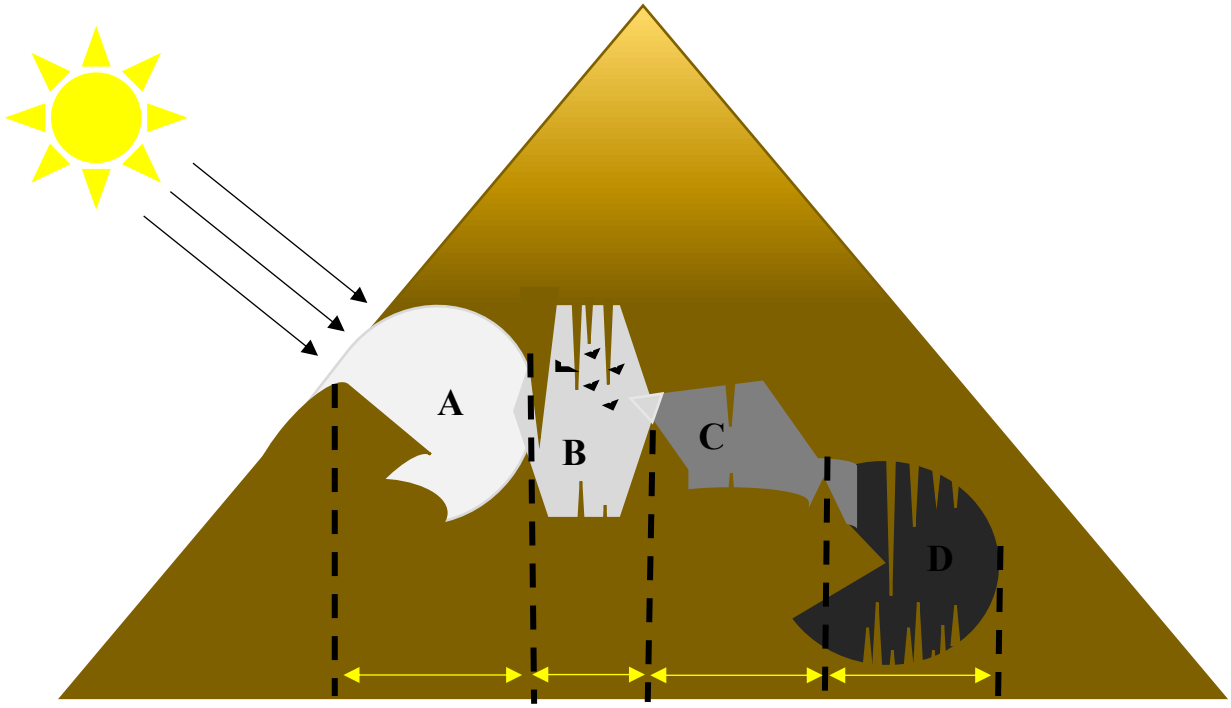

Figure 3. Schematic representation of the 4 common zones within a cave (A: Entrance zone; B: Twilight zone; C: Transition zone; D: Deep zone), (Adapted from Lee *et al*., 2012).

1.3. Microbial diversity of cave systems

The occurrence and structure of microbial communities in caves can be influenced by pH level, nutrient availability, light intensity, humidity, oxygen, sulphur as well as by other compounds and metals that contribute for substrate colonization (Jones et al., 2014; Tomczyk-Żak and Zielenkiewicz, 2016). Therefore, variation of the physico-chemical parameters could ultimately influence community composition, which leads to a broad range of biodiversity in cave systems. Currently, it is known that typical colonizers of cave habitats comprise several members belonging to *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *δ-Proteobacteria*, *ε-Proteobacteria*, *Nitrospirae*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, *Chloroflexi*, *Gemmatimonadetes*, *Spirochetes*, *Cyanobacteria*, *Chlorobi*, and *Fibrobacteres* (Borsodi et al., 2012; Macalady et al., 2007; Tomczyk-Żak and Zielenkiewicz, 2016). A review regarding microbial diversity in caves has recently been published by Tomcyzk-zak *et al.* (2016), therefore the most representative phyla will only be briefly discussed.

Among the vast diversity of microorganisms reported in cave systems, the phylum *Proteobacteria* is very abundant, mainly the classes *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, and *δ-Proteobacteria*. The success of these groups for the colonization of caves can be related to the ability to degrade a wide range of organic compounds (Tomczyk-Żak and Zielenkiewicz, 2016). Interestingly, *Proteobacteria* have been found in one of the most pristine and oligotrophic caves (Lechughilha cave, New Mexico, USA) as well as in caves with tourism activities (human impact) (Spider cave, New Mexico, USA) (Northup et al., 2003). Moreover, the diversity and the ratio of the classes belonging to *Proteobacteria* is variable in the cave system (Tomczyk-Żak and Zielenkiewicz, 2016). For example, the class *ε-Proteobacteria* dominated the streams of the Lower Kane Cave (Wyoming, USA), where the water contains sulphur in high concentration and low concentration of dissolved oxygen. In contrast, when the water flows out of the cave, the dissolved oxygen increases and the concentration of sulphur decreases, the class *γ-Proteobacteria* was the most abundant (Engel et al., 2010; Tomczyk-Żak and Zielenkiewicz, 2016). The study performed by Ikner *et al.* (2007) in Kartchner Caverns (Arizona, USA) reported that *Proteobacteria* was dominant in sites highly impacted by tourist, contrasting with the dominance of *Firmicutes* in areas with low anthropogenic impact (Ikner et al., 2007). The comparison of iron-manganese deposits from Lechuguilla Cave (closed to tourists) and from Spider Cave (highly explored by tourists) revealed an increase of nitrogen-fixing *Proteobacteria* in the last cave (Northup et al., 2003). Indeed, these nitrogen-fixing genera (*e.g.*, *Bosea*, *Bradyrhizobium*, *Burkholderia*, *Cupriavidus*, *Devosia*, *Methylobacter*, *Methylococcus*, *Mesorhizobium*, *Ochrobactrum*, *Rhizobium*, *Sinorhizobium*) are found in the deepest and most nutrient-limited caves around the world (Barton, 2015).

Another Gram-positive bacteria phylum, well represented in cave systems, is *Actinobacteria*, which present remarkable biodegrading enzymes that allows them to thrive in several soil niches (Barton, 2015). In caves, the colonization is observed in soil but also in rock walls, stalactites, and stalagmites. While *Actinobacteria* are best known for their secondary metabolic products, it is reported that some of these bacteria are also involved in biomineralization processes in caves (Barton et al., 2007; Chater, 2016, 2006; Cuezva et al., 2009; Tomczyk-Żak and Zielenkiewicz, 2016; Wafaa Haggag et al., 2014). Several genera of *Actinobacteria* have been found in cave systems: *Pseudonocardia*, *Propionibacterium*, *Corynebacterium*, *Gordonia*, *Frankia*, *Arthrobacter*, *Curtobacterium*, *Promicromonospora*,

Rhodococcus, *Nocardia*, *Micrococcus*, *Blastococcus*, *Streptomyces*, *Kribbella* and *Streptomyces* (Tomczyk-Żak and Zielenkiewicz, 2016; Urzì et al., 2010). *Firmicutes* is a widely diverse phylum found in several ecosystems and are able to grow in the presence of several organic compounds (Barton, 2015), as well as to sporulate upon nutrient limitation. The following genera from this phylum have been found in different caves around the world: *Lactobacillaceae*, *Leuconostoc*, *Streptococcus*, *Sulfobacillus*, *Bacillus*, *Peanibacillus* and *Staphylococcus* (Macalady et al., 2007; Northup et al., 2003; Pawlowski et al., 2016; Urzì et al., 2010). In addition to these main groups (*Actinobacteria*, *Firmicutes* and *Proteobacteria*), environmental sequence analysis in cave systems has demonstrated a broad phylogenetic diversity in *Planctomycetes* and *Chloroflexi* and *Acidobacteria* even when compared with *Proteobacteria* or *Actinomycetales* (Barton, 2015). Despite the wide diversity reported among different caves, the organisms from these groups are yet to be cultured from cave systems. Taking into consideration the diverse cave microbiomes and the lack of knowledge regarding the ecological roles of several groups of bacteria, a lot of work is still to be done and caves present an enormous reservoir of bacterial strains with unknown biosynthetic potential to produce antibacterial metabolites (Barton, 2015).

1.4.The cave habitat as a source of novel antibiotic-producing bacteria

Nowadays, the need for novel therapeutic agents is evident and efforts are being made to tackle pathogens responsible for severe infectious diseases, some of them comprising several distinct resistance mechanisms (Alanis, 2005; Davies and Davies, 2010; Giddings and Newman, 2015; Rossiter et al., 2017). The search for novel drug producer strains from natural environments has lead to the exploration of more remote and extreme ecosystems such as caves, in order to find novel biodiversity and new metabolites (Bérdy, 2012; Ghosh et al., 2017; Sánchez and Demain, 2015). As discussed above, various studies on cave environments have demonstrated the wide diversity of microbial populations found inside these ecosystems (Engel et al., 2010; Mulec, 2015; Schabereiter-Gurtner et al., 2002; Tomczyk-Żak and Zielenkiewicz, 2016; Urzì et al., 2010). However, few studies have been made regarding the potential to find novel drug producer strains from these ecosystems (Ghosh et al., 2017). Nevertheless, it was demonstrated that *Actinobacteria*, *Firmicutes,* and *Proteobacteria* possess antibacterial activity against several Gram-positive and Gram-

negative human pathogens including multidrug resistant strains (Jiang et al., 2015; Klusaite et al., 2016; Lamprinou et al., 2015; Maciejewska et al., 2016; Nakaew et al., 2009a, 2009b; Rajput, Yogita Biswas and Rai, 2012; Tomova et al., 2013; Yasir, 2017; Yücel and Yamaç, 2010). Overall, several studies with *Actinobacteria* members (*e.g. Streptomyces* genus) have demonstrated antibacterial activity (Adam et al., 2018; Axenov-Gribanov et al., 2016; Jiang et al., 2015; Maciejewska et al., 2016; Nakaew et al., 2012, 2009a, 2009b; Rajput, Yogita Biswas and Rai, 2012; Stankovic et al., 2012; Yücel and Yamaç, 2010). For instance, *Streptomyces tendae* (strain HKI 0179) isolated from Grotta dei Cervi cave system (Lecce, Italy), was found to produce Cervimycins A–D polyketide glycosides that exhibited strong antibiotic capacities against, *Staphylococcus aureus* SG511, multidrug resistant *S. aureus* 134/93 (MRSA), efflux-mediated resistance *Staphylococcus aureus* EfS4 and vancomycin-resistant *Enterococcus faecalis* 1528 (VRE), among other strains such as *Bacillus subtilis* ATCC 6633 (Herold et al., 2005). In addition, *Streptomyces* sp. CC8-201, isolated from the soil of a remote karst cave near Chongqing city (China), was found to produce Xiakemycin A, a molecule that exhibits activity against multidrug resistant strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Enterococcus faecium* (Jiang et al., 2015). In a more general screening, several *Streptomyces* strains, isolated from caves, have been found to produce compounds against *Acinetobacter baumannii*, *Micrococcus luteus*, *Bacillus subtilis*, *Xanthomonas oryzae*, *Peseudomonas auroginosa*, *Staphylococcus aureus*, *Mycobacterium* sp., *Echerichia coli*, *Peseudomonas putida*, *Enterococcus faecalis*, *Enterococcus faecium* and *Staphylococcus epidermidis* (Adam et al., 2018; Axenov-Gribanov et al., 2016; Jiang et al., 2015; Maciejewska et al., 2016; Nakaew et al., 2012, 2009a, 2009b; Rajput, Yogita Biswas and Rai, 2012; Stankovic et al., 2012; Yücel and Yamaç, 2010). Despite the few studies performed in cave environments regarding this topic, the results seem promising with several species/strains with high antibiotic capacities against a wide array of human pathogens (Ghosh et al., 2017). The study of cave microbiomes is still in its infancy and the exploration of these habitats is highly recommended, being still an unknown reservoir regarding drug producing strains.

1.5. Objectives and structure of the dissertation

The main aim of the present study was to prospect the potential of Cerâmica Cave microbiome belonging to the Sicó karst system of Portugal as well as to evaluate their bioactivity against human pathogenic bacteria. In addition, modulation of pigmentation as well as the antimicrobial activity of *Cupriavidus* sp. CC166 isolated from Cerâmica Cave was performed. The specific objectives addressed in the different chapters of the present document were:

1) To review the methods used in the collection of cave cultivable members with antimicrobial activity, as well as the methodology employed in the screening of *in vitro* antibacterial activity from cave bacterial strains (Chapter 2);

2) To identify the bacterial strains collected in the Cerâmica cave and screening the antimicrobial activity of the most important bacterial strains (Chapter 3);

3) To modulate and evaluate the antimicrobial activity of each phenotype that exhibits pigmentation from *Cupriavidus* sp. CC166 isolated from Cerâmica cave against Gram-positive and Gram-negative pathogenic bacteria (Chapter 4).

This dissertation is organized in five chapters: (1) the current chapter (Chapter 1) deals with a general introduction that holds an overview covering the main topics of the subject addressed in this dissertation; (2) Chapter 2 is comprised by a review of the methods used in the screening of antimicrobial activity from cave bacteria strains as well as the methodology employed in the acquisition of drug producer strains from cave systems, highlighting the unexplored reservoir of potential novel antibiotic producing strains; (3) Chapter 3 entails the identification and screening of antimicrobial activity from bacterial strains collected from Cerâmica Cave, Portugal; (4) Chapter 4 covers the modulation of pigment production of Cupriavidus sp. CC166 isolated from Cerâmica cave and the evaluation of the antimicrobial activity of each pigment phenotype against Gram-positive and Gram-negative pathogenic bacterias; (5) Chapter 5 represents the final wrap-up of the major discussion items and provides the final remarks summarising the findings.

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Chapter 2: Prospecting cave environments for drug producing strains: Current status and methodologies

Chapter 2. Prospecting cave environments for drug producing strains: Current status and methodologies

2.1. Abstract

The increasing resistance to antibiotics observed among several pathogenic strains and the failure in providing adequate treatment has become alarming in the past decades. Natural sources have provided a wide range of complex and structurally diverse compounds, and extreme and unexplored habitats are now becoming a new niche of research. The study of extreme ecosystems such as caves has proven to be a potential way to find novel drug producing bacterial strains, although it is still in its early stage. This review aims to highlight cave microbiomes as a source of drug producing strains and to elucidate the methodologies involved in the acquisition of strains with antimicrobial activity from cave environments, as well as the methods to evaluate the antibacterial activity. It is possible to emphasise several challenges linked to sample processing, as well as to isolating and maintaining the cave bacterial strains under laboratory conditions. Moreover, the employment of *in vitro* screening assays for antimicrobial activity is yet to be standardized for cave bacteria organisms. Therefore, we intend to promote a visual flow of the difficulties and advantages of each method based on the literature. Cave microbiomes may indeed be a reservoir of novel drug producer strains and most caves of the world remain unexplored emphasising the need for more studies in this natural and extreme ecosystem.

2.2. Introduction

In the last decades an obvious decrease in the discovery of new classes of compounds with antibiotic properties has made a huge impact in modern medicine (Bérdy, 2012). This, allied with the continuous increase observed in resistance and tolerance mechanisms to most widely available antibiotics, and with the emergence and dissemination processes that are yet to be fully characterized to most resistant mechanisms, has become a serious threat to human health (Ahmad *et al*., 2017; Alanis, 2005; Bérdy, 2012; Davies and Davies, 2010; Dias *et al*., 2012; Hong, 2012; Sass, 2017; Walsh, 2013; Walsh and Duffy, 2013). Currently,

the treatment failures associated with multidrug resistance bacteria are alarming and search for solutions has become widespread in several research areas (Tanwar *et al*., 2014; Wright, 2017). The exploration of natural resources have provided a wide range of complex and structurally diverse compounds (e.g. microbial secondary metabolites) that encompass several functional groups, stereochemistry and unique scaffolds with a vast array of biological activities (Giddings and Newman, 2015; Bérdy, 2012, 2005; Dias *et al*., 2012; Hong, 2012). Therefore, a solution to cope with this crisis may rely on the exploration of the unknown microbial diversity from extreme and unexplored environments in the search for novel therapeutic agents with unique mechanisms of action (Bérdy, 2012). Isolation of novel bacteria can be an appellative way for drug discovery and the focus in extreme habitats have boosted in the last decades, from which caves are not an exception (Ghosh *et al*., 2017a).

Caves constitute an enormous world reservoir that is widely remote (some are still unknown and most are of difficult access) and persist as one of the harshest oligotrophic systems in the biosphere (Barton and Jurado, 2007). Microbial species in these ecosystems are constrained to several distinct ecological and environmental forces leading to the devolvement of unique mechanisms to sustain the survival of the species (Barton, 2015; Barton and Jurado, 2007; Engel, 2010; Gabriel and Northup, 2013; Ghosh *et al*., 2017a). The heterogeneous nature of the soil of each cave and the variation in biotic and abiotic features of each zone within the cave ultimately lead to the development of several strategies from the microbial species in order to adapt, survive and thrive in these ecosystems (Barton, 2015; Barton and Jurado, 2007; Engel, 2015; Lee *et al*., 2012; Northup and Lavoie, 2001). For instance, cave systems can be divided in different zones such as entrance, the twilight zone and the deep zone. In each zone of the cave the biotic and abiotic constrains are quite different leading to an observable variation in the microbial community along the cave (Cheeptham, 2013; Engel *et al*., 2010; Gabriel and Northup, 2013; Lee *et al*., 2012). Moreover, unlike other nutrient deprived environments [oligotrophic habitats are conventionally defined to have less than 2 mg of total organic carbon (TOC) per litre], caves are devoid of sunlight (mainly in transition and deep zone) and usually isolated from external input (from the surface) with low fluctuations in humidity and temperature (Montano and Henderson, 2013; Tomczyk-Żak and Zielenkiewicz, 2016). In the last decades, research in cave microbiomes using phylogenetic methods have shown an outstanding bacterial diversity with several members belonging to *Proteobacteria* (including α-*Proteobacteria*, β-

Proteobacteria, γ-*Proteobacteria*, δ-*Proteobacteria*, ε-*Proteobacteria*), *Nitrospirae*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, *Chloroflexi*, *Gemmatimonadetes*, *Spirochetes*, *Cyanobacteria*, *Chlorobi* and *Fibrobacteres* (Tomczyk-Żak and Zielenkiewicz, 2016). However, when focusing in cave culturable representatives the same is not observed, and members of the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* often dominate the plates (Barton, 2015). Notwithstanding, several members of these groups of bacteria, which have been isolated from cave environments, often exhibit a broad range of activities including antimicrobial action against both Gram-positive and Gram-negative pathogenic bacteria, including multidrug resistant strains (Balkwill, 2015; Ghosh *et al*., 2017b; Herold *et al*., 2005; Lamprinou *et al*., 2015; Lee *et al*., 2000; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a; Riquelme *et al*., 2017; Rule and Cheeptham, 2013).

In this energetically unfavourable environment, when studying more endemic cave representatives (usually bacteria from cave envirounments that have not been disturbed by human impact) with or without reported activities, several problems arise. Usually, these bacteria are slow growers and require specialized nutritional aspects or cooperative behaviour with other bacteria ultimately leading to their failure in cultivation (Barton, 2015; Cheeptham, 2013; Ghosh *et al*., 2017a). Nonetheless, in order to enhance the odds to culture novel species with antimicrobial capacities and to prospect the endemic diversity of unexplored environments, selective treatments and a vast array of culture options are usually employed (Adam *et al*., 2018; Ghosh *et al*., 2017b, 2017a; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a).

To study *in vitro* antimicrobial activity a wide variety of methods can be used, however, for cave bacteria which entail more specialized requirements no standardization assay has been proposed yet. Here, we review the studies conducted in these systems and the techniques applied in both acquisition and screening for drug producer strains from cave habitats while summarizing the different challenges and options when prospecting this unexplored bacterial reservoir. Moreover, we intend to promote a visual flow of the challenges as well as the advantages of each method based on the literature, aiming to highlight the benefits of each screening technique and to incentive the exploration of cave microbiomes, as a way to cope with multidrug resistance pathogens.

2.3. The multidrug resistance crisis

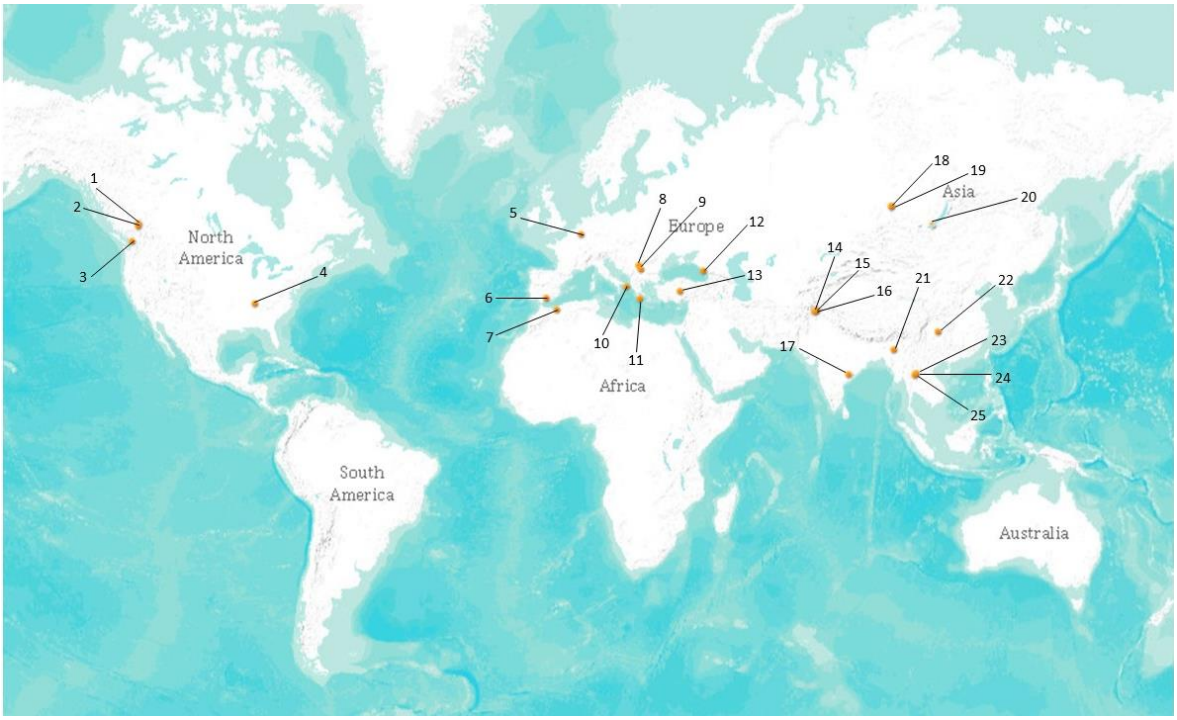
Since the discovery of the first antibiotic in the 40's, modern medicine has improved remarkably, and several previous deadly infections are now able to be treated (Wright, 2017). However, the lack of knowledge regarding how resistance mechanisms are developed for each specific strain, and how they are disseminated, has resulted in a huge misusage of antibiotics leading to a worldwide increase of multidrug resistant strains (Ahmad *et al*., 2017; Alanis, 2005; Bérdy, 2012; Davies and Davies, 2010; Dias *et al*., 2012; Hong, 2012; Sass, 2017; Walsh, 2013; Walsh and Duffy, 2013). Nowadays, several major pathogenic Gram-positive and Gram-negative bacteria, specially strains associated with health care installations, have become resistant to several antibiotic classes (Prestinaci *et al*., 2015). Moreover, last resource antibiotics such as vancomycin are now unable to provide adequate treatment due to resistance acquirement by strains such as *Staphyloccocus aureus* (Boneca and Chiosis, 2003; Koch *et al*., 2015).

In the last decades, with the improvement of several molecular techniques, it became of knowledge that resistance mechanisms can be intrinsic to the genetic makeup of the species, or can be acquired through mobile elements such as plasmids (Munita1 and Arias, 2016; Partridge *et al*., 2018). The second one is considered one of the major ways for resistance mechanisms dissemination (by "horizontal gene transfer") from one bacterial strain to another (Partridge *et al*., 2018; Rossiter *et al*., 2017). Moreover, different types of resistance are developed by the bacteria, being the major ones the following: (1) limitation in the permeability by efflux pumps resulting in a reduction of the penetration by the drug, (2) modification or mutation by the bacteria on the binding target of the specific antibiotic, and (3) degradation of the drug by the bacteria under attack (Rossiter *et al*., 2017). Efflux pumps allow bacteria to cope with some antibiotics by actively transporting small molecules to the external system of the bacterial cell. For instance, in *S. aureus* more than ten multidrug efflux pumps have been found and characterized (encoded in the chromosomes or/and in plasmids) (Costa *et al*., 2013; Rossiter *et al*., 2017). Nevertheless, bacterial resistance is a normal trait for the survival of the species in any ecosystem (Rossiter *et al*., 2017). For instance, in one of the most pristine environments, Lechuguilla Cave (New Mexico, USA), isolated for over 4,000 million years and where human impact is neglectable, Pawlowski and colleagues

(2012) found a strain of the genus *Paenibacillus* designated as *Paenibacillus* sp. LC231, resistant to 26 of 40 screened antibiotics and with five new resistance mechanisms (Pawlowski *et al*., 2016). This puts in evidence not only that in oligotrophic habitats such as caves, antibiotics may be used as weaponry by bacterial populations for their survival but also that a vast range of biomolecules may be present in the dynamics of cave microbial populations (Bhullar *et al*., 2012). Therefore, the study of cave microbiomes may provide not only new insights on natural resistance mechanisms but also on new molecules with antibiotic capacities. One example is *Streptomyces* sp. CC8-201, isolated from the soil of a remote karst cave near Chongqing city (China), found to produce Xiakemycin A, a novel pyranonaphthoquinone antibiotic that exhibits activity against multidrug resistant strains of *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *E. faecium* (Jiang *et al*., 2015). Moreover, several studies have provided a first glimpse of cave habitats as a source of drug producing bacteria against several relevant Gram-positive and Gram-negative pathogenic bacteria (Axenov-Gribanov *et al*., 2016; Herold *et al*., 2005; Maciejewska *et al*., 2016; Rajput, Yogita Biswas and Rai, 2012; Yasir, 2017).

2.4. Cave systems as sources of novel microorganisms with antimicrobial activity

Caves constitute a unique shelter for several groups of extremophilic microbes including oligotrophic, acidophilic, thermophilic and sulfidophilic communities (Engel, 2010). Moreover, studies in geomicrobiology have demonstrated the ability of several cave dwelling microorganisms to alter and produce several mineralogic matrices (Barton *et al*., 2007; Chater, 2016, 2006; Cuezva *et al*., 2009; Tomczyk-Żak and Zielenkiewicz, 2016; Wafaa Haggag *et al*., 2014). Within cave microbial diversity, several members with remarkable antimicrobial capacities as well as antibiotic resistance have been found (Adam *et al*., 2018; Herold *et al*., 2005; Jiang *et al*., 2015; Stankovic *et al*., 2012). The study of cave microbiomes regarding the discovery of novel therapeutic agents is still a field on its early stages, and in the last decades an increase in studies prospecting this extreme natural reservoir is evident.



Number	Cave name, Country	Reference
1	Wells Gray Provincial Park, Canada	Rule and Cheeptham, 2013
2	Wells Gray Provincial Park, Canada	Kay et al., 2012
3	Iron Curtain Cave, Canada	Ghosh et al. 2018
4	Hardin's Cave system, Tennessee, USA	Derewacz et al., 2014
5	Springtails' Cave, Comblain-au-Pont, Belgium	Maciejewska et al. 2016
6	Cueva de los Murciólagos, Murcia, Spain	Gálvez et al., 1993
7	Chaabe Cave, Tlemcen, Algeria (34°53'N, 1°19'W)	Belyagoubi et al., 2018
8	Miroc Mountain, Serbia	Stankovic et al., 2012
9	Magura Cave, Serbia	Tomova et al. 2013
10	Grotta dei Cervi , Porto Badisco, Italy	Herold et al., 2005
11	'Francthi' Cave, Peloponnese, Greece, (37°25'21.01"N, 22°17'51.18"N)	Lamprinou et al., 2015
12	Krubera-Voronja Cave, Caucasus	Klusaite et al., 2016
13	Turkey (19 caves)	Yucel and Yamaç, 2011
14	Koat Maqbari Ghaar, Pakistan, (34°49'13.06'' N, 72°30'41.81'' E)	Yasir, 2015
15	Smasse-Rawo Ghaar, Pakistan, (34°33'38.1'' N, 71°51'03.4'' E)	Yasir, 2015
16	Kashmir Smast, Khyber Pakhtunkhwa, Pakistan, (34°25'42.12"N, 72°13'10.82"E)	Zada et al., 2016
17	Kotumsar Cave, India, (18°52'09'' N; 81°56'05'' E)	Rajput et al., 2012
18	Bolshaya Oreshnaya Cave, Siberia, Russia, (55°17'34.8''N, 93°44'8.52'' E)	Axenov-Gibanov et al., 2016
19	Badzheyskaya Cave, Krasnoyarsk, Russia, (55°14'32'', N 93°46'32'' E)	Voytsekhovskaya et al. 2018
20	Okhotnichya Cave, Irkutsk, Russia, (52°8'18"N, 105°27'49"E)	Voytsekhovskaya et al. 2018
21	Loktak Lake, Manipur, India	Ningthoujam et al., 2009
22	Chongqing city, China	Jiang et al., 2015
23	Phanangkoi Cave, Phrae, Thailand	Nakaew et al., 2009a
24	Phatup Cave Forest Park, Thailand	Nakaew et al., 2009a
25	Phanangkoi Cave, Phrae, Thailand	Nakaew et al., 2009b

Figure 4. World map with the cave locations that were screened positive for *in vitro* antimicrobial activity of cave bacterial strains.

Until date, at least 24 studies with focus in antimicrobial activity of cave bacterial strains have been published covering several caves around the world (**Figure 4**) including Khangkhui cave and Kotumsar cave (India), Phatup cave Forest Park and Phanangkhoi cave (Thailand), Grotta dei Cervi (Italy), Magura cave (Bulgaria), Hardin's cave (Tennessee, USA), Koat Maqbari Ghaar cave, Smasse-Rawo Ghaar cave and Kashmir cave (Pakistan),

Francthi' cave (Greece), Krubera-Voronja cave (Gerogia), Springtails' cave (Belgium), Chaabe cave (Algeria), Iron Curtain cave and Helmcken Falls cave (Canada) as well as Bolshaya Oreshnaya cave, Badzheyskaya cave and Okhotnichya cave (Siberia), Murcié1agos cave (Spain), 19 Turkish caves, a gold mine and a remote karst cave in China (**Figure 4**) (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Belyagoubi *et al*., 2018; Derewacz *et al*., 2014; Ghosh *et al*., 2017b; Herold *et al*., 2005; Jiang *et al*., 2015; Kay *et al*., 2013; Klusaite *et al*., 2016; Lamprinou *et al*., 2015; Lee *et al*., 2000; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a, 2009b; Ningthouja *et al*., 2009; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Stankovic *et al*., 2012; Tomova *et al*., 2013; Voytsekhovskaya *et al*., 2018; Yasir, 2017; Yücel and Yamaç, 2010; Zada *et al*., 2016).

Although most of the caves worldwide are yet to be explored regarding this topic, it is possible to infer based on the studies performed that caves present microbiomes with drug producing strains across the world. Among the literature several cave bacterial strains have been found to inhibit Gram-positive (*Bacillus megaterium*, *B. subtilis*, *B. cereus*, *B. thuringiensis, Corynebacterium glutamicum, Enterococcus faecium, E. faecalis, Enterococcus faecium, Listeria monocytogenes, Micrococcus luteus*, *Mycobacterium phlei, M. smegmatis, Paenibacillus larvae, Rhodococcus equi, Staphylococcus aureus, S. epidermidis* and *Streptomyces murinus*) and Gram-negative bacteria (*Acinetobacter baumannii*, C*itrobacter freundii*, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, P. putida*, *Salmonella typhi* and *Xanthomonas oryzae*) bacteria (**Table 1**).

Table 1. Studies of bioactivities in caves over the last 40 years.

Cave	Sample type	Isolation techniques	Taxon Found	Bioactivity	Bioassay	Reference
PhaTup Cave Forest Park, Thailand	Soil	Samples were placed in oven at 120 ℃ for 1 h and treated with phenol. Soil suspension was spread onto humic acid vitamin agar containing nystatin and cycloheximide.	*Nonomuraea monospora* (strain PT708)	Antibacterial activity against *Bacillus cereus* TISTR 687, methicillin-resistant *Staphylococcus aureus* and *Peanibacillus larvae* LMG 9820^{T}	Disk diffusion	(Nareeluk Nakaew *et al*., 2012)
Phanangkhoi cave in northern Thailand	Soil	Soil samples were pre-treated by dry heat followed by phenol treatment and then spread on humic acid-vitamin agar, humic acid-vitamin gellan gum media and starch casein agar. All media were supplemented with cycloheximide and nystatin	*Micromonospora chersina* (strain PNK 404)	Antibacterial activity against *B. cereus* and *P. larvae*	Agar well method	(Nakaew *et al*., 2009b)
Phanangkhoi cave in northern Thailand	Soil	Soil samples were pre-treated by dry heat followed by phenol treatment and then spread on humic acid-vitamin agar, humic acid-vitamin gellan gum media and starch casein agar. All media were supplemented with cycloheximide and nystatin	*Spirillospora albida* (strains PNK470 and CMU-PNK470)	Antibacterial activity against *B. cereus*, methicillin-resistant *S. aureus* and *P. larvae*	Agar well method	(Nakaew *et al*., 2009b, 2009a)
PhaTup Cave Forest Park, Thailand	Soil	Soil samples were pre-treated by dry heat followed by phenol treatment and then spread on humic acid-vitamin agar, humic acid-vitamin gellan gum media and starch casein agar. All media were supplemented with cycloheximide and nystatin	*Actinocorallia aurantiaca* (strain PT 725)	Antibacterial activity against *P. larvae*	Agar well method	(Nakaew *et al*., 2009b)
PhaTup Cave Forest Park, Thailand	Soil	Soil samples were pre-treated by dry heat followed by phenol treatment and then spread on humic acid-vitamin agar, humic acid-vitamin gellan gum media and starch casein agar. All media were supplemented with cycloheximide and nystatin	*Nonomuraea roseola* (strain PT 708)	Antibacterial activity against *B. cereus*, methicillin-resistant *S. aureus* and *P. larvae*	Agar well method	(Nakaew *et al*., 2009b)
Iron Curtain Cave, Canada	Wall	Swabs of wall samples were inoculated on to the R2A and Actinomycete Isolation agar media for 9 months inside the cave with temperature ranging from 4 to 8 ℃	Unknown actinomycete (strain ICC1)	Antibacterial activity against *Escherichia coli* (NDM type carbapenemase) 15-102 and *E. coli* (oxa48 type carbapenemase) 15-124.	Seeded agar method (Disk diffusion)	(Ghosh *et al*., 2017b)
Iron Curtain Cave, Canada	Wall	Swabs of wall samples were inoculated on to the R2A and Actinomycete Isolation agar media for 9 months inside the cave with temperature ranging from 4 to 8 ℃	Unknown actinomycete (strain ICC4)	Antibacterial activity against non-resistant strain of *E. coli* and *S. aureus*, along with *E. coli* (New Delhi strain)15-318, *E. coli* (oxa48 type carbapenemase) 15-124, *E. coli* (NDM type carbapenemase) 15-102, and *Pseudomonas aeruginosa.*	Seeded agar method (Disk diffusion)	(Ghosh *et al*., 2017b)

A gold mine cave in Kongju, Korea	Soil samples	Soil samples were diluted on tap water agar and oligotrophic media (M5)	*Saccharothrix violacea*	Antibacterial activity against *B. subtilis* IMSNU 10011, *Micrococcus luteus* IMSNU 20371, *Streptomyces murinus* IMSNU 20248T and *Staphylococcus aureus* subsp. *aureus* IMSNU 11089	Overlay technique	(Lee *et al*., 2000)
Smasse-Rawo Ghaar, Pakistan	Sediment	Samples were serially diluted in two different concentrations of R2A media and three different concentrations of diluted nutrient broth supplemented with agar and aqueous extract of the collected cave sediment. Samples were incubated for 1 week at 17 ºC and 37 ºC.	*Fictibacillus nanhaiensis* (strain MY-CB9) and *Paenibacillus lautus* (strain MY-CB14)	Antibacterial activity against *S. aureus* and *Salmonella typhi*.	Confrontation bioassay using paper discs	(Yasir, 2017)
Smasse-Rawo Ghaar, Pakistan	Sediment	Samples were serially diluted in two different concentrations of R2A media and three different concentrations of diluted nutrient broth supplemented with agar and aqueous extract of the collected cave sediment. Samples were incubated for 1 week at 17 ºC and 37 ºC.	*Bacillus niacini* (strain MY-CB144), *Bacillus timonensis* (strain MY-CB128) and *Caulobacter vibrioides* (strain MY-CB65)	Antibacterial activity against *S. aureus*	Confrontation bioassay using paper discs	(Yasir, 2017)
Smasse-Rawo Ghaar, Pakistan	Sediment	Samples were serially diluted in two different concentrations of R2A media and three different concentrations of diluted nutrient broth supplemented with agar and aqueous extract of the collected cave sediment. Samples were incubated for 1 week at 17 ºC and 37 ºC.	*Bacillus muralis* (strain MY-CB146), *Bacillus simplex* (strains MY-CB11 and MY-CB152), *Bacillus timonensis* (strain MY-CB12), *Paenibacillus lautus* (strain MY-CB145) and *Pseudomonas mosselii* (strain MY-CB149)	Antibacterial activity against *S. typhi*	Confrontation bioassay using paper discs	(Yasir, 2017)
Koat Maqbari Ghaar, Pakistan	Sediment	Samples were serially diluted in two different concentrations of R2A media and three different concentrations of diluted nutrient broth supplemented with agar and aqueous extract of the collected cave sediment. Samples were incubated for 1 week at 17 ºC and 37 ºC.	*Microbacterium oleivorans* (strain MY-CA97), *Pseudomonas graminis* (strain MY-CA27) and *Pseudomonas koreensis* (strains MY-CA28 and MY-CA50)	Antibacterial activity against *S. aureus* and *S. typhi*.	Confrontation bioassay using paper discs	(Yasir, 2017)
Koat Maqbari Ghaar (KMG), Pakistan	Sediment	Samples were serially diluted in two different concentrations of R2A media and three different concentrations of diluted nutrient broth supplemented with agar and aqueous extract of the collected cave sediment. Samples were incubated for 1 week at 17 ºC and 37 ºC.	*Bacillus humi* (strain MY-CA167), *Bacillus mycoides* MY-CA84 *Bacillus thaonhiensis* MY-CA168 *Carnobacteriuminhibens* MY-CA29 *Pseudomonas baetica* MY-CA85 *Pseudomonas cremoricolorata* MY-CA66 *Pseudomonas koreensis* MY-CA169 *Pseudomonas mosselii* MY-CA17 *Pseudomonas simiae* MY-CA99	Antibacterial activity against *S. aureus*	Confrontation bioassay using paper discs	(Yasir, 2017)

			Staphylococcus warneri MY-CA92			
Koat Maqbari Ghaar (KMG), Pakistan	Sediment	Samples were serially diluted in two different concentrations of R2A media and three different concentrations of diluted nutrient broth supplemented with agar and aqueous extract of the collected cave sediment. Samples were incubated for 1 week at 17 ºC and 37 ºC.	*Bacillus humi* (strain MY-CA172), *Bacillus tequilensis* (strain MY-CA3) and *Brevibacillus borstelensis* (strain MY-CA2)	Antibacterial activity against *S. typhi*	Confrontation bioassay using paper discs	(Yasir, 2017)
Kotumsar cave, India	Red laterite sediment	Dilution plate and direct plate techniques using starch casein agar as media supplemented with cycloheximide and nystatin prior to wet-heat treatment. Incubation was performed at 28 ºC for 7 to 15 days.	*Streptomyces prasinosporus* (strain KCA 3 and 8)	Antibacterial activity against *Escherichia coli* MTCC 1667 and *Pseudomonas aeruginosa* JNMC	Cup plate diffusion method	(Rajput, Yogita Biswas and Rai, 2012)
Kotumsar cave, India	Guano mixed sediment	Dilution plate and direct plate techniques using starch casein agar as media supplemented with cycloheximide and nystatin prior to wet-heat treatment. Incubation was performed at 28 ºC for 7 to 15 days.	*Streptomyces prasinosporus* (strain KCA 22)	Antibacterial activity against *E. coli* MTCC 1667, *P. aeruginosa* JNMC and *Staphylococcus aureus* MTCC 96	Cup plate diffusion method	(Rajput, Yogita Biswas and Rai, 2012)
Kotumsar cave, India	Guano	Dilution plate and direct plate techniques using starch casein agar as media supplemented with cycloheximide and nystatin prior to wet-heat treatment. Incubation was performed at 28 ºC for 7 to 15 days.	*Streptomyces longisporoflavus* (strain KCA18)	Antibacterial activity against *E. coli* MTCC 1667, *P. aeruginosa* JNMC and *S. aureus* MTCC 96	Cup plate diffusion method	(Rajput, Yogita Biswas and Rai, 2012)
Kotumsar cave, India	Red laterite sediment	Dilution plate and direct plate techniques using starch casein agar as media supplemented with cycloheximide and nystatin prior to wet-heat treatment. Incubation was performed at 28 ºC for 7 to 15 days.	*Streptomyces aurantiacus* (strain KCA6), *Streptomyces luridus* (strain KCA23) and *Streptomyces roseus* (strain KCA13)	Antibacterial activity against *E. coli* MTCC 1667, *P. aeruginosa* JNMC and *S. aureus* MTCC 96	Cup plate diffusion method	(Rajput, Yogita Biswas and Rai, 2012)
Magura Cave, Bulgaria	Guano	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14 ºC	*Pseudomonas fragi* (strain B2)	Antibacterial activity against *Pseudomonas aeruginosa* NBIMCC 1390	Agar well diffusion method	(Tomova *et al*., 2013)
Magura Cave, Bulgaria	Guano	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14 ºC	*Sphingobacterium* sp. (strain B7)	Antibacterial activity against *P. aeruginosa* NBIMCC 1390 and *Xanthomonas oryzae*	Agar well diffusion method	(Tomova *et al*., 2013)
Magura Cave, Bulgaria	Wall drawings	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14 ºC	*Pseudomonas fluorescens* (strain B3)	Antibacterial activity against *P. aeruginosa* NBIMCC 1390 and *X. oryzae*	Agar well diffusion method	(Tomova *et al*., 2013)

Magura Cave, Bulgaria	Wall drawings	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14 ºC	Unknown (strain B4), *Sphingobacterium* sp. (strain B13 and CB15), *Serratia* sp. (strain B14), and *Bacillus amyloliquefaciens* (strain CB16)	Antibacterial activity against *P. aeruginosa* NBIMCC 1390	Agar well diffusion method	(Tomova *et al*., 2013)
Magura Cave, Bulgaria	Wall drawings	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14 ºC	*Serratia* sp. (strain B5)	Antibacterial activity against *X. oryzae*	Agar well diffusion method	(Tomova *et al*., 2013)
Magura Cave, Bulgaria	Rock wall	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14 ºC	*Arthrobacter* sp. (strain IM6 and IM10), *Pseudomonas fluorescens* (strain IM5), *Obesumbacterium proteus* (strain IM7), *Serratia sp.* (strain B6, IM1and IM4), *Serratia proteamaculans* (strain IM8) and *Stenotrophomonas* sp. (strain IM17 and IM19)	Antibacterial activity against *P. aeruginosa* NBIMCC 1390	Agar well diffusion method	(Tomova *et al*., 2013)
Magura Cave, Bulgaria	Rock wall	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14ºC	*Pseudomonas putida (*strain IM15)	Antibacterial activity against *Bacillus subtilis* ATCC 6633 and *X. oryzae*	Agar well diffusion method	(Tomova *et al*., 2013)
Magura Cave, Bulgaria	Rock wall	Each sample was incubated aerobically at 14 ºC for 10 days in nutrient broth (NB) followed by cultivation in nutrient agar for 1 week at 14ºC	*Serratia sp.* (strain IM16), *Comamonas* sp. (strain IM18) and *Pseudomonas plecoglossicida* (strain IM20)	Antibacterial activity against *B. subtilis* ATCC 6633, *P. aeruginosa* NBIMCC 1390 and *X. oryzae*	Agar well diffusion method	(Tomova *et al*., 2013)
Helmcken Falls, Canada	Soil	Dilution plating on Hickey-Tresner agar, Starch agar and Sodium Caseinate agar. The incubation period was one week at 22°C.	*Streptomyces durmitorensis* (strain E9)	Antibacterial activity against *Paenibacillus larvae* ATCC 13537^{T}	Disk diffusion	(Kay *et al*., 2013)
Helmcken Falls, Canada	Unknown	Unknown	*Streptomyces durmitorensis* (strain E9)	Antibacterial activity against *Acinetobacter baumannii* strain 14394, (ESBL) *Escherichia coli* strain 1841 JVC1195, *Micrococcus luteus, Mycobacterium smegmatis* strain JVC1213 and multidrug Resistant (MDR) *Staphylococcus aureus*	Agar plug method	(Rule and Cheeptham, 2013)
Helmcken Falls, Canada	Unknown	Unknown	*Streptomyces microflavus* (strain PM 106)	Antibacterial activity against *A. baumannii* strain 14394 and *M. smegmatis* JVC1213	Agar plug method	(Rule and Cheeptham, 2013)
Helmcken Falls, Canada	Unknown	Unknown	*Streptomyces* sp. (strain E25)	Antibacterial activity against (ESBL) *E. coli* strain 1841 JVC1195, *M. luteus,* and (MDR) *S. aureus*	Agar plug method	(Rule and Cheeptham, 2013)

Helmcken Falls, Canada	Unknown	Unknown	*Streptomyces coelescens* (strain NC-18)	Antibacterial activity against *A. baumannii* strain 14394 *M. luteus,* and (MDR) *S. aureus*	Agar plug method	(Rule and Cheeptham, 2013)
Helmcken Falls, Canada	Unknown	Unknown	*Streptomyces phaeochromogenes* (strain NC-39)	Antibacterial activity against *M. luteus* and *M. smegmatis* JVC1213	Agar plug method	(Rule and Cheeptham, 2013)
A cave on National Park Djerdap region, Serbia	Soil	Starch casein agar containing soluble starch and bacteriological agar supplemented with cycloheximide. The incubation period was one week at 30 °C.	*Streptomyces* sp. (strain JS520)	Antibacterial activity against *Bacillus subtilis* ATCC 6633*, Micrococcus luteus* ATCC 379 and *Pseudomonas aeruginosa* ATCC 27853	Disc diffusion and microtiter plate-based assay	(Stankovic *et al*., 2012)
Krubera-Voronja Cave, Georgia	White clay	Sediment samples were suspended and diluted in saline solution and further spread over Tryptic Soy agar and incubated at 30 ºC for 3 days.	*Bacillaceae* bacterium (strain 1350R2-TSA30-6)	Antibacterial activity against *Bacillus cereus* ATCC 10876, *Bacillus thuringiensis, Brevibacillus* sp., *Enterococcus faecalis* 29212, *Listeria monocytogenes* 7644, *Micrococcus luteus*, *Paenibacillus* sp., *Rhodococcus equi* ATCC 6939, *Staphylococcus aureus* subsp. *aureus* ATCC 25923 and *Streptomyces* sp.	Agar-well diffusion method	(Klusaite *et al*., 2016)
Krubera-Voronja Cave, Georgia	Water	Water sample (100 µL) was spread over Tryptic Soy agar and incubated at 30 ºC for 3 days.	*Bacillus* sp. (strain 1410WF1-TSA30-2)	Antibacterial activity against *B. thuringiensis*, *Brevibacillus* sp., *M. luteus* and *Paenibacillus* sp.	Agar-well diffusion method	(Klusaite *et al*., 2016)
Kashmir Smast (cave), Pakistan	Soil	1 g of each soil sample was serially diluted in saline solution and further spread on nutrient-agar plates and incubated for 24h at 35 ºC	*Bacillus licheniformis* (strain KC2-MRL)	Antibacterial activity against *Micrococcus luteus* (ATCC 10240), *Klebsiella* sp., *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli*	Agar-well diffusion assay	(Zada *et al*., 2016)
Francthi' Cave, Greece	Scrapped mats and pieces of rocks	Enrichment cultures were obtained in flasks and petri dishes with culture media BG11o and BG11 under specific conditions (23 ºC, 80% RH, 7 µmols·s^{-1}·m^{-2})	*Toxopsis calypsus* (type strain: ATHU-CY 3314)	Antibacterial activity against Methicillin-Resistant *S. aureus* (MRSA) 1629, Methicillin-Susceptible *S. aureus* (MSSA) 1646, *Enterococcus faecalis* ATCC 29212, Vancomycin-Resistant *E. faecalis* (VRE) 880 and Vancomycin-Resistant *E. faecium* (VRE) 1291	Disk diffusion and microdilution method	(Lamprinou *et al*., 2015)
Francthi' Cave, Greece	Scrapped mats and pieces of rocks	Enrichment cultures were obtained in flasks and petri dishes with culture media BG11o and BG11 under specific conditions (23 ºC, 80% RH, 7 µmols·s^{-1}·m^{-2})	*Phormidium melanochroun* (type strain: ATHU-CY 3315)	Antibacterial activity against Methicillin-Resistant *S. aureus* (MRSA) 1629, Methicillin-Susceptible *S. aureus* (MSSA) 1646, *Enterococcus faecalis* ATCC 29212, Vancomycin-Resistant *E. faecalis* (VRE) 880 and Vancomycin-Resistant *E. faecium* (VRE) 1291	Disk diffusion	(Lamprinou *et al*., 2015)
Turkey caves	Rock wall, speleothems surfaces and soil samples	Using starch casein media supplemented with cycloheximide and rifampicin at 27º C	*Streptomyces* sp. Strain 1492	Antibacterial activity against *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Enterococcus faecium* (VRE) and *Acinetobacter baumanii*	Serial dilution method	(Yücel and Yamaç, 2010)
Bolshaya Oreshnaya cave, Russia	Moonmilk speleothem	Sample aliquots were preheated for 5 minutes at 50 n°C and diluted in a 1%	*Streptomyces* sp. (strains IB 2014 /I/78–1, IB 2014 /I/78–3,	Antibacterial activity against *Bacillus subtilis* ATCC 66337	Disk diffusion	(Axenov-Gribanov *et al*., 2016)

		sterile saline solution and further plated on the solid nutrient media and agarised DNPM supplemented with cycloheximide and phosphomycin for 30 days at 28 °C	IB 2014 /I/78–9 and IB 2014 /I/78–12, IB 2014 /I/78–11)			
Bolshaya Oreshnaya cave, Russia	Moonmilk speleothem	Sample aliquots were preheated for 5 minutes at 50 °C and diluted in a 1% sterile saline solution and further plated on the solid nutrient media MS and agarised DNPM supplemented with cycloheximide and phosphomycin for 30 days at 28 °C	*Streptomyces* sp. (strain IB 2014 /I/78-6)	Antibacterial activity against *B. subtilis* ATCC 66337, *Escherichia coli* ATCC 25922	Disk diffusion	(Axenov-Gribanov *et al*., 2016)
Bolshaya Oreshnaya cave, Russia	Moonmilk speleothem	Sample aliquots were preheated for 5 minutes at 50 °C and diluted in a 1% sterile saline solution and further plated on the solid nutrient media MS and agarised DNPM supplemented with cycloheximide and phosphomycin for 30 days at 28 °C	*Streptomyces* sp. (strain IB 2014/I/78–8)	Antibacterial activity against *B subtilis* ATCC 66337*, E coli* ATCC 25922 and *Pseudomonas putida* KT 2440	Disk diffusion	(Axenov-Gribanov *et al*., 2016)
Grotta dei Cervi (cave), Italy	Rock wall covered with Neolithic red paintings	Dilution plating on peptone yeast extract brain-heart-infusion media incubated at 28 ºC	*Streptomyces tendae* (strain HKI 0179)	Antibacterial activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* SG511, multidrug resistant *S. aureus* 134/93 (MRSA), efflux-resistant *S. aureus* EfS4 and vancomycin-resistant *Enterococcus faecalis* 1528 (VRE).	Disk diffusion	(Herold *et al*., 2005)
Cueva de los Murciélagos (cave), Spain	Unknown	Unknown	*Bacillus licheniformis* (strain A12)	Antibacterial activity against (*Bacillus megaterium*, *Corynebacterium glutamicum* CECT 78, *Mycobacterium phlei* and *Sarcina* sp.	Agar well diffusion method	(Gálvez *et al*., 1993)
A karst cave in Chongqing city, China	Soil	Unknown	*Streptomyces* sp. (strain CC8-201)	Antibacterial activity against *Staphylococcus aureus* ATCC 29213 (MSSA), *S. aureus* ATCC 33591 (MRSA), *S. aureus* 15 (MSSA), *S. aureus* 12-28 (MSSA), *S. aureus* 12-33 (MRSA), *Staphylococcus epidermidis* ATCC 12228 (MSSE) *S. epidermidis* 12-6 (MSSE), *S. epidermidis* 12-8 (MRSE), *Enterococcus faecalis* ATCC 29212 (VSE), *E. faecalis* ATCC 51299 (VRE) *E. faecalis* 12-5 (VSE), *E. faecalis* 09-9 (VRE), *E. faecalis* ATCC 700221 (VRE), *Enterococcus faecium* 12-1 (VRE) and *E. faecium* 12-3 (VSE)	Disk diffusion	(Jiang *et al*., 2015)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on ISP media, starch nitrate media, B-4agar, and minimal media with 1% chitin. All media were supplemented with nalidixic acid	*Streptomyces* sp. (strain MM99)	Antibacterial activity against Antibacterial activity against *Bacillus subtilis* ATCC 19659, *Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* ATCC 9341	Cross streak method and Disk Diffusion Assay	(Maciejewska *et al*., 2016)

		and nystatin. Incubation was performed at 17 ºC for 1 month				
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on minimal media with 1% chitin supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM48, MM12, MM111)	Antibacterial activity against *B. subtilis* ATCC 19659 and *M. luteus* ATCC 9341	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on starch nitrate media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM12, MM107)	Antibacterial activity against *B. subtilis* ATCC 19659 and *M. luteus* ATCC 9341	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on starch nitrate media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM1)	Antibacterial activity against *Bacillus subtilis* ATCC 19659, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341 and *Citrobacter freundii* ATCC 43864	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on starch nitrate media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM17)	Antibacterial activity against *Bacillus subtilis* ATCC 19659, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341, *Citrobacter freundii* ATCC 43864 and *Klebsiella pneumoniae* ATCC 13883	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on starch nitrate media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM6, MM59)	Antibacterial activity against *Bacillus subtilis* ATCC 19659, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341, *Citrobacter freundii* ATCC 43864 and *Klebsiella pneumoniae* ATCC 13883	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on ISP6 supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM104, MM10)	Antibacterial activity against *B. subtilis*	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on starch nitrate media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM24)	Antibacterial activity against *Escherichia coli* (ATCC25922), *Bacillus subtilis* (ATCC19659), *Staphylococcus aureus* (ATCC25923) and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated starch nitrate media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM100, MM109)	Antibacterial activity against *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Staphylococcus aureus* (ATCC25923) and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on ISP7	*Streptomyces* sp. (strain MM117)	Antibacterial activity against *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae*	Cross streak method	(Maciejewska *et al*., 2016)

		media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month		(ATCC13883), *Bacillus subtilis* (ATCC19659) and *Micrococcus luteus* (ATCC9341)		
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM122)	Antibacterial activity against *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Staphylococcus aureus* (ATCC25923), *Citrobacter freundii* (ATCC43864), and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM14)	Antibacterial activity against *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Staphylococcus aureus* (ATCC25923), *Citrobacter freundii* (ATCC43864), and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on ISP6 media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM105, MM128,)	Antibacterial activity against *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Escherichia coli* (ATCC25922), *Citrobacter freundii* (ATCC43864), and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM23)	Antibacterial activity against *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Citrobacter freundii* (ATCC43864), and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM21)	Antibacterial activity against *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC 27853), *Citrobacter freundii* (ATCC43864), and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM3)	Antibacterial activity against *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Pseudomonas aeruginosa* (ATCC 27853), and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM19 and MM7)	Antibacterial activity against *Escherichia coli* (ATCC25922), *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Pseudomonas aeruginosa* (ATCC 27853), *Citrobacter freundii*	Cross streak method	(Maciejewska *et al*., 2016)

				(ATCC43864), and *Micrococcus luteus* (ATCC9341)		
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM90)	Antibacterial activity against *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659), *Pseudomonas aeruginosa* (ATCC 27853), and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Springtails' Cave, Belgium	Moonmilk deposits	Moonmilk suspensions were serially diluted in PBS and inoculated on B-4 agar media supplemented with nalidixic acid and nystatin. Incubation was performed at 17 ºC for 1 month	*Streptomyces* sp. (strain MM108, MM106, MM5, MM44)	Antibacterial activity against *Klebsiella pneumoniae* (ATCC13883), *Bacillus subtilis* (ATCC19659) and *Micrococcus luteus* (ATCC9341)	Cross streak method	(Maciejewska *et al*., 2016)
Khangkhui cave, India	Soil	Soil was sieved and air dried for 1 week and further treated with 0.1 g $CaCO_3$ and incubated for a week. Serial dilution was performed in SCNA media with the presence or absence of nystatin and/or cycloheximide	Unknown actinomycete (strain KC1-7)	Antibacterial activity against *Micrococcus luteus* MTCC 106 and *Bacillus subtilis* MTCC 121	Disk diffusion	(Ningthouja *et al*., 2009)
Khangkhui cave, India	Soil	Soil was sieved and air dried for 1 week and further treated with 0.1 g $CaCO_3$ and incubated for a week. Serial dilution was performed in SCNA media with the presence or absence of nystatin and/or cycloheximide	Unknown actinomycete (strain KC1-10)	Antibacterial activity against *M. luteus* MTCC 106, *B. subtilis* MTCC 121 and *Escherichia coli* MTCC 739	Disk diffusion	(Ningthouja *et al*., 2009)
Hardin's cave, USA	highly decomposed bark	Soil samples vortexed with 1 mL of sterile water, and the supernatant was subjected to serial dilution and plated on the agars selective for actinobacteria (unknown). Agar plates were then incubated at 30 °C for 3 weeks.	*Nonomuraea specus*	Antibacterial activity against *Bacillus subtilis*	Unknown	(Derewacz *et al*., 2014)
Chaabe Cave, Algeria	Soil	Sediment samples were air dried for 1 week and further crushed in a sterile mortar. Isolation was performed by the dilution plate thecnique onto one of the following media: Chitin-B vitamin agar media, Olson's media, Bennett's media and Tryptic soy agar media. The four media were supplemeted with cycloheximide and nystatin	*Streptomyces celluloflavus* (strain A5)	Antibacterial activity against *Staphylococcus aureus* ATCC 6538 and *Micrococcus luteus* ATCC 9341	Disk diffusion	(Belyagoubi *et al.*, 2018)
Chaabe Cave, Algeria	Soil	Sediment samples were air dried for 1 week and further crushed in a sterile mortar. Isolation was performed by the dilution plate technique onto one of the following media: Chitin-B vitamin agar media, Olson's media, Bennett's media and Tryptic soy agar media. The four	*Streptomyces celluloflavus* (strain 11)	Antibacterial activity against *S. aureus* ATCC 6538 *M. luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 8739	Disk diffusion	(Belyagoubi *et al*., 2018)

		media were supplemented with cycloheximide and nystatin				
Chaabe Cave, Algeria	Soil	Sediment samples were air dried for 1 week and further crushed in a sterile mortar. Isolation was performed by the dilution plate technique onto one of the following media: Chitin-B vitamin agar media, Olson's media, Bennett's media and Tryptic soy agar media. The four media were supplemented with cycloheximide and nystatin	*Streptomyces celluloflavus* (strain A16)	Antibacterial activity against *S. aureus* ATCC 6538 *M. luteus* ATCC 9341 and *E. coli* ATCC 8739	Disk diffusion	(Belyagoubi *et al*., 2018)
Chaabe Cave, Algeria	Soil	Sediment samples were air dried for 1 week and further crushed in a sterile mortar. Isolation was performed by the dilution plate technique onto one of the following media: Chitin-B vitamin agar media, Olson's media, Bennett's media and Tryptic soy agar media. The four media were supplemented with cycloheximide and nystatin	*Streptomyces aureoverticillatus* (strain A22)	Antibacterial activity against *S. aureus* ATCC 6538 *M. luteus* ATCC 9341, *B. subtilis* ATCC 6633 and *Listeria monocytogenes* ATCC 19111	Disk diffusion	(Belyagoubi *et al*., 2018)
Chaabe Cave, Algeria	Soil	Sediment samples were air dried for 1 week and further crushed in a sterile mortar. Isolation was performed by the dilution plate technique onto one of the following media: Chitin-B vitamin agar media, Olson's media, Bennett's media and Tryptic soy agar media. The four media were supplemented with cycloheximide and nystatin	*Streptomyces* sp. (strain A45)	Antibacterial activity against *S. aureus* ATCC 6538 *M. luteus* ATCC 9341, *B. subtilis* ATCC 6633	Disk diffusion	(Belyagoubi *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Nocardia* sp. (strain MMun 129)	Antibacterial activity against *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883 and *Bacillus subtilis* ATCC 19659	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Nocardia* sp. (strain MMun 133)	Antibacterial activity against *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, *Micrococcus luteus* ATCC 9341 and *Citrobacter freundii* ATCC 43864	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN)	*Streptomyces* sp. (strain MMun 135) and *Nocardia soli* (strain MMun 136)	Antibacterial activity against *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC	Cross streak method	(Adam *et al*., 2018)

		supplemented with nalidixic acid, nystatin and $FeCl_3$. The plates were incubated for 2 months at 15 ºC		13883, *B. subtilis* ATCC 19659, *M. luteus.* ATCC 9341 and *C. freundii* ATCC 43864		
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and desferrioxamine B. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 137)	Antibacterial activity against *P. aeruginosa* ATCC 27853*, B. subtilis* ATCC 19659, *M. luteus* ATCC 9341 and *Staphylococcus aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 138)	Antibacterial activity against *P. aeruginosa* ATCC 27853*, K. pneumoniae* ATCC 13883, *B. subtilis* ATCC 19659, *M. luteus* ATCC 9341 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 139)	Antibacterial activity against *P. aeruginosa* ATCC 27853*, K. pneumoniae* ATCC 13883, *M. luteus* ATCC 9341 and *C. freundii* ATCC 43864	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Nocardia* sp. (strain MMun 140)	Antibacterial activity against *P. aeruginosa* ATCC 27853*, K. pneumoniae* ATCC 13883,	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 141)	Antibacterial activity against*, B. subtilis* ATCC 19659, *M. luteus* ATCC 9341 and *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and bovine liver catalase. The plates were incubated for 2 months at 15 ºC	*Streptomyces lunaelactis* (strain MMun 143)	Antibacterial activity against*, P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, *C. freundii* ATCC 43864, *B. subtilis* ATCC 19659 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and bovine liver catalase. The plates were incubated for 2 months at 15 ºC	*Nocardia soli* (strain MMun 144)	Antibacterial activity against*, P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, *C. freundii* ATCC 43864 and *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)

Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and desferrioxamine B. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 146)	Antibacterial activity against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. freundii* ATCC 43864, *K. pneumoniae* ATCC 13883, *B. subtilis* ATCC 19659, *S. aureus* ATCC 25923 and *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and desferrioxamine B. The plates were incubated for 2 months at 15 ºC	*Streptomyces xiamenensis* (strain MMun 147)	Antibacterial activity against *K. pneumoniae* ATCC 13883 and *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and desferrioxamine B. The plates were incubated for 2 months at 15 ºC	*Streptomyces xiamenensis* (strain MMun 148)	Antibacterial activity against *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Micrococcus* sp. (strain MMun 149)	Antibacterial activity against *K. pneumoniae* ATCC 13883 and *B. subtilis* ATCC 19659	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 150)	Antibacterial activity against *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, *B. subtilis* ATCC 19659, *S. aureus* ATCC 25923 and *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 151)	Antibacterial activity against *K. pneumoniae* ATCC 13883, *B. subtilis* ATCC 19659 and *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Streptomyces lunaelactis* (strain MMun 152)	Antibacterial activity against *B. subtilis* ATCC 19659 and *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and	*Streptomyces* sp. (strain MMun 154)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659 and *K. pneumoniae* ATCC 13883	Cross streak method	(Adam *et al*., 2018)

		nystatin. The plates were incubated for 2 months at 15 ºC				
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Rhodococcus* sp. (strain MMun 155)	Antibacterial activity against *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 156)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin and bovine liver catalase. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 157)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 158)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Kocuria rhizophila* (strain MMun 160)	Antibacterial activity against *M. luteus* ATCC 9341, *C. freundii* ATCC 43864, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 161)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$.	*Streptomyces* sp. (strain MMun 162)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853	Cross streak method	(Adam *et al*., 2018)

		The plates were incubated for 2 months at 15 ºC				
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 163)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in Starch nitrate (SN) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 164)	Antibacterial activity against *M. luteus* ATCC 9341, *C. freundii* ATCC 43864, *B. subtilis* ATCC 19659 and *K. pneumoniae* ATCC 13883	Cross streak method	(Adam et al., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 166)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853	Cross streak method	(Adam et al., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid, nystatin and bovine liver catalase. The plates were incubated for 2 months at 15 ºC	*Agromyces cerinus* (strain MMun 167)	Antibacterial activity against *B. subtilis* ATCC 19659	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid, nystatin and bovine liver catalase. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 168)	Antibacterial activity against *M. luteus* ATCC 9341 and *C. freundii* ATCC 43864	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 170)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)

Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Amycolatopsis* sp. (strain MMun 171)	Antibacterial activity against *M. luteus* ATCC 9341, *C. freundii* ATCC 43864, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid, nystatin along with desferrioxamine B and $FeCl_3$. The plates were incubated for 2 months at 15 ºC	*Micromonospora maoerensis* (strain MMun 172)	Antibacterial activity against *M. luteus* ATCC 9341 and *K. pneumoniae* ATCC 13883	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid, nystatin and bovine liver catalase. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 174)	Antibacterial activity against *M. luteus* ATCC 9341, *B. subtilis* ATCC 19659 and *S. aureus* ATCC 25923	Cross streak method	(Adam *et al*., 2018)
Springtails' Cave, Belgium	Moonmilk deposits	Samples were pre-treated by rehydration-centrifugation method and further inoculated in International Streptomyces Project (ISP) media number 5 (ISP5) supplemented with nalidixic acid and nystatin. The plates were incubated for 2 months at 15 ºC	*Streptomyces* sp. (strain MMun 176)	Antibacterial activity against *M. luteus* ATCC 9341	Cross streak method	(Adam *et al*., 2018)
Badzheyskaya cave, Russia	Water, underground lake	To isolate actinobacteria 100 uL of each water samples preheated for 5 min at 50 ºC were plated onto solid nutrient media and mannitol soy flour agar supplemented with the cycloheximide and phosphomycin. Incubation was performed for 30 days at 28 ºC	*Streptomyces* sp. (strains IB 2014I88-1, IB 2014I88-2HS, IB 2014I88-2, IB 2014I88-4HS, IB 2014I88-4, IB 2014I88-6, IB 2014I88-7 and *Streptomyces* sp. IB 2014I88-8)	Antibacterial activity against *Bacillus subtilis* ATCC 66337, *Staphylococcus carnosus* ATCC 51365, *Pseudomonas putida* KT 2440 and *Escherichia coli* ATCC 25922	Disk diffusion	(Voytsekhovskaya *et al*., 2018)
Badzheyskaya cave, Russia	Water, underground lake	To isolate actinobacteria 100 uL of each water samples preheated for 5 min at 50 ºC were plated onto solid nutrient media and mannitol soy flour agar supplemented with the cycloheximide and phosphomycin. Incubation was performed for 30 days at 28 ºC	*Streptomyces* sp. (strain IB 2014I88-6HS)	Antibacterial activity against *Staphylococcus carnosus* ATCC 51365, *Pseudomonas putida* KT 2440 and *Escherichia coli* ATCC 25922	Disk diffusion	(Voytsekhovskaya *et al*., 2018)

2.4.1. *In vitro* antibacterial activity against Gram-positive bacteria

Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Enterococcus faecium* and *Listeria monocytogenes* are known to cause severe types of infections and high rates of mortality (Bottone, 2010; Hernandez-Milian and Payeras-Cifre, 2014; Higuita and Huycke, 2014; Ikeda *et al*., 2015; Noskin *et al*., 1995; Nurjadi *et al*., 2015; Sowash and Uhlemann, 2014; Suppli *et al*., 2011; Thakur *et al*., 2018; Vergis *et al*., 2001). Among these pathogens, *S. aureus* is found in ~30 to 50 % of the mucosal surfaces and skin of humans and is able to cause a wide spectrum of diseases that encompass skin and soft tissue infections, endocarditis, pneumonia and bacteraemia, among others (Nurjadi *et al*., 2015; Sowash and Uhlemann, 2014). Nowadays several human pathogenic strains of *S. aureus* are known, among them some are resistant to the available antibiotics such as the methicillin resistance S*taphylococcus aureus* (MRSA) affecting hospital healthcare settings (Walker, 2014). In caves, researchers have found different bacterial strains, the majority belonging to *Actinomycetes* group, to be able to inhibit MRSA (**Table 1**) such as: *Streptomyces tendae* (strain HKI 0179), *Spirillospora albida* (strains CMU-PNK470), *Nonomuraea roseola* (strain PT708), *Streptomyces* sp. (strain 1492), *Nonomuraea monospora* (strain PT708), *Streptomyces durmitorensis* (strain E9), *Streptomyces* sp. (strain E25), *Streptomyces coelescens* (strain NC-18), *Nocardia* sp., *Bacillus* sp., *Erwinia* sp., *Pseudomonas* sp. and two cyanobacteria *Toxopsis calypsus* (strain ATHU-CY 3314) and *Phormidium melanochroun* (strain ATHU-CY 3315) (Arroyo *et al*., 1997; Herold *et al*., 2005; Lamprinou *et al*., 2015; Nakaew *et al*., 2009a, 2009b; Nareeluk Nakaew *et al*., 2012; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Yasir, 2017). In contrast, only one study has shown a cave bacterium, *Bacillaceae* (strain 1350R2-TSA30-6) able to inhibit *Listeria monocytogenes* (Klusaite *et al*., 2016). *Listeria monocytogenes* is a foodborne pathogen that may cause listeriosis with severe symptoms and high rates of hospitalization with significant morbidity levels associated (Hernandez-Milian and Payeras-Cifre, 2014; Thakur *et al*., 2018). Great efforts have been done to understand *L. monocytogenes* virulence and pathogenicity, as well as in the improvement of the detection methods and on the epidemiologic studies. However, *L. monocytogenes* remains a problem in need of constant surveillance and risk management (Hernandez-Milian and Payeras-Cifre, 2014; Thakur *et al*., 2018).

In the past decades, the *Enterococcus* genus has been recognized to encompass several important healthcare-associated pathogens, including *E. faecium* and *E. faecalis* which are opportunistic pathogens causing life threatening infections (Higuita and Huycke, 2014; Noskin *et al*., 1995). Moreover, resistance to a last resort antibiotic, vancomycin, by *Enteroccocus* (VRE) has been observed in several health care installations, highlighting the need for new ways to prevent and fight these pathogens (Higuita and Huycke, 2014; Noskin *et al*., 1995; Suppli *et al*., 2011). In cave systems, *Streptomyces tendae* (strain HKI 0179) isolated in Grotta dei Cervi, Italy, and two cyanobacteria, *Toxopsis calypsus* (strain ATHU-CY 3314) and *Phormidium melanochroun* (strain ATHU-CY 3315) both isolated from Francthi Cave in Greece, were found to inhibit VRE *faecalis* (**Table 1**) (Herold *et al*., 2005; Lamprinou *et al*., 2015). The two cyanobacterias were also able to inhibit VRE *faecium* along with *Streptomyces* sp. (strain 1492) isolated in a karst cave from Turkey (**Table 1**) (Herold *et al*., 2005; Yücel and Yamaç, 2010).

Bacillus cereus is another Gram-positive pathogen, found to be inhibited by cave bacterial strains. This pathogen is associated with food poisoning and can cause serious and potentially fatal non-gastrointestinal-tract infections (Bottone, 2010; Ikeda *et al*., 2015). The pathogenicity of *B. cereus* is linked to the production of tissue-destructive exoenzymes (Bottone, 2010; Ikeda *et al*., 2015). From caves, a *Bacillaceae* bacterium (strain 1350R2-TSA30-6), *Nonomuraea monospora* (strain PT708), *Nonomuraea roseola* (strain PT70), *Spirillospora albida* (strains CMU-PNK470 and PNK470) and *Micromonospora chersina* (strain PNK404) have been screened positive against *B. cereus* (**Table 1**) (Klusaite *et al*., 2016; Nakaew *et al*., 2009b, 2009a; Nareeluk Nakaew *et al*., 2012).

Regarding other economically relevant pathogens, American foulbrood disease in honeybees is caused by *Paenibacillus larvae* and is a major threat to bee industries (Ebeling *et al*., 2016; Kay *et al*., 2013). This pathogen causes gut infection in larval honeybees ultimately causing death in larval stage (Ebeling *et al*., 2016; Kay *et al*., 2013). The screening of cave microorganisms has revealed success in the inhibition of *Paenibacillus larvae* (Kay *et al*., 2013; Nakaew *et al*., 2009b, 2009a; Nareeluk Nakaew *et al*., 2012). For instance, *Setreptomyces durmitorensis* strain E9 revealed strong inhibitory power against this honeybee pathogen (**Table 1**) (Kay *et al*., 2013).

Several bacterial strains isolated from caves have demonstrated the ability to inhibit Gram-positive bacteria that cause several infections to human health, as well as others that

compromise the economic sector (Belyagoubi *et al*., 2018; Ghosh *et al*., 2017b; Kay *et al*., 2013; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a; Rajput, Yogita Biswas and Rai, 2012; Yücel and Yamaç, 2010; Zada *et al*., 2016). The exploration of caves in more detail and the screening of a wide array of pathogens are of significant interest to uncover the potential that cave microbial population may have to tackle down this modern health crises.

2.4.2. *In vitro* antibacterial activity against Gram-negative bacteria

Infections caused by antibiotic resistant Gram-negative bacteria have become prevalent, constituting a major concern to public health worldwide (Exner, 2017). The lack of therapeutic agents to fight back these deadly infectious diseases has become evident. In Gram-negative bacteria, the difference in the structure of cell wall results in higher difficulty in penetration and retention of chemical agents, in contrast to Gram-positive bacteria (Richter and Hergenrother, 2018). The increase of resistance from Gram-negative pathogens specially in hospitalized patients has become alarming, among them several multidrug resistant strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Escherichia coli* have been reported (Center for Food Security and Public Health, 2016; Exner, 2017; Hong, 2012; Kaye and Pogue, 2015).

Peseudomonas aeruginosa is a pathogenic Gram-negative bacteria which is difficult to treat, with limited therapeutic agents available due to frequent antibiotic resistance. Moreover, high mortality rate associated to this species makes it a major threat to human health that cannot be overlooked (Theuretzbacher, 2017). Efforts to treat infections caused by this agent are constantly being made and one way could be the exploration of cave bacteria. A study performed in Kotumsar Cave (India), revealed that among the screened cave bacteria, several strains from the genus *Streptomyces* had antibacterial activity against *P. auruginosa* (**Table 1**) (Rajput, Yogita Biswas and Rai, 2012). Other strains from the genera *Pseudomonas*, *Serratina*, *Sphingobacterium* and *Bacillus*, among others, from different geographic caves also revealed positive activity against *P. aeruginosa* strains (**Table 1**) (Rajput, Yogita Biswas and Rai, 2012; Tomova *et al*., 2013). These activities emphasize the possible chance of metabolites produced by cave bacteria to be able to circumvent *P. aeruginosa* resistance, perhaps revealing new modes of actions (Conway and Cohen, 2015).

Although *E. coli* is an important bacterium in natural human flora, it is also a major pathogen causing high morbidity and mortality worldwide, and a reliable treatment still constitutes a major quest to the scientific community (Conway and Cohen, 2015). In caves, several members of *Actinobacteria* mainly from the *Streptomyces* genus have demonstrated inhibition against *E. coli* strains, among them *E. coli* MTCC 1667, *E. coli* strain 1841 JVC1195 and extended spectrum beta-lactamase (ESBL) *E. coli* (Lamprinou *et al*., 2015; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013).

The *Citrobacter* genus belong to the *Enterobacteriaceae* family and is also known to be responsible for several types of infections affecting the biliary tract, urinary tract, respiratory tract, intestines, liver, among others (Liu *et al*., 2018). In hospital settings, *Citrobacter* species may account for 3 to 6% of the isolates belonging to the *Enterobacteriaceae* (Liu *et al*., 2018). Two studies conducted in Springtails' Cave (Belgium) on moonmilk samples have found several *Actinobacteria*, manly *Streptomyces*, with the ability to inhibit *Citrobacter freundii* ATCC 43864 (**Table 1**) (Adam *et al*., 2018; Maciejewska *et al*., 2016).

Another *Enterobacteriaceae* that constitutes a major threat to public health is the genus *Salmonella* with several foodborne pathogens accounting for millions of illnesses every year with high rates of mortality (Eng *et al*., 2015). *Salmonela typhi* causes typhoid fever and it can be acquired by consuming contaminated water and food (Eng *et al*., 2015; Paul and Bandyopadhyay, 2017). A study performed by Yasir and co-workers (2017), has found several members belonging to *Proteobacteria*, *Firmicutes* and *Actinobacteria* to inhibit *Salmonella typhi* (Yasir, 2017).

As it can be observed by the bibliography and **Table 1**, several cave microbiomes may indeed have potential drug producing strains that have proven to be bioactive against a wide range of human pathogens. Nevertheless, it is important to fully characterize the bioactive molecules produced by cave strains and only a few studies have revealed the causative agent for the demonstrated bioactivity (Ghosh *et al*., 2017a). Therefore, more studies need to be conducted in order to understand the chemical potential of cave bacterial by-products as well as the screening of a broader range of pathogenic microorganisms.

2.5. Prospection of cave microorganisms with potential antimicrobial activity

The first step for prospecting any kind of environment for microbial culture representatives is the selection of the type of samples to be screen. Overall, bacterial strains with antibacterial activity in caves have been sampled from soil, sediments, walls, guano, guano-sediments mixtures, wall drawings, moonmilk deposits and a piece of bark (Axenov-Gribanov *et al*., 2016; Derewacz *et al*., 2014; Ghosh *et al*., 2017b; Klusaite *et al*., 2016; Lamprinou *et al*., 2015; Maciejewska *et al*., 2016; Rajput, Yogita Biswas and Rai, 2012; Tomova *et al*., 2013; Yasir, 2017). Throughout the literature, in the search of cave drug producing strains, the screening of the *Actinobacteria* phylum has been widely observed while only few studies have reported a broader screening encompassing member of *Proteobacteria* and *Firmicutes* phyla (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Ghosh *et al*., 2017b, 2017a; Maciejewska *et al*., 2016; Rule and Cheeptham, 2013; Stankovic *et al*., 2012; Tomova *et al*., 2013; Voytsekhovskaya *et al*., 2018; Yasir, 2017). This is expectable, since *Actinobacteria* species, specifically the genus *Streptomyces*, account for a great number of the discovered antibiotics until date (Bérdy, 2005; de Lima Procópio *et al*., 2012; Genilloud, 2017). With focus on *Actinobacteria*, Rule and Cheepmant (2013) have screened 400 isolates from Helmcken Falls Cave and have found that 26.5% exhibited activity against *Klebsiella pneumoniae*, 10.25% against *Micrococcus luteus*, 9.25% against *Mycobacterium smegmatis*, 6.25% against *Pseudomonas aeruginosa*, 2.25% against *Acinetobacter baumannii*, 2% against MRSA, 1.75% against *Escherichia coli*, and 1% against (ESBL) *E. coli* (Rule and Cheeptham, 2013). Another study performed by Herold and colleagues (2005), isolated *Streptomyces tendae* (strain HKI 0179) (**Table 1**) from Grotta dei Cervi, and found that it produced Cervimcycins A-D polyketide glycosides with strong antimicrobial capacities against efflux-resistant *S. aureus* EfS4 and vancomycin-resistant *Enterococcus faecalis* 1528 (VRE) within a MIC range of 1.6–12.5 $\mu g\ mL^{-1}$ (Herold *et al*., 2005). Moreover, in cave systems, *Actinobacteria* members with antimicrobial capacities have been isolated from several different samples including guano mixed sediment, guano, sediment, soil, water, decomposed bark, red laterite sediment, rock wall and moonmilk deposits (Axenov-Gribanov *et al*., 2016; Derewacz *et al*., 2014; Ghosh *et al*., 2017b; Herold *et al*., 2005; Rajput, Yogita Biswas and Rai, 2012; Voytsekhovskaya *et al*., 2018).

The exploration of mineral formations and deposits can be on the way to finding novel microbial strains with interesting biochemistry (Cheeptham and Saiz-Jimenez, 2015). For instance, deposits rich in iron and manganese have been associated to a by-product formation by several types of cave dwelling microorganisms, mainly Fe-/Mn-oxidizing bacteria (Carmichael *et al*., 2013; Cheeptham and Saiz-Jimenez, 2015; Hurst, 2016). Among these intriguing formations, moonmilk are secondary mineral deposits with variable composition found in subterranean environments such as karstic caves and lava tubes (Miller *et al*., 2018; Portillo and Gonzalez, 2011). Several constituents have been reported in its nature such as calcite, hydromagnesite, aragonite, vaterite and huntite as well as some nitrates and sulphates, nonetheless, the origin of moonmilk remains mysterious and several authors suggest that biogenic processes may be linked to its formation (Hurst, 2016; Miller *et al*., 2018; Montano and Henderson, 2013). Interestingly, moonmilk deposits have been long used by humans for its medicinal properties and perhaps its associated microbiomes may be linked to the observable bioactivities (Maciejewska *et al*., 2016). For instance, Marta Maciejewska and colleagues (2016) isolated several *Streptomyces* strains from moonmilk deposits in Springtails' Cave (Belgium) that demonstrated antimicrobial activity against *B. subtilis*, *M. luteus*, *S. aureus*, *K. pneumoniae*, *E. coli, C. freundii* and *P. aeruginosa* (Maciejewska *et al*., 2016). In moonmilk deposits from the same cave, Delphine Adam and colleagues (2018) also found several *Actinomycetes* from the *Micrococcaceae*, *Nocardiaceae*, *Rhodobacteraceae* and *Streptomycetaceae* families to possess antibacterial activity against Gram-positive and Gram-negative bacteria (Adam *et al*., 2018). Another study, performed by Axenov-Gibanov and colleagues (2016) found several *Streptomyces* from moonmilk deposits of Bolshaya Oreshnaya cave to possess antimicrobial activity against *Bacillus subtilis* ATCC 66337, *Pseudomonas putida* KT 2440, *Escherichia coli* ATCC 25922 (Axenov-Gribanov *et al*., 2016). Despite the increasing research in this area, more studies need to be performed in order to fully understand the potential of moonmilk microbiomes as possible sources of drug producing strains, being an interesting niche for sample strategy (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Maciejewska *et al*., 2016).

Guano in caves is a source of high nutritional input especially of nitrogen, carbon, and phosphorus, being a selective microbial niche within the cave system (Gabriel and Northup, 2013). In Kotumsar cave (India), from guano/sediment and guano samples, *Streptomyces*

prasinosporus KCA 22 and *Streptomyces longisporoflavus* KCA18 respectively, have been found to inhibit *E. coli* MTCC 1667, *P. auruginosa* JNMC and *S. aureus* MTCC 96 (Rajput, Yogita Biswas and Rai, 2012). In another study, in Magura cave (Bulgaria), *Pseudomonas fragi* (strain B2) and *Sphingobacterium* sp. (strain B7) isolated from guano samples were both found to be bioactive against *P. aeruginosa* while the latter was also able to inhibit *Xanthomonas oryzae* (Tomova *et al.*, 2013).

At last, in most studies performed on caves, sampling sediment and soil is the most common approach and have reveal several groups of bacteria from the genera *Bacillus, Fictibacillus, Nonomuraea, Microbacterium, Paenibacillus, Pseudomonas, Saccharothrix, Spirillospora, Staphylococcus* and *Streptomyces* to possess antimicrobial ability against a wide list of Gram-positive and Gram-negative bacteria (Jamil *et al.*, 2017; Lee *et al.*, 2000; N. Nakaew *et al.*, 2012; Nakaew *et al.*, 2009a, 2009b; Stankovic *et al.*, 2012; Tomova *et al.*, 2013). Nevertheless, it is important to note that if prospecting for more endemic cave bacteria, selection of the samples in low anthropogenic areas of the cave is highly advised.

2.6. Isolation of cave bacteria: Challenges and methods

As discussed previously, the groups of *Actinobacteria*, *Firmicutes* and *Proteobacteria* have demonstrated a huge potential of cave microbiomes in inhibiting several pathogens that cause life-threatening infectious diseases (Belyagoubi *et al.*, 2018; Ghosh *et al.*, 2017b; Kay *et al.*, 2013; N. Nakaew *et al.*, 2012; Nakaew *et al.*, 2009a; Rajput, Yogita Biswas and Rai, 2012; Yücel and Yamaç, 2010; Zada *et al.*, 2016). Nevertheless, among them, *Actinobacteria* have been far more explored in cave ecosystems (Ghosh *et al.*, 2017a). After the selection of the samples to be screened, the second step encompasses the isolation of cave bacterial strains. When concerning the prospection of culturable diversity in caves, several challenges are noted such as the low nutritional habitat that contrasts to the usual media culture (which is highly nutritional), and the lack of specific requirements such as signalling molecules, cooperative relationships between other species, among others (Barton, 2015; Cheeptham, 2013; Cheeptham and Saiz-Jimenez, 2015). Therefore, some approaches have been developed in order to circumvent some of these challenges such as the employment of diluted media formulations, the use of pre-treatments and the application

of antibiotics to inhibit bacterial and fungal species that are undesired (Adam *et al*., 2018; Ghosh *et al*., 2017a; Nakaew *et al*., 2009a; Nareeluk Nakaew *et al*., 2012; Yasir, 2017).

2.6.1. Challenges in the isolation of cave bacterial strains

One of the main challenges when accessing an oligotrophic habitat such as caves, is culturing microorganisms adapted to nutrient deprived environments. The challenge arises when using traditional nutrient rich media formulations to acquire microorganisms whose pathways are adapted to low nutrient availability usually leading to their death by osmotic pressure (Barton, 2015). Moreover, after the first round of isolation from cave environmental samples, significant losses during purification step are usually observed since microbial communities in oligotrophic environments require a certain degree of nutrient exchange between individuals of the microbiome as a cooperative strategy that is in fact absent when purifying the bacterial strain (Maciejewska *et al*., 2016). Thus, strains obtained by purification steps are directly linked to the ability to feed on nutrients present in the synthetic media formulation (Adam *et al*., 2018). Moreover, it is known that only ~1% of the microbial diversity in any sample is possible to be isolated using culture-dependent approaches (Cheeptham, 2013). Therefore, low bacterial abundance from certain strains in cave samples are usually outcompeted by more abundant communities and/or more adapted strains to the employed synthetic media formulation (Barton, 2015). Some cave bacterial strains may also shift from viable cells to a non-cultivable state due to several stresses (Cheeptham, 2013; Ghosh *et al*., 2017a).

In caves, several abiotic conditions such as pH level and the presence and type of organic carbon play an important role in shaping microbial community structure from these systems (Cheeptham and Saiz-Jimenez, 2015; Ghosh *et al*., 2017a; Tomczyk-Żak and Zielenkiewicz, 2016). Moreover, variable temperatures are found between caves and even the chemistry of the cave itself is absent in the culture media formulation chemistry, which influences the isolation of cave bacterial strains (Barton, 2015, 2006; Cheeptham and Saiz-Jimenez, 2015; Ghosh *et al*., 2017b)

Studies in caves that relied in the use of cultivation methods with no target objective, usually obtain fast-growing species belonging, on the overall, to *Proteobacteria*, *Actinobacteria*, and *Firmicutes* (Barton, 2015). These species can shift from nutrient

deprived systems to rich-nutrient systems which is generally lethal to oligotrophic species. The ability to separate organisms well adapted to laboratory culture conditions (generally the fast-growing species) from the rest, would allow to tackle the unculturable diversity (Barton, 2015). For instance, members of the *Planctomycetes, Chloroflexi, Acidobacteria, Nitrospirae, Gemmatimonadetes*, or *Verrucomicrobia* are still uncultured in cave systems even though phylogenetic studies reveal a great diversity of these phyla among different caves (Barton, 2015). Although the vast majority of microbial species remain unculturable and the unavailability to find proper growth conditions is highly observed, the exploration of these novel species regarding the discovery of antimicrobial compounds may be one of the next frontiers to find novel biochemistry, as well as metabolic active compounds (Barton, 2015; Cheeptham, 2013; Ghosh *et al*., 2017a).

2.6.2. Isolation of the main cave bacteria groups with antimicrobial activity

Herein we focus exclusively in the media, pre-treatments and supplements used in the acquisition of cave strains that revealed antimicrobial capacities. Based on the literature, isolation techniques that have proven efficient often rely on target media or in a vast array of media as culture option (Adam *et al*., 2018; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a, 2009b; Yasir, 2017). Moreover, several studies have applied pre-treatments in the samples, such as dry-heat, in order to potentially promote the isolation of hard to culture bacteria (Nakaew *et al*., 2017, 2009b, 2009a). The employment of supplements such as antibiotics is widely observed among the bibliography, since these compounds inhibit the growth of undesired bacteria and fungi species (Fang *et al*., 2017; Nakaew *et al*., 2009b; Rajput, Yogita Biswas and Rai, 2012; Stankovic *et al*., 2012). Overall, nystatin, cycloheximide, phosphomycin, rifampicine and nalidixic acid have been applied as supplements in different studies. Nonetheless, the first two (nystatin and cycloheximide) were the selected options in most of the performed studies (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Belyagoubi *et al*., 2018; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a, 2009b; Ningthouja *et al*., 2009; Voytsekhovskaya *et al*., 2018; Yücel and Yamaç, 2010). Other supplements such as soil extract collected from the environment were also observed in the isolation of some cave bacterial strains with

antimicrobial potential from Smasse-Rawo Ghaar cave and Koat Maqbari Ghaar cave (Pakistan) (Yasir, 2017).

Incubation periods and temperatures are variable between studies and, overall, the range of temperature selected in the different studies ranged from 4 to 35ºC, while in the case of the incubation period applied it varied from 3, 7, 14, 30 days and 2 months (**Table 1**) (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Belyagoubi *et al*., 2018; Derewacz *et al*., 2014; Ghosh *et al*., 2017b; Herold *et al*., 2005; Jiang *et al*., 2015; Kay *et al*., 2013; Klusaite *et al*., 2016; Lamprinou *et al*., 2015; Lee *et al*., 2000; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a, 2009b; Ningthouja *et al*., 2009; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Stankovic *et al*., 2012; Tomova *et al*., 2013; Voytsekhovskaya *et al*., 2018; Yasir, 2017; Yücel and Yamaç, 2010; Zada *et al*., 2016). Nevertheless, each study is unique, for instance in the acquisition of *Streptomyces* with bioactivity from moonmilk deposits on Springtails' Cave (Belgium), 17 ºC was the incubation temperature applied while in moonmilk deposits from Oreshnaya cave (Serbia) the selected temperature for the prospecting of cave actinobacteria with antibacterial activity was 28 ºC (**Table 1**) (Axenov-Gribanov *et al*., 2016; Maciejewska *et al*., 2016). In both studies the incubation period was of 1 month while the temperatures chosen were different (Axenov-Gribanov *et al*., 2016; Maciejewska *et al*., 2016). Nevertheless, longer periods of time are usually applied to allow slow growing bacteria to grow in the culture conditions (Cheeptham and Saiz-Jimenez, 2015; Ghosh *et al*., 2017a).

As briefly discussed above, one of the major focuses when prospecting cave environments is the screening for *Actinobacteria,* especially *Streptomyces* strains. In caves, several media have been successfully applied in the acquisition of *Streptomyces* members with reported activities, namely: International Streptomyces Project (ISP) medium number 6 (ISP6) and ISP7 media, starch nitrate media, B-4 agar, minimal media with 1% chitin, peptone yeast extract, brain-heart-infusion media, solid nutrient media, agarised DNPM, starch casein agar, bacteriological agar, Hickey-Tresner agar, Starch agar, Sodium Caseinate agar and R2A agar media (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Belyagoubi *et al*., 2018; Derewacz *et al*., 2014; Ghosh *et al*., 2017b; Herold *et al*., 2005; Jiang *et al*., 2015; Kay *et al*., 2013; Maciejewska *et al*., 2016; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Stankovic *et al*., 2012; Voytsekhovskaya *et al*., 2018; Yücel and Yamaç, 2010). As for other members of the *Actinobacteria* phylum, the following media were used:

nutrient broth, starch nitrate, ISP5, humic acid-vitamin agar, humic acid-vitamin gellan gum media, starch casein agar, tap water agar and oligotrophic media (M5) (Adam *et al*., 2018; Maciejewska *et al*., 2015; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a). Regarding *Proteobacteria* and *Firmicutes*, cave representatives' media such as TSA, Nutrient broth, diluted nutrient broth, R2A agar media, have been employed in the isolation of cave strains with antibacterial activity (Tomova *et al*., 2013; Yasir, 2017; Zada *et al*., 2016).

Despite the vast array of media used, it is highly advised to use more oligotrophic media formulations or dilution of the nutritional source, in order to mimic the low nutrient environment observed in caves, especially in the deep zone. Nevertheless, each study has its own set of parameters and the different media are allied with a diverse range of settings such as incubation period, temperature, employment of pre-treatments and supplements which will also contribute for the isolation of the cave bacterial strain (Ghosh *et al*., 2017a).

At last, pre-treatments are one of the employed strategies when prospecting cave environments and it has been applied with focus on *Actinomycetes* group including rare or hard to culture *Actinobacteria* members (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Ghosh *et al*., 2017b; Maciejewska *et al*., 2016; Rajput, Yogita Biswas and Rai, 2012; Stankovic *et al*., 2012). For instance, Nakaew and co-workers (2009a, 2009b and 2012) have used dry-heat at 120 ºC for 1 hour, and further exposure to phenol (inoculated in several media), and were able to isolate strains from the genera *Nonomuraea, Micromonospora, Spirillospora* and *Actinocorallia* with a range of activities against *B. cereus* TISTR 687, methicillin-resistant *Staphylococcus aureus* and *Peanibacillus larvae* LMG 9820^{T} (Nakaew *et al*., 2012; Nakaew *et al*., 2009a, 2009b). Using wet-treatment by applying a water bath for 16 hours at 45 ºC, Rajput and colleagues (2012) were able to isolate several *Streptomyces* strains with acitivity against *E. coli* MTCC 1667, *P. aeruginosa* JNMC and *S. aureus* MTCC 96 (Rajput, Yogita Biswas and Rai, 2012). By applying 5 minutes at 50 ºC treatment in water samples from the underground lake of Badzheyskaya cave, Voytsekhovskaya and co-workers (2018) were able to isolate several streptomyces with activity agaisnt *Bacillus subtilis* ATCC 66337, *Staphylococcus carnosus* ATCC 51365, *Pseudomonas putida* KT 2440 and *Escherichia coli* ATCC 25922 (Voytsekhovskaya *et al*., 2018). More recently, through dehydration and centrifugation method, Adam and colleagues (2018) have successfully isolated several strains belonging to the genera *Agromyces, Amycolatopsis, Kocuria, Micrococcus, Micromonospora, Nocardia,*

Rhodococcus and *Streptomyces* with different bioactivities against *M. luteus* ATCC 9341, *C. freundii* ATCC 43864, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 (Adam *et al*., 2018). It is important to note that in all cases where the pre-treatments were employed, the media used were supplemented with antibiotics such as cycloheximide.

Although several variables can be modified when prospecting cave habitats for drug producing strains, the application of low temperatures, longer incubation periods, wide array of culture media including oligotrophic formulations, supplements (antibiotics, e.g. nystatin and cycloheximide) and pre-treatments (dry-heat, phenol, among others) have been successful in the culture of cave strains with antibacterial activity against a wide range of Gram-positive and Gram-negative pathogens, therefore the employment of these settings in prospecting cave environmental samples is advisable (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Belyagoubi *et al*., 2018; Derewacz *et al*., 2014; Ghosh *et al*., 2017b; Herold *et al*., 2005; Jiang *et al*., 2015; Kay *et al*., 2013; Klusaite *et al*., 2016; Lamprinou *et al*., 2015; Lee *et al*., 2000; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a, 2009b; Ningthouja *et al*., 2009; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Stankovic *et al*., 2012; Tomova *et al*., 2013; Voytsekhovskaya *et al*., 2018; Yasir, 2017; Yücel and Yamaç, 2010; Zada *et al*., 2016).

2.7. Antimicrobial screening in cave systems: Past, present and future methodologies

In the search for antimicrobial metabolites it is important to understand the methodologies performed in cave environments. Since few studies have explored the antimicrobial activity of bacterial isolates retrieved from cave systems, the following sections will cover in detail the bioassays conducted in the different geographic caves with focus on the antibacterial activity of cave bacterial strains. Other approaches that could be of beneficial use when exploring drug producing strains from cave will also be briefly summarized. After the acquisition of the cave bacterial pure isolates, the next step relies on the selection of the method to evaluate *in vitro* antibacterial activity. In general, through the researched literature, the most used bioassays were disk diffusion method, agar plug diffusion method, agar well diffusion method and cross streak method (Ghosh *et al*., 2017b; Herold *et al*., 2005; Jiang *et al*., 2015; Klusaite *et al*., 2016; Maciejewska *et al*., 2016; N.

Nakaew *et al*., 2012; Nakaew *et al*., 2009b, 2009a; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Tomova *et al*., 2013; Yasir, 2017; Yücel and Yamaç, 2010; Zada *et al*., 2016). Each assay has its own features presenting both advantages and disadvantages, however, all of them are widely employed in the screening of *in vitro* antimicrobial activity from a wide diversity of environmental samples.

2.7.1. Bioassays employed for *in vitro* evaluation of antimicrobial activity from cave bacteria

2.7.1.1. Disk diffusion method

Agar disk diffusion assay is a traditional method widely performed for antimicrobial susceptibility testing in several clinical microbiology laboratories (Balouiri *et al*., 2016) being approved for bacteria and yeasts since it is a standardized method and has been published by the Clinical and Laboratory Standards Institute (CLSI) (Balouiri *et al*., 2016). The standard procedure protocol works as follows (**Figure 5**): (1) agar plates (usually Mueller Hinton agar, MHA) are inoculated with a standardized inoculum of the test microorganisms (usually a density equivalent to 0.5 McFarland) and evenly spread across the agar plate to ultimately achieve a lawn of the test organism; (2) sterile filter paper discs (about 6 mm in diameter) containing the desired compound or mixture to be tested for the activity, at chosen concentrations, are placed in the agar surface and incubated under suitable conditions (dependent on the lawn strain usually, 24h to 48h). (3) After the incubation period inhibition growth zones are measured (Balouiri *et al*., 2016).

This method offers advantages such as simplicity, low cost and ability to test a wide number of microbial strains as well as antimicrobial agents with easy to interpret results. Nevertheless, the disk diffusion assay cannot reliably determine the minimum inhibitory concentration (MIC), since quantification of the amount of antimicrobial agent that has diffused into the media cannot be done (Balouiri *et al*., 2016).

In caves, disk diffusion assay is the most common approach and has been applied in the screening of cave bacterial strain from several caves, among them including, Chaabe Cave (Algeria), Springtails' Cave (Belgium), Francthi' Cave (Greece), PhaTup Cave Forest Park, (Thailand), Badzheyskaya cave (Russia), Khangkhui cave (India) and a cave in China (**Table**

1) (Belyagoubi *et al*., 2018; Ghosh *et al*., 2017b; Jiang *et al*., 2015; Lamprinou *et al*., 2015; Maciejewska *et al*., 2016; N. Nakaew *et al*., 2012; Ningthouja *et al*., 2009; Voytsekhovskaya *et al*., 2018). Prior to the application of the disk diffusion assay, bacterial strains are cultured in broth media to promote the production of metabolites (including antibacterial metabolites) allowing the screening of several broth formulations in the modulation of metabolite production, for instance, Soumya Ghosh and co-workers screened two unknow *Actinomycetes* strains (ICC1 and ICC4) using disk diffusion method and found that strain ICC1 only had activity when grown on **R2A** while ICC4 only exhibit activity when grown in V8 and HT media (Ghosh *et al*., 2017b). In Chaabe Cave (Algeria), MHB (Mueller-Hinton broth) was used as fermentation media for the screening of antimicrobial metabolites from cave strains. In this study several *Streptomyces* exhibited inhibition against *E. coli, S. aureus M. luteus, B. subtilis* and *L. monocytogenes* (**Table 1**) (Belyagoubi *et al*., 2018). Using more complex and elaborated formulation, Jiang and co-workers (2015) found a novel pyranonaphthoquinone antibiotic xiakemycin A, produced by the *Streptomyces* sp. CC8-201, bioactive against several type strains of *S. aureus*, *S. epidermidis*, *E. faecalis* and *E. faecium* (Jiang *et al*., 2015).

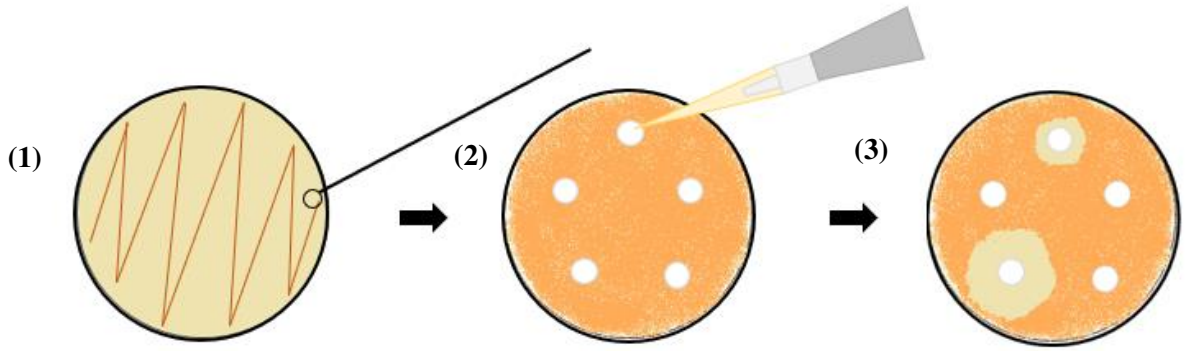


Figure 5. Visual representation of the disk diffusion assay**.**

2.7.1.2.Cross streak method

The principal advantage to employ cross streak method is the quick screening of microorganisms for antagonism abilities (**Figure 6**) (Balouiri *et al*., 2016). The standard procedure protocol works as follows: (1) the microbial strain (strain used to assess antimicrobial production) is seeded by a single streak in the centre of the agar plate; (2) After

the incubation period (dependent to the strain of interest) the plate is further seeded with the microorganisms to be tested (e.g. pathogenic bacteria) by performing a single streak perpendicular to the central streak; (3) after further incubation usually 24h to 48h, antimicrobial interaction is observed by analysing the inhibition size (usually in mm) (**Figure 6**) (Balouiri *et al*., 2016).

In oligotrophic bacterial strains, growth periods are usually longer, therefore, methods such as cross streak that allow variation in the incubation conditions (e.g. temperature) may be a reliable first approach when assessing this type of strains from cave habitats (Cheeptham and Saiz-Jimenez, 2015; Ghosh *et al*., 2017a; Maciejewska *et al*., 2016). Moreover, variation in the media formulations used may enhance the production of higher quantities of the inhibitory compound/mixture that are linked to specific biosynthetic pathways from the screened bacteria (Adam *et al*., 2018; Maciejewska *et al*., 2016). In caves, two studies relying on cross streak method to evaluate antimicrobial activity of bacterial isolates have been performed in moonmilk samples from Springtails' Cave, Belgium. According to the study carried out by Marta Maciejewska (2016), *Streptomyces* species isolated from moonmilk deposits have shown a vast array of biosynthetic gene clusters with antimicrobial potential (Maciejewska *et al*., 2016). However, it is hard to activate the expression of these clusters since specific cues and triggers must be present. One way is the use of different media and supplements to modulate their activation (Maciejewska *et al*., 2016). Therefore, in their study, five media for bacterial screening were employed including: MHA, TSA, ISP7, starch nitrate (SN) media, and minimal media supplemented with 25 mM *N*-acetyl glucosamine (MM plus GlcNAc) as well as two media for antifungal activity screening: MHA and MM + GlcNAC. GlcNAC is a known elicitor of antimicrobial metabolites under poor culture conditions (Maciejewska *et al*., 2016). From the screened *Streptomyces*, 94% were found to be active against *B. subtilis*, 87% to *M. luteus*, and 36% against *S. aureus*. In the case of Gram-negative 65% were found to show activity against *K. pneumoniae*, while 39% were active against both *E. coli,* 39% and *C. freundii* and 16% against *P. aeruginosa* (Maciejewska *et al*., 2016). In another study performed by Daphine Adam and colleagues (2018), several *Actinobacteria* members were found to inhibit *M. luteus* ATCC 9341, *C. freundii* ATCC 43864, *B. subtilis* ATCC 19659, *K. pneumoniae* ATCC 13883, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 while using different ISP2, S-ISP5, ISP7, and TSA agar to elicit antibacterial production (Adam *et al*., 2018). In

the different studies performed, cross streak method has revealed to be a reliable method for the preliminary screening of antimicrobial metabolites produced by cave bacterial strains, allowing a versatility of option settings by changing the media, the supplements, the temperature and incubation periods while only performing one assay (Adam *et al*., 2018; Maciejewska *et al*., 2016). This approach allows a broader screening of the proper culture conditions to elicit the production of cryptic antibiotic pathways in cave bacterial organisms (Adam *et al*., 2018; Maciejewska *et al*., 2016). Nevertheless, some disadvantages are also associated with the cross streak method being the most important the lack of evaluation of MIC and MLC (minimal inhibitory concentration and minimal lethal concentration, respectively) due to the uncertainty of the real quantities of compound produced by the screened strain (Balouiri *et al*., 2016).

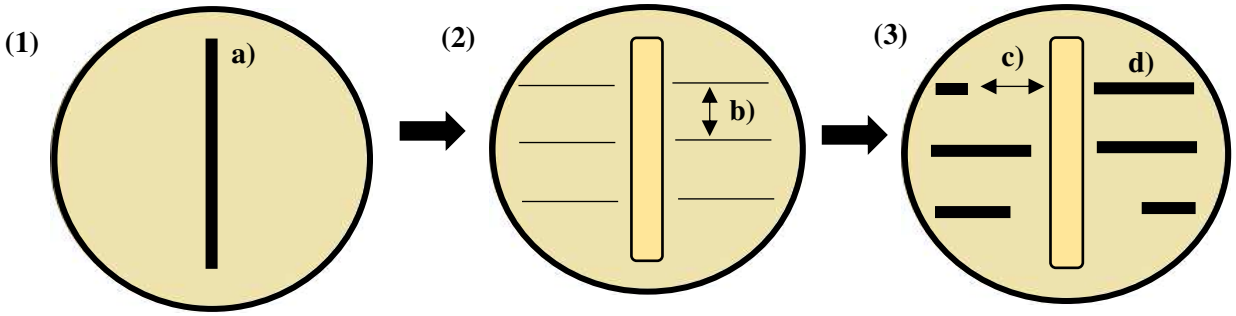


Figure 6. Visual representation of cross streak technique. **a)** cave bacterial strain; **b)** Indicator strain to be inhibit (e.g. pathogenic bacteria); **c)** positive inhibition and **d)** negative inhibition.

2.7.1.3. Other techniques

Several other techniques have also been applied in the screening of antimicrobial activity from cave bacterial strains such as agar well diffusion method and agar plug diffusion method (Klusaite *et al*., 2016; N. Nakaew *et al*., 2012; Nakaew *et al*., 2009a, 2009b; Rule and Cheeptham, 2013; Tomova *et al*., 2013). In short, agar well diffusion method works as follow: (1) The agar plate surface is inoculated by spreading a known volume of the test microbial strain over the entire surface; (2) After the inoculation a hole (with 6 to 8mm diameter) is punched aseptically and a volume of 20 to 100 mL of the antimicrobial agent or extract at known concentrations is poured in to the well; (3) Plates are

further incubated at appropriated conditions for the test strain and the antimicrobial agent diffuses into the media inhibiting the test strains (Balouiri *et al*., 2016). This method has been employed to screen several cave bacteria from Phanangkhoi cave, PhaTup Cave (Thailand), Magura Cave (Bulgaria), Cueva de los Murciélagos (cave in Spain) Krubera-Voronja Cave (Gerogia) and Kashmir Smast (cave in Pakistan) (**Table 1**) (Klusaite *et al*., 2016; Nakaew *et al*., 2009b, 2009a; Rule and Cheeptham, 2013; Tomova *et al*., 2013; Zada *et al*., 2016). By employing this method several cave bacterial strains from the phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria* have successfully found to inhibit the following pathogens: *Bacillus cereus* ATCC 10876, *B. megaterium, B. thuringiensis, B. subtilis* ATCC 6633, *Corynebacterium glutamicum* CECT 78, *Enterococcus faecalis* 29212, *Escherichia coli, Listeria monocytogenes* 7644, *Micrococcus luteus* ATCC 10240, *Pseudomonas aeruginosa* NBIMCC 1390, *Paenibacillus larvae, Rhodococcus equi* ATCC 6939, *Staphylococcus aureus* ATCC 6538 and *S. aureus* subsp. *aureus* ATCC 25923 (**Table 1**) (Klusaite *et al*., 2016; Nakaew *et al*., 2009b, 2009a; Rule and Cheeptham, 2013; Tomova *et al*., 2013; Zada *et al*., 2016).

In brief, agar plug diffusion method work out as follow: (1) the strain of interested its incubated in the appropriated media until cells secrete molecules which end up diffusing in the agar media; (2) After an appropriated incubation period an agar-plot or cylinder is cut aseptically and deposited over the surface of a previously inoculated agar plate with the test organisms; (3) antimicrobial activity from the molecules that diffused from the plug to the agar media is detected by the presence of inhibition zones around the plug (Balouiri *et al*., 2016). With this method, Rule and Cheeptham (2013) screened several *Streptomyces* strains with antagonist properties against *Acinetobacter baumannii*, *Mycobacterium smegmatis*, ESBL *Escherichia coli, Micrococcus luteus*, MRSA *Staphylococcus aureus* (Rule and Cheeptham, 2013).

In caves, disk diffusion method has been widely used followed by agar well diffusion assay, allowing fermentation conditions using broth media which are also used for large scale quantity production prior to the isolation of the pure compounds. Nevertheless, several other options are available such as the cross streak method, which allows a wider variability of culture settings in a single assay and fermentation conditions using agar media formulations, or agar plug diffusion method, which has been used in fewer instances (Balouiri *et al*., 2016). Other techniques such as TLC-bioautography, direct bioautography,

agar overlay bioassay and dilution methods including broth dilution (micro and macro broth dilution assay) and agar dilution method are also applied in the *in vitro* evaluation of antimicrobial activity from bacterial strains (Balouiri *et al*., 2016). In caves microdilution technique has been employed to screen the bioactivity of *Toxopsis calypsus* a cyanobacteria found in Francthi' Cave (**Table 1**), while thin-layer chromatography (TLC)-bioautograph has been employed by Aiste Klusaite and colleagues (2016) in two cave bacterial strains namely a *Bacillaceae* bacterium (strain 1350R2-TSA30-6) and *Bacillus* sp. (strain 1410WF1-TSA30-2), both revealing activity against *Micrococcus luteus* (**Table 1**) (Klusaite *et al*., 2016; Lamprinou *et al*., 2015) Nevertheless, macro and microdilution techniques are recommended since, in contrast to other techniques, they allow the determination of MIC values (the lowest concentration tested of the screened agent that causes a full inhibition of the microbial strain tested) (Balouiri *et al*., 2016). Moreover, approved standards by the CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have also been performed and the application of these methodologies on the screening for bioactive metabolites from cave bacterial strains is highly incentivized (Balouiri *et al*., 2016).

2.7.2. Potential for future work in the screening of antimicrobial metabolites in cave systems

2.7.2.1. Genome mining cave bacteria: search for PKS/NRPS biosynthetic gene clusters

Microorganisms contain significantly more biosynthetic pathways for the production of metabolites, than those found to be involved in producing the isolated compounds when screening for *in vitro* activity (Chooi *et al*., 2011). However, these genes usually remain undiscovered since one either screens strains only for a specific activity or, new genes/pathways are unable to be activated under the conditions used, becoming dormant/sleeping genes (Chooi *et al*., 2011; Maciejewska *et al*., 2016; Ziemert *et al*., 2016). Therefore, many unknown valuable metabolites might get unnoticed when working with culturing microorganisms under standardized laboratory conditions. In fact, over 90% of the genes or gene clusters that are accountable for secondary metabolite biosynthesis are

overlooked (Ghosh *et al*., 2017a; Hill *et al*., 2017; Hodges *et al*., 2012). One way to tackle this issue it to turn to genomics in order to identify the cryptic (or orphan) biosynthetic gene clusters. For instance, many of the bioactive secondary metabolites produced by actinomycetes (and other organisms) belong to the classes of polyketide synthases (PKS) that are divided into types I, II, and III or nonribosomal peptide synthetases (NRPS) (Adam *et al*., 2018; Maciejewska *et al*., 2016). Although in caves we are not yet close to predicting secondary metabolite clusters using genomic data alone, some studies have found isolates which are able to produce type II polyketides with remarkable activities e.g. antibacterial activity and anticancer activity (Ghosh *et al*., 2017a; Hill *et al*., 2017). An isolate from the *Streptomyces* genus found in Groto de Cervi cave in Southern Italy was able to produce four type II polyketides named Cervimycins A–D (Herold *et al*., 2005). These compounds were able to inhibit multidrug resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus faecalis* (Herold *et al*., 2005). In Hardin's Cave, in Tennessee, another *Actinomycete* belonging to the genus *Nonomuraea* was found to produce type II polyketides (Derewacz *et al*., 2014). *Nonomuraea specus* was isolated from a piece of decomposed bark inside the cave and was able to produce hypogeamicin A, the dimeric product revealed to be toxic to TCT-1 colon cancer cell line (Derewacz *et al*., 2014). Another three non-dimeric precursors were also isolated and revealed low toxicity to *Bacillus subtilis* (Derewacz *et al*., 2014). Hodges *et al*. (2012) were also able to effectively apply target primers to PKS and NRPS gene clusters in bacterial isolates from Mystery Cave and Norman's Cave (Bahamas). These clusters were found in *Streptomyces* sp. strains G140 and G141, *Nocardiopsis* sp. strain G142, *Solwaraspora* sp. strains G146 and G165, as well as *Micromonospora* sp. strain G153 (Hodges *et al*., 2012). By using reference strains for PKS gene products it was possible to classify several obtained PKS sequences and translate into functional chemical classes. For instance, PKS and NRPS gene sequences were predicted to represent angucycline (an anti-tumour antibiotic), the antibiotics naphthoquinone and b-lactam, as well as cyclohexadepsipeptide and siderophore (Hodges *et al*., 2012).

At last, Maciejewska and colleagues (2016), carried out a large scale genome mining investigation in isolates from cave moonmilk deposits (Springtails' Cave located in Comblainau Pont in Belgium) highlighting that 100% of the evaluated strains encoded at least two NRPS genes, while 97 %, 94% and 48 % were found to own PKS type I, II and III, respectively (Maciejewska *et al*., 2016). In addition, no significant correlation from

individual antimicrobial activity analyses and biosynthetic genes analysed was possible to be achieved. These findings put in evidence the lack of adequate culture conditions that enable the expression of biosynthetic gene clusters even though more than 5 culture media were used for the analyses of individual antimicrobial activity (Maciejewska *et al*., 2016).

The biosynthetic potential of microbes is far from being explored and cave systems microorganisms are almost unexplored. Genome mining has become an important part of drug discovery, being a reliable approach to unveil different aspects such as ecological function and evolution of these compounds. Additionally, whole genome sequences of cave bacterial strains are starting to be available such as the ones of *Beutenbergia cavernae* from Guangxi, China, *Pseudomonas fluorescens* from the Guiana Shield, South America, *Gloeobacter kilaueensis* from a biofilm in Kılauea Caldera, Hawaii (Barton *et al*., 2013; Hki *et al*., 2009; Saw *et al*., 2013). The genomes of *Devosia* sp., *Sphingopyxis* sp., *Bosea* sp., *Massilia* sp. and *Sphingopyxis* sp. from Lechuguilla Cave, *Streptomyces lunaelactis from* Grotte des Collemboles, Belgium and eight *Ensifer* sp. from Wind Cave, South Dakota, and Lechuguilla Cave, New Mexico are also available (Gan *et al*., 2014; Kumar *et al*., 2017; Naômé *et al*., 2018). The study of cave bacterial genomes will allow for a more in-depth knowledge of these cave bacterial species and with the evolution and accuracy of the computational tools available, researchers from other fields might be able to mine cave bacterial genomes, not only for secondary metabolites but also to understand the ecological role of these genus in cave systems, allowing the development of this research field related in caves (Blin *et al*., 2017; Ghosh *et al*., 2017a; Weber and Kim, 2016; Ziemert *et al*., 2016).

2.7.2.2.The OSMAC (one strain many compounds) approach

When exploring strains from unknown environments, a possible approach could be the employment of the OSMAC approach (One strain many Compounds). As it is known, the production of secondary metabolites is often constrained to the ability of the organisms to produce them under laboratory settings (Romano *et al*., 2018). The amount of gene clusters identified that are linked to the production of secondary metabolites is inconsistent with the observable products by any screened microorganism. To unlock this unreachable chemical diversity, key factors are needed to trigger the expression of the designated “silent” clusters (Romano *et al*., 2018). The OSMAC framework consists on the screen of a single strain for

many metabolites by modulating several parameters such as nutrient content, temperature, rate of aeration, pH level, light quality and quantity while other extents of the approach could rely in co-cultivation strategies, as well as the supplement of chemical elicitors that serve as cues to activate silent gene clusters (Rateb *et al*., 2011; Romano *et al*., 2018). This approach has been effectively applied in several microorganisms revealing in vast extent the capability of a single strain to produce several bioactive metabolites (Bode *et al*., 2002; Bode and Müller, 2005; Hewage *et al*., 2014; Liu *et al*., 2017; Rateb *et al*., 2011; Romano *et al*., 2018). For instance, Rateb *et al*. (2011) applied the OSMAC approach using *Streptomyce* sp. C34 isolated from desert soils and was able to elicit the production of four new ansamycin-type compounds when cultivated on two different media (Rateb *et al*., 2011). Helge Bjorn Bode and colleagues (2002) were able to isolate more than 100 compounds belonging to more than 25 different structural classes from only 6 different microorganisms when applying the OSMAC approach (Bode *et al*., 2002). In cave systems studies, it has already been indicated the presence of several biosynthetic gene clusters from cave *Streptomyces* strains that are still cryptic despite the application of several media cultures (Maciejewska *et al*., 2016). The OSMAC approach brings countless advantages and its employment in cave bacterial strains may unlock the cryptic biochemistry housed in these organisms.

2.8. Conclusions

Caves comprise a reservoir of unknown bacterial strains as well as potential for unknown secondary metabolite production. In fact, with the evident lack of studies regarding this system, only few researches have attempted to fully characterize the microbial communities from cave environments regarding the potential for drug producing strains. The acquisition of culturable diversity in caves may rely in the use of broad media formulation (with special interest on oligotrophic ones) and pre-treatments in order to cope with fast-growing microbes, allowing access to more oligotrophic species as well as hard to culture ones. Moreover, classical antimicrobial activity screen approaches are dependent on cultivation conditions highlighting the need for a broader screen of pathogenic agents as well as culture options to understand the biosynthetic potential of cave bacterial species. Beyond the preliminary screening, few researches attempted to fully indicate the responsible agent for the observed bioactivity. It is imperative to further screen the drug producing strain taking

into account the variability of settings that influence the metabolite production and its final identification and characterization of the chemical structure of the metabolite. Furthermore, molecular methods may provide new insights on the screening of bioactive molecules such as the search for biosynthetic gene cluster (e.g. PKS and NRPS). Nevertheless, it is important to find the pre-requisites that unlock the metabolite production of the cryptic biosynthetic pathways and one way could be the application of the OSMAC approach. The exploration of cave microbial niches is still in its early stages and the application of multifactorial approaches are of the most importance to effectively screen cave habitats for drug producer strains unveiling the potential of cave microbiomes.

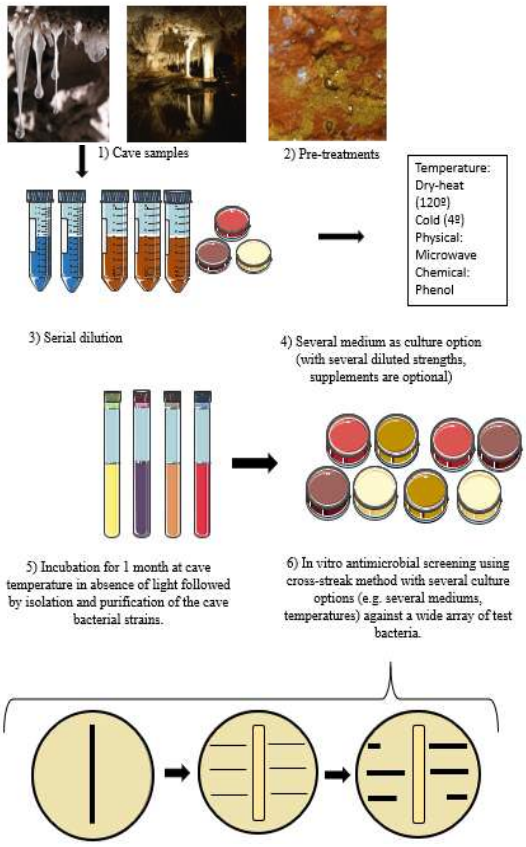


Figure 7. Proposed workflow for *in vitro* evaluation of antimicrobial activity from cave bacteria.

Herein, we suggest a possible standardized work flow for the preliminary screening of drug producer strains in cave habitats encompassing bacterial strains acquisition and *in vitro* evaluation of antimicrobial activity. In short, the following work flow is designed as follows (**Figure 7**): (1) Several types of samples (soil, moonmilk, guano, among others) prioritizing

low anthropogenic input sites, the application of several media cultures is advised if the applied technique is swabbing the walls. (2) After sample collection, it is recommended the application of several pre-treatments (e.g. dry-heat, wet-heat, among others) depending on the target approach; (3) Serial dilutions are imperative when tackling samples from oligotrophic environments; (4) After dilution of the samples, the employment of several media including more oligotrophic formulations is highly advised, as well as the application of supplements such as cycloheximide; (5) Incubation periods with higher lengths are recommended (e.g. 1 month) in absence of light at cave temperature. (6) *In vitro* screening of antimicrobial activity using one of the following four methods described above as, e.g. cross streak method with several media (at least 3) and several incubation periods (including longer ones e.g. 7 to 14 days) against a wide variety of test organisms (e.g. Gram-positive and Gram-negative). Overall, each employed setting can be adapted for the target approach to be conducted, therefore the described work flow is a general idea on how to prospect cave microbiomes based on the literature. We hope with this schematic approach to facilitate the study of cave microbiomes aiming at finding new ways to cope with the recent antibiotic crises.

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<u>Chapter 3:</u> Antimicrobial activity of the culturable bacteria from Cerâmica Cave, Portugal

Chapter 3. Antimicrobial activity of the culturable bacteria from Cerâmica Cave, Portugal

3.1. Abstract

The treatment failure associated with multidrug resistant bacteria infections has become alarming and nowadays the search for solutions to cope with this crisis is prevalent in multiple bioresearch fields. Natural sources have provided a wide range of complex and structurally diverse metabolites and several niches are yet to be explored. In order to find microorganisms able to provide new bioactive metabolites, the prospection of unknown reservoirs such as extreme environments are highly advised. Cave habitats are considered extreme environments for microbial populations and encompass a novel and unexplored reservoir of microbial populations with potential bioactive metabolites. Herein, we report both the bacteria sampling from a Portuguese karstic cave, as well as, the application, for the first time, of several pre-treatments and media in the acquisition of several microbial isolates belonging to the phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria*. Following their isolation, the antimicrobial activity of the screened bacteria revealed that 37.5% of the isolates exhibited antagonist activity against at least one of the following pathogens: *Aeromonas salmonicida* ATCC 33658 *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. Portugal karstic complex comprises more than 1000 caves and, as far as we known, no studies regarding the potential for antimicrobial activity of their microbiome have been reported until today. This study highlights the efficiency in the application of several culture options on the acquisition of numerous groups of bacteria and their potential to produce antimicrobial metabolites. Furthermore, this is the first report on the microbial community from Portugal karstic caves as well as the first insight on their antimicrobial activity.

3.2. Introduction

Recently, cave microbiomes have become a hot spot for the search of novel microbial diversity as well as the potential to find novel drug metabolites (Belyagoubi *et al*., 2018; Cheeptham *et al*., 2013; Ghosh *et al*., 2017a, 2017b). Since caves are an extreme and oligotrophic environment, microbial diversity housed in these ecosystems was forced to develop several adaptations that differ from the ones found in surface organisms (Cheeptham and Saiz-Jimenez, 2015; Gabriel and Northup, 2013). Although caves may be extreme

environments for microorganisms survival, a vast array of bacterial diversity is found in these systems with several representatives belonging to the groups of *Acidobacteria*, *Actinobacteria*, *Cyanobacteria*, *Firmircutes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Bacteroidetes* (Barton, 2015; Tomczyk-Żak and Zielenkiewicz, 2016). Nevertheless, when focusing in cultivation procedures to obtain these organisms, several challenges are imposed such as incubation temperature, nutritional media and the need for cooperative growth (Barton, 2015; Ghosh *et al*., 2017a). In general, when employing cultivation methods with rich culture media, fast-growing strains dominate the agar plates. Recent studies in caves are now attempting to isolate more oligotrophic strains from these habitats by using oligotrophic medias such as R2A or diluted rich culture media (Barton, 2015, 2006; Ghosh *et al*., 2017a; Ortiz *et al*., 2015). The employment of several pre-treatments such as dry-heat and phenol treatment has proven to be effective in the isolation of strains with antimicrobial activities (Nakaew *et al*., 2009a, 2009b; 2012). At last, the use of several supplements such as cycloheximide and nystanin also proved to influence positively the isolation of novel bacterial strains from cave habitats and some strains with bioactive compounds (Fang *et al*., 2017; Nakaew *et al*., 2009b; Rajput, Yogita Biswas and Rai, 2012; Stankovic *et al*., 2012).

Studies regarding antimicrobial potential from cave bacterial strains have shown remarkable results. Overall, inhibition of Gram-positive and Gram-negative pathogens has been reported including the following species: *Bacillus cereus*, *B. subtilis*, *Enterococcus faecalis*, *E. faecium*, *Listeria monocytogenes*, *Mycobacterium phlei*, *Paenibacillus larvae*, *Staphylococcus aureus*, *S. epidermidis*, *Acinetobacter baumanii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Xanthomonas oryzae* (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Derewacz *et al*., 2014; Ghosh *et al*., 2017b; Herold *et al*., 2005; Jiang *et al*., 2015; Kay *et al*., 2013; Klusaite *et al*., 2016; Lamprinou *et al*., 2015; Maciejewska *et al*., 2016; Nakaew *et al*., 2009a, 2009b; 2012; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Stankovic *et al*., 2012; Tomova *et al*., 2013; Varela *et al*., 2009; Zada *et al*., 2016). Nonetheless, few studies regarding antimicrobial potential from cave microbiome have been performed, having however, revealed the potential of caves to harbour several unknown or barely studied bacteria. The study of drug producing strains from cave habitats may in fact be one of the next frontiers of exploration regarding new therapeutic agents against multidrug resistant pathogens (Ghosh *et al*., 2017a).

Considering the lack of knowledge regarding the diversity of cultivable bacteria in Portuguese Karsic caves, as well as the need to discover new molecules with antimicrobial activity, our study aims at providing new information on these questions. Herein, we report the cultivation-based bacterial diversity of Cerâmica Cave Portugal, as well as the antimicrobial activity of a set of isolates, being, as far as we are aware, the first study regarding this topic in karstic caves of mainland Portugal. In order to carry out the bacterial diversity study, a set of different culture media and pre-treatments were used and the isolates were identified through 16S rRNA gene profiling techniques. Regarding the antimicrobial activity, selected bacterial isolates were screened against pathogenic bacteria using a modified disk diffusion method.

3.3. Material and methods

3.3.1. Investigated cave

Portugal is located in southwestern of Europe and comprises two archipelagos (Madeira and Azores). In mainland Portugal there are several relevant karst massifs, such as the massifs of Estremenho (Serra d'Aire and Candeeiros), Arrábida, Sicó-Condeixa-Alvaiázere, Montejunto and Algarve (Reboleira *et al*. 2011). Sicó massif is located south of Coimbra and encompasses approximately three hundred caves, with different dimensions and speleological characteristics, being the most diverse and complex karst system in Portugal (Cunha & Dimuccio, 2014; Cunha, 2005; Cunha, 2003).

Fieldwork was conducted in Cerâmica Cave (Sicó massif), mainland Portugal. The cave is on the overall 120 meters in length, and 6 meters wide (**Figure 8**). Inside the cave, gallery walls are found mostly wet and roots can be observed growing in the cealing (**Figure 8**). Since this cave is not considered touristic, human impact is low. Sampling strategy included active search of sites with the lowest anthropogenic input and difficult access.

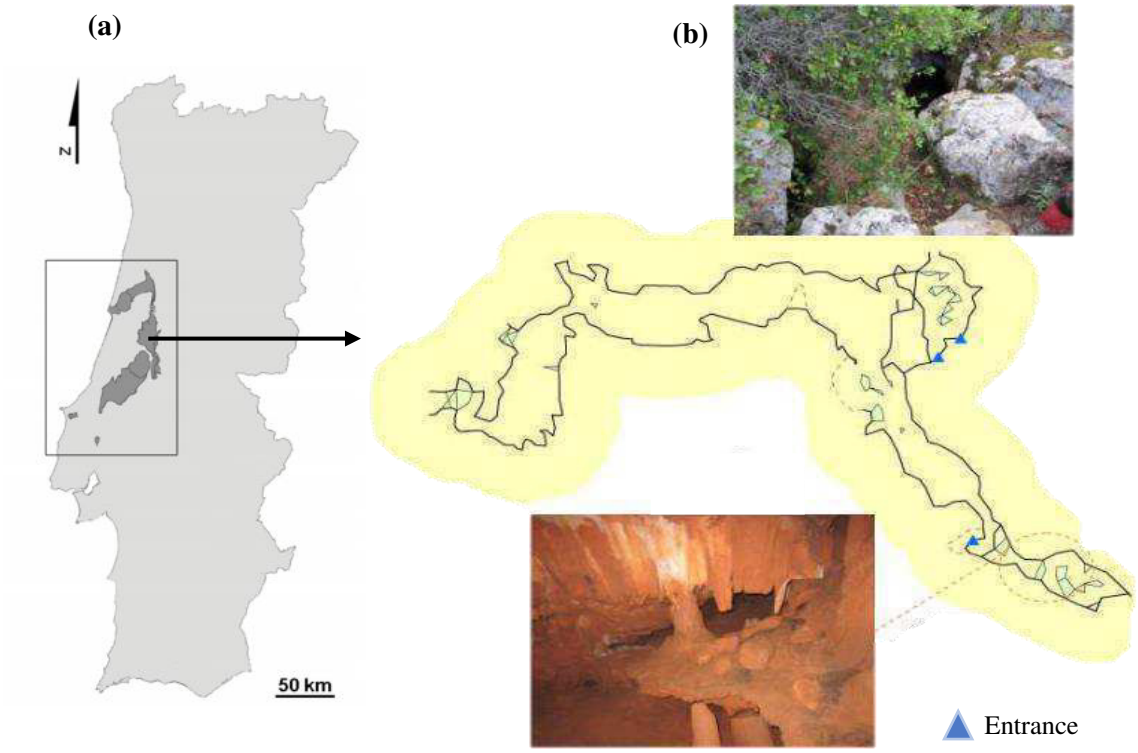

Figure 8. Location of Cerâmica Cave (Portugal) in Leiria (39°55'36.57''N, 8°31'03.63''W). **a)** Portugal map with dark grey representing some of the main karst units in Portugal (Rocha *et al*., 2016); **b)** Cerâmica Cave representation and pictures (Santos, 2012).

3.3.2. Sample collection and isolation of Cerâmica Cave bacteria

Isolation of cave bacterial strains used in this study, was previously performed from soil samples obtained from Cerâmica Cave, Portugal. Briefly, regarding sample collection, a total of 6 cave sediment samples were collected aseptically at 10 cm depth by scooping the soil sediment into sterile bags and air-dried for 30 days. The isolation of the strains was carried out by processing each sample using different chemical and physical pre-treatments regimes: no treatment (**NT**), dry heat (**DH**), dry heat plus phenol (1,5%) (**DHP**), phenol (1,5%) (**P**), wet heat (**WH**), microwave (**MW**) and rehydration centrifugation (**RC**) (**Figure 9**). After each regime, dilution 10^{-2}, 10^{-3} and 10^{-4} of each sample were further inoculated onto 3 different media: actinomycetes isolation agar (**AIA**) (Himedia, Mumbai, India), R2A agar (**R2A**) (Oxoid, Hampshire, United Kingdom) and yeast-malt agar (**YM**) (Himedia, Mumbai, India) and incubated at room temperature for 30 days (**Figure 9**). Visual selection of colonies

was performed based on structural morphology, colour, shape and further purified using the same agar media where growth occur. Stock isolates were maintained using 30% glycerol solution with Triptic Soy Broth (**TSB**) (Liofilchem, Roseto degli Abruzzi, Italy) and were kept at -20°C.

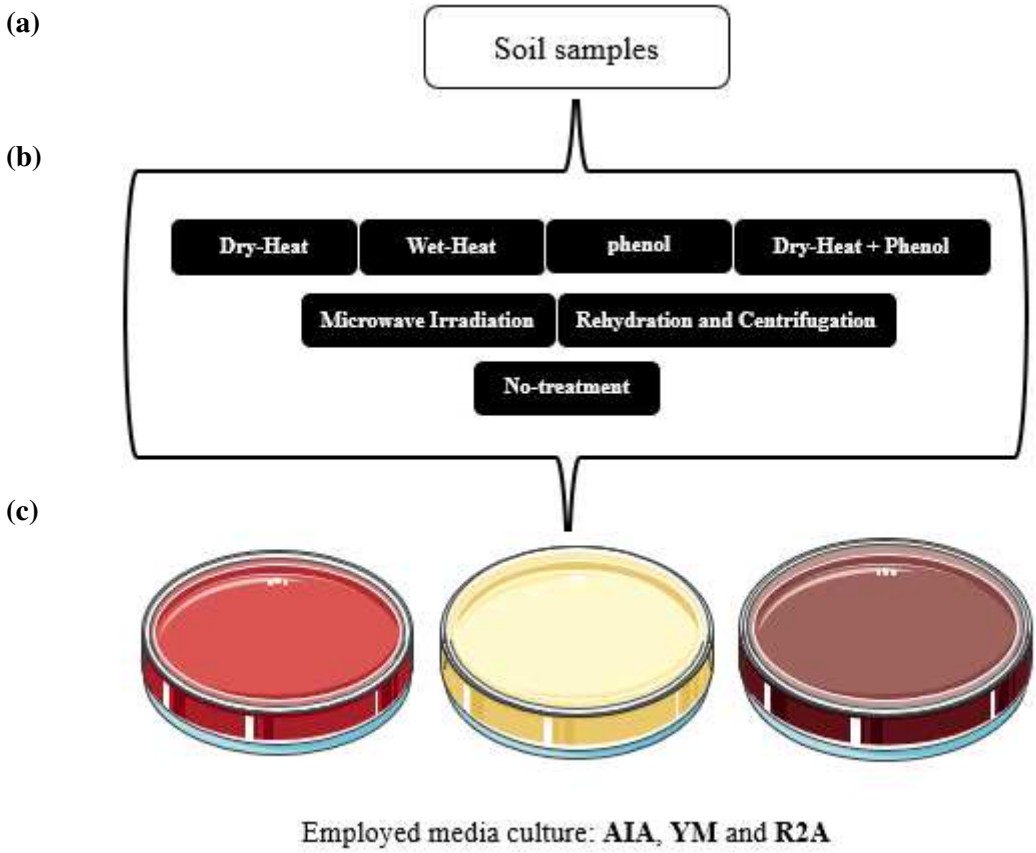

Figure 9. Visual work flow of the employed screening for the isolation of Cerâmica Cave bacterial strains. **(a)** Soil sampling; **(b)** Pre-treatments (regimes); **(c)** Agar media (**AIA:** actinomycetes isolation agar, **YM:** yeast-malt agar and **R2A:** R2A).

3.3.3. Culture conditions of cave bacteria isolates for phylogenetic analysis

The preparation of the obtained isolates (**Table 2**) for 16S rRNA sequencing involved the culture in the respective appropriated media, **R2A**, **AIA** and **YM** (**Table 2**), for 3 days at room temperature. Colonies of each bacterial isolate were collected with a sterilized toothpick and suspended in 1 mL of **TSB** in a 1.5 mL centrifuge tube and incubated overnight for further use in DNA extraction procedure.

3.3.4. DNA extraction, sequence alignment and phylogenetic analysis of cave isolates

The extraction of DNA was performed on 55 cave bacterial pure cultures (previously incubated, as described above) by using GF-1 Bacterial DNA Extraction Kit (Frilabo, Maia, Portugal) according to the instructions of the manufacturer. The 16S rRNA gene was amplified using universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGG(CT)TACCTTGTTACGACTT-3'). the reactions for PCR amplification were performed in 25 µL reactions containing 0.5 µL each primer (27F and 1492R), 12.5 µL of PCR master mix (Thermo Fisher Scientific, Waltham, USA), 9.5 µL of ultra-pure water and 2 µl of cell lysate as template DNA in a final reaction volume of 25 µL. PCR was conducted with a MJ thermal cycler (Bio Rad iCycler iQ™5 Real-Time PCR Detection System) as follows: a first cycle of 3 min at 95°C (initial denaturation), followed by 30 cycles of 95ºC (denaturation), followed by 30 s annealing at 56°C and 1 min at 72°C (extension), ending with final extension step of 10 min at 72°C.

The PCR products were further analysed by electrophoresis on an agarose gel (1%) containing green safe (nzytech, Lisboa, Portugal) to confirm the amplification of the 16S rRNA of the bacterial isolates. The sequencing of the PCR products was made by STAB VIDA through sanger sequencing. The DNA sequences obtained were analysed using the BLAST (Basic Local Alignment Search Tool) algorithm with the available sequences in the GenBank at National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences were identified with the >98% identity and >98% coverage to the closest relative in the GenBank. GenBank accession numbers of the closest related are given in **Table 3**.

3.3.5. Culture conditions of cave bacteria strains for antimicrobial activity screening

Seed cultures from each cave isolate that grows within 24 hours was cultured in their adequate culture medium (media of origin, **Table 2**) for 1 day at room temperature in absence of light. Pure colonies were picked into 25 mL falcon tubes with 5 mL of saline solution (NaCl 0.9 %) and diluted until a final suspension equivalent to an inoculum density of 0.5 McFarland was reached, in order to further use in antimicrobial susceptibility assays.

The following bacterial strains were used as test microorganism in antagonistic assays: *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Klesbiella pneumoniae* ATCC 13883, *Enterococcus faecalies* ATCC 29212, *Aeromonas salmonicida* ATCC 33658 and *Bacillus cereus* ATCC 14579. Each tested human pathogenic strain was inoculated in tryptone soya agar (**TSA**) (Himedia, Mumbai, India) plates and incubated at 37º for 24 hours. Pure colonies were collected onto 25 mL falcon tubes with 5 mL of saline solution (NaCl 0.9 %) and diluted until an absorbance of equivalent to an inoculum density of 0.5 McFarland was reached. The final suspensions were used as seed cultures of test bacteria for antimicrobial activity assays.

Table 2. Broth and agar media constituents used in this study.

TSB	MHA	TSA	AIA	R2A	YMA
Final pH (at 25°C) 7.3±0.2	Final pH (25ºC) 7.3 ± 0.1	Final pH (25ºC) 7.3 ± 0.2	Final pH (25ºC) 8.1 ± 0.2	Final pH (25ºC) 7.2 ± 0.2	Final pH (25ºC) 6.2 ± 0.2
Pancreatic digest of casein 17.0g	Beef, infusion from 300.0g	Pancreatic digest of casein 15.0g	Sodium caseinate 2.00g	Yeast extract 0.5g	Peptone 5.0g
Papaic digest of soyabean meal 3.0g	Casein and Hydrolisate 17.5g	Papaic digest of soyabean meal 5.0g	L-Asparagine 0.10g	Proteose peptone 0.5g	Yeast extract 3.0g
Sodium chloride 5.0g	Starch 1.5g	Sodium chloride 5.0g	Sodium propionate 4.00g	Casein hydrolysate 0.5g	Malt extract 3.0g
Dibasic potassium phosphate 2.5g	Agar 17.0g	Agar 15.0g	Dipotassium phosphate 0.50g	Glucose 0.5g	Dextrose 10.0g
Dextrose 2.5g			Magnesium sulfate 0.10g	Starch 0.5g	Agar 20.0g

	Ferrous sulphate 0.001g	Di-potassium phosphate 0.3g
	Agar 15.0g	Magnesium sulphate 0.024g
		Sodyum pyrante 0.3g
		Agar 15.0g

Note: Media abbreviations: **TSB** (Tryptic Soy Broth), **TSA** (Tryptone Soya Agar), **AIA** (Actinomycetes Isolation Agar), **YMA** (Yeast Malt Agar), **MHA** (Mueller-Hinton Agar), **R2A** (R2A agar).

3.3.6. Screening for antibacterial activity from cave bacteria

From the identified isolates, the ones that grow in under 24h (**Table 3**) were selected to be screened for antimicrobial production. This was achieved thought modified disk diffusion method against Gram-positive and Gram-negative human pathogens. Pure colonies of both pathogenic and cave bacteria were prepared, as previously described, and employed for the screening of the antimicrobial activity. For this, seed cultures of the human pathogenic bacteria were inoculated in Mueller-Hinton agar (**MHA**) (Oxoid, Hampshire, United Kingdom) plates and left at room temperature for 30 minutes. These plates were thereafter inoculated with 20 µL of the cave bacterial suspension in NaCl 0.9 % (0.5 McFarland) and incubated at room temperature for 48 hours in the absence of light to mimic the cave environment condition. The antimicrobial activity was recorded as positive by the presence of a halo surrounding the bacterial growth, or negative on its absence. The bioassays were performed in triplicate (with exception of *E. coli* and *E. faecalis* screening which were only performed one time) and saline solution was used as negative control (Rocha *et al*., 2016).

3.4. Results and discussion

3.4.1. Phylogenetic affiliation to the closest relatives in the GenBank

Cave bacteria were isolated from soil samples recovered from Cerâmica Cave, Portugal, using three media (**R2A**, **YMA** and **AIA**) with prior application of seven pre-treatments (**Figure 9**). After selection of distinct colonies, 55 isolates were further identified by using 16S rRNA gene sequence analysis. BLAST search using either full or nearly full-length 16S rRNA gene sequences revealed that the closest hits belonged to members of the phyla

Proteobacteria, *Actinobacteria* and *Firmicutes*. Taxonomic classification yielded matches with similarity, ranging from 98.00 to 100 %.

The identified isolates belonging to *Proteobacteria* phylum included the classes alpha- and gamma-*Proteobacteria* which included the genera *Cupriavidus* (isolate CC166), *Ensifer* (isolates, CC15, CC145, CC205, CC376), *Methylobacterium* (isolate C100) and *Pseudomonas* (isolates CC2, CC101, CC181, CC292, CC293, CC340 and CC397) (**Table 3**). Cultivable members of these genera have been previously reported to be present in cave microbiomes (Barton, 2015; Yasir, 2017).

Regarding the *Actinobacteria* phylum, identified isolates were represented by the genera *Agromyces* (CC116), *Arthrobacter* (CC64 and CC109), *Microbacterium* (CC294), *Rhodococcus* (CC87, CC148 and CC170), and *Streptomyces* (CC70, CC74, CC104, CC129, CC138, CC153, CC167, CC195, CC320 and CC391) (**Table 3**). *Actinobacteria* is a widely diversified phylum found in cave systems and several cave microbiomes have been investigated regarding the potential to harbour actinomycetes that are able to produce bioactive molecules against multi drug resistant pathogens, and have found several strains with promising activities (Adam *et al*., 2018; Belyagoubi *et al*., 2018; Derewacz *et al*., 2014; Jiang *et al*., 2015; Nakaew *et al*., 2012; Stankovic *et al*., 2012; Tomczyk-Żak and Zielenkiewicz, 2016). For instance, cervimycin A–D a polyketide glycoside complex produced by *Streptomyces tendae* that was isolated in Grotta dei Cervi (cave), in Italy, was found to have strong activity against multidrug resistant *Staphylococcus aureus* and, most remarkably, against vancomycin-resistant *Enterococcus faecalis* strains (Herold *et al*., 2005).

At last, the isolates belonging to the *Firmicutes* phylum included the genera *Bacillus* (CC45, CC46, CC50, CC69, CC75, CC80, CC91, CC108, CC119, CC126, CC127, CC134, CC196, CC254, CC277, CC282, CC318 and CC362), *Paenibacillus* (CC304 and CC316) and *Sporosarcina* (CC20, CC76 and CC172) (**Table 3**). The genus *Bacillus* has been found in several caves when employing culture dependent approaches (Barton, 2015; Yasir, 2017). Yasir (2017) has found several *Bacillus* strains isolated from Koat Maqbari Ghaar cave and Smasse-Rawo Ghaar cave with antimicrobial activity against *Salmonella typhi* and *Staphylococcus aureus* (Yasir, 2017). *Paenibacillus* genus is widely diversified and novel strains have been found in several distinct type of samples even from cave s(Dong Lee, 2016; Osman *et al*., 2006; Sáez-Nieto *et al*., 2017). For instance, in Lechuguilla Cave, authors

found an interesting strain of this genus denominated as *Paenibacillus* sp. LC231, that exhibited resistance to 23 of 40 tested commercially available antibiotics. Within mechanisms of resistance *Paenibacillus* sp. LC231, was found to have 3 new mechanisms that were unknown to be involved in resistance until date (Pawlowski *et al*., 2016). Moreover, a recent study was able to isolate a novel strain of this genus, *Paenibacillus cavernae* sp. nov., isolated from the soil of a natural cave in Jeju, Republic of Korea (Dong Lee, 2016).

The search for culturable diversity from extreme and unexplored environments still constitutes one of the major reservoirs of unknow biochemistry and biosynthetic pathways from natural environments (Bérdy, 2005; Ghosh *et al*., 2017a; Wright, 2017). Despite the application of several pre-treatments and culture media, the pool of genera belonged to widely found phyla (*Proteobacteria, Actinobacteria* and *Firmicutes*) in cave environments. This is still expected since several members of these phyla are known to be able to sustain both oligotrophic and highly nutritional media as it is observed in conventional agar formulations (Barton, 2015). Nevertheless, several of the genera are known for remarkably bioactivities and/or resistance. For instance, *Cupriavidus* genus is known for several of its members presenting tolerance to high concentrations of metals (Cuadrado *et al*., 2010; Hajdu *et al*., 2010; Seccareccia *et al*., 2016) while the *Streptomyces* genus is widely known for their secondary metabolite production with antibiotic activity (Bérdy, 2005; Chater, 2006; Lima Procópio *et al*., 2012). It is important to note that common soil organisms found in extreme environments had to adapt to that habitat, therefore strains found in these ecosystems may possess unknown capacities such as the wide resistance observed by *Paenibacillus* isolated from a pristine and remote cave (Ghosh *et al*., 2017a; Pawlowski *et al*., 2016).

3.4.2. Culture conditions and pre-treatments

In total, 55 strains belonging to Gram-positive and Gram-negative bacteria were identified and isolated from Cerâmica Cave (Figure 1). When performing assays from cave samples, a selection of an adequate media is advised to successfully enhance the conditions for oligotrophic organisms to grow, or if a specific target organism is desired (such as screening for actinomycetes) (Barton, 2015; Ghosh *et al*., 2017a). After the identification of

the bacteria through 16S rRNA the affiliation with blast closest related, the highest array of species identified (17 species) belonged to **R2A** with genera including: *Ensifer*, *Bacillus*, *Arthrobacter*, *Streptomyces*, *Sporosarcina*, *Methylobacterium*, *Pseudomonas*, *Agromyces*, *Rhodococcus* and *Peaenibacillus*. Followed by **AIA**, with 16 bacterial species identified within 6 pre-treatments (with exception of phenol), belonging to the genera *Bacillus, Pseudomonas, Microbacterium, Arthrobacter* and *Cupriavidus*. In the **YM** case, when employing the following pre-treatments: **NT**, **MW** and **P,** only 7 bacterial species were identified, belonging the genus *Bacillus, Nocardia* and *Streptomyces*.

Several studies in cave environments have employed R2A agar in order to isolate more oligotrophic microorganisms, since this media was formulated to marine oligotrophic environments (Ghosh *et al*., 2017a; Snider *et al*., 2009). **AIA** media was formulated for the isolation and proliferation of actinomycetes, therefore, it is expected the isolation of rare-actinomycetes and *Streptomyces* members when using this media (Jayashree *et al*., 1991; Pathalam *et al*., 2017). In the present study, despite the application of several treatments and culture media options, the identified isolates belonged only to *Proteobacteria*, *Actinobacteria*, and *Firmicutes*, which have been widely cultured in several caves (Barton, 2015; Tomczyk-Żak and Zielenkiewicz, 2016). Usually species from these phyla are able to shift from nutrient deprived system to rich-nutrient systems which is generally lethal to oligotrophic species (Barton, 2015). **Table 3** lists the closest hits for members of our collection identified by sequence homology from BLAST searches, and their corresponding treatment and acquisition media. In respect to the species diversity within the genus, *Bacillus* (*B. simplex*, *B. mycoides*, *B. niacin* and *B. pocheonensis*) and *Streptomyces* (*S.spororaveus*, *S. exfoliates*, *S. vinaceus* and *S. avidinii*), were the most diverse genera, with four species each, followed by *Pseudomonas* (*P. alkylphenolica, P. jessenii* and *P. poae*) genus with three different species and *Paenibacillus* (*P. pabuli* and *P. macquariensis*) with two species. Besides these, only one bacterial species was recovered from the genera *Cupriavidus* (*C. necator)*, *Microbacterium* (*M. oxydans*), *Nocardia* (*N. globerula*), *Ensifer* (*E. adhaerens*), *Arthrobacter* (*A. methylotrophus*) and *Methylobacterium* (*M. goesingense*). Remarkably, 5 of the identified isolates (CC304, CC254, CC116, CC109 and CC20) exhibited a 16S rRNA gene sequence similarity of 98% from the closest related homologue strains, representing possibly novel strains or species since 16S rRNA based phylogeny is known to be insufficient for discriminating between closely related species, underestimating the true

identity (Abbott and Janda, 2007; Hong and Farrance, 2015; Rajendhran and Gunasekaran, 2011).

This study reveals the potential of unexplored caves regarding novel microbial culturable populations, highlighting the exploration of these habitats as a prolific reservoir of unknow microbiota. Regarding the employed pre-treatments, 25 of the identified isolates were recovered from microwave (**MW**) treatments, belonging to the genera *Arthrobacter*, *Bacillus, Brevibacterium, Ensifer, Paenibacillus, Pseudomonas*, *Sporosarcina* and *Streptomyces*; followed by 13 isolates in the no treatment (**NT**) regime, belonging to the genera *Bacillus, Cupriavidus, Ensifer, Nocardia, Pseudomonas*, *Rhodococcus* and *Streptomyces*. From wet-heat (**WH**) treatment, 6 isolates were identified, affiliated to the genera *Agromyces, Bacillus*, *Paenibacillus* and *Sporosarcina*; while using phenol (**P**) treatment, 5 isolates from the genera *Bacillus*, *Methylobacterium*, *Pseudomonas* and *Streptomyces* were recovered. Besides these treatments, from rehydration and centrifugation (**RC**) regime, four isolates were identified and affiliated to the genera *Arthrobacter*, *Ensifer*, *Microbacterium* and *Pseudomonas* while using dry-heat (**DH**) treatment only two isolates from the *Bacillus* genus were acquired. As for dry-heat plus phenol (**DHP**) treatment, one isolate belonging to the genus *Sporosarcina* was identified.

Throughout the literature, several pre-treatments have been applied in cave systems in order to prospect cave microbiomes. For instance, microwave (**MW**) treatment has been employed to enhance actinomycetes isolation by Niyomvong and co-workers (2012), which demonstrated to be a suitable treatment to isolate rare actinomycetes from limestone cave soil using HV agar supplemented with nalidixic acid and ketoconazole as culture media (Niyomvong *et al*., 2012). Recently, Adam and colleagues (2018) have applied rehydration and centrifugation (**RC**) in order to isolate actinobacteria species with bioactivates from cave samples and were able to identify several *Streptomyces* species (Adam *et al*., 2018). In respect to the other regimes, dry-heat (**DH**) and dry-heat plus phenol (**DHP**) have proven to enhance the isolation of novel species, since many bacterial groups are unable to withstand such high temperatures. In caves, Nakaew and coworkers (2009) have used dry-heat at 120 ºC for 1 hour and further exposure with phenol and were able to isolate strains from the genera *Nonomuraea, Micromonospora, Spirillospora* and *Actinocorallia* with bioactivity against Gram-positive and Gram-negative bacteria (Nakaew *et al*., 2009b, 2009a). Although in this study the employment of antibiotics was avoided to understand if the media itself

enabled the isolation of novel strains, the use of antibiotics, such as cycloheximide, is highly recommendable (Fang *et al*., 2017; Nakaew *et al*., 2009b; Rajput, Yogita Biswas & Rai, 2012; Stankovic *et al*., 2012). For instance, cycloheximide inhibits a wide diversity of fungal species which ultimately constrain the development of slow growing microorganisms (Belyagoubi *et al*., 2018). Nevertheless, in our study the application of selective media allied with pre-treatments have proven to be an effective, although time consuming, strategy for the isolation of several groups of bacteria including strains with antimicrobial capacities from cave systems.

Table 3. Identification of the isolates and closest hits according to BLAST search and their corresponding treatment and acquisition media.

Source/site	Isolates Nº	Pre-treatment	Media	Nucleotide nº	Query Score (%)	Ident (%)	Closest related strains, Name, Sequence ID
Gallery IV	CC2	MW	AIA	1367	100	99	*Pseudomonas alkylphenolica* KL28| NR_145644.1; *Pseudomonas donghuensis* HYS| NR_136501.2; *Pseudomonas putida* NBRC 14164| NR_113651.1.
Gallery IV	CC15	RC	R2A	1346	100	99	*Ensifer adhaerens* NBRC 100388| NR_113893.1; *Ensifer adhaerens* LMG 20216| NR_042482.1; *Ensifer sesbaniae* CCBAU 65729| NR_133053.1.
Gallery IV	CC20	DP	AIA	1401	100	98	*Sporosarcina psychrophila* strain NBRC 15381| NR_113752.1; *Sporosarcina globispora* strain NBRC 16082| NR_113837.1; *Sporosarcina globispora* strain 785| NR_029233.1.
Gallery IV	CC45	MW	R2A	1377	100	100	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821 NR_024697.1.
Gallery IV	CC46	MW	R2A	1407	100	99	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821 NR_024697.1.
Gallery IV	CC50	DH	AIA	1402	100	99	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821 NR_024697.1.
Gallery III	CC64	RC	R2A	1344	100	99	*Arthrobacter methylotrophus* TGA| NR_025083.1; *Arthrobacter bambusae* THG-GM18| NR_133968.1; *Arthrobacter gyeryongensis* DCY72| NR_133699.1.
Gallery III	CC69	MW	YM	1423	100	99	*Bacillus paramycoides* MCCC 1A04098| NR_157734.1 *Bacillus tropicus* MCCC 1A01406 NR_157736.1| *Bacillus nitratireducens* MCCC 1A00732 | NR_157732.1.
Gallery III	CC70	MW	YM	1341	100	99	*Streptomyces spororaveus* NBRC 15456| NR_112469.1; *Streptomyces nojiriensis* NBRC 13794| NR_112414.1; *Streptomyces avidinii* NBRC 13429| NR_041132.1.
Gallery III	CC74	MW	R2A	1383	100	99	*Streptomyces spororaveus* NBRC 15456| NR_112469.1; *Streptomyces nojiriensis* NBRC 13794| NR_112414.1; *Streptomyces avidinii* NBRC 13429| NR_041132.1.
Gallery III	CC75	MW	R2A	1408	100	99	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821 NR_024697.1.

Gallery III	CC76	MW	R2A	1396	100	99	*Sporosarcina globispora* strain NBRC 16082| NR_113837.1; *Sporosarcina psychrophila* strain NBRC 15381| NR_113775.2; *Sporosarcina globispora* strain 785| NR_029233.1.
Gallery III	CC80	MW	R2A	1400	100	100	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821| NR_024697.1.
Gallery III	CC87	NT	YM	1374	100	99	*Nocardia globerula* DSM 44596| NR_104795.1*; Rhodococcus globerulus* DSM 43954| NR_026184.1; *Rhodococcus globerulus* DSM 43954| NR_119617.1.
Gallery III	CC91	NT	R2A	1371	100	100	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821| NR_024697.1.
Gallery III	CC100	P	R2A	1330	100	99	*Methylobacterium goesingense* iEII3| NR_115219.1; *Methylobacterium gossipiicola* Gh-105| NR_108243.1; *Methylobacterium adhaesivum* AR27| NR_125482.1.
Gallery III	CC101	P	R2A	1380	100	99	*Pseudomonas jessenii* CIP 105274| NR_024918.1; *Pseudomonas reinekei* MT1| NR_042541.1; *Pseudomonas koreensis* Ps 9-14| NR_025228.1.
Gallery III	CC104	P	R2A	1382	100	99	*Streptomyces avidinii* NBRC 13429| NR_041132.1; *Streptomyces avidinii* NRRL 3077| NR_115452.1; *Streptomyces spororaveus* NBRC 15456| NR_112469.1.
Gallery III	CC108	MW	AIA	1407	100	100	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821 NR_024697.1.
Gallery III	CC109	MW	AIA	1369	100	98	*Arthrobacter methylotrophus* TGA| NR_025083.1; *Arthrobacter bambusae* THG-GM18| NR_133968.1; *Arthrobacter alkaliphilus* LC6| NR_041401.1.
Gallery III	CC111	DH	R2A	1404	100	99	*Bacillus simplex* LMG 11160| NR_114919.1; *Bacillus simplex* NBRC 15720| NR_112726.1; *Bacillus simplex* DSM 1321| NR_042136.1.
Gallery III	CC116	WH	R2A	1381	100	98	*Agromyces subbeticus* strain Z33| NR_043149.1; *Agromyces iriomotensis* strain IY07-20| NR_133690.1; *Agromyces ramosus* strain DSM 43045| NR_026165.1.
Gallery III	CC119	WH	R2A	1403	100	99	*Bacillus simplex* LMG 11160| NR_114919.1; *Bacillus simplex* NBRC 15720| NR_112726.1; *Bacillus simplex* DSM 1321| NR_042136.1.
Gallery III	CC126	MW	R2A	1381	100	99	*Bacillus niacini* NBRC 15566| NR_113777.1; *Bacillus niacini* IFO15566| NR_024695.1; *Bacillus drentensis* NBRC 102427| NR_114085.
Gallery III	CC127	MW	R2A	1398	100	100	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821 NR_024697.1.
Gallery III	CC129	MW	R2A	1341	100	100	*Streptomyces exfoliatus* NBRC 13191| NR_041117.1; *Streptomyces zaomyceticus* NRRL B-2038| NR_044144.1*; Streptomyces zaomyceticus* NBRC 13348| NR_112376.1.

Gallery III	CC134	MW	R2A	1402	100	99	*Bacillus simplex* LMG 11160| NR_114919.1; *Bacillus simplex* NBRC 15720| NR_112726.1; *Bacillus simplex* DSM 1321| NR_042136.1.
Gallery III	CC138	NT	R2A	1362	100	100	*Streptomyces exfoliatus* NBRC 13191| NR_041117.1; *Streptomyces zaomyceticus* NRRL B-2038| NR_044144.1*; Streptomyces zaomyceticus* NBRC 13348| NR_112376.1.
Gallery III	CC145	NT	R2A	1349	100	99	*Ensifer adhaerens* NBRC 100388| NR_113893.1; *Ensifer adhaerens* LMG 20216| NR_042482.1; *Sinorhizobium fredii* NBRC 14780| NR_133053.1.
Gallery III	CC148	NT	R_2A	1377	100	99	*Rhodococcus maanshanensis* strain M712| NR_025190.1*Rhodococcus maanshanensis* strain DSM 44675| NR_118601.1; *Rhodococcus jostii* strain IFO 16295| NR_118421.1.
Gallery III	CC153	MW	YM	1364	100	99	*Streptomyces gardneri* NBRC 12865| NR_1125751.1; *Streptomyces exfoliatus* NBRC 13191| NR_041117.1; *Streptomyces narbonensis* NBRC 12801| NR_112282.1.
Gallery III	CC166	NT	AIA	1378	100	99	*Cupriavidus necator* N-1|NR_102851.1; *Cupriavidus numazuensis* NBRC 100056| NR_113877.1; *Cupriavidus oxalaticus* NBRC 13593| NR_113619.1.
Gallery III	CC167	NT	AIA	1384	100	99	*Streptomyces spororaveus* NBRC 15456| NR_112469.1; *Streptomyces nojiriensis* NBRC 13794| NR_112414.1; *Streptomyces avidinii* NBRC 13429| NR_041132.1.
Gallery III	CC170	NT	AIA	1381	99	99	*Rhodococcus maanshanensis* strain M712| NR_025190.1; *Rhodococcus maanshanensis* strain DSM 44675| NR_118601.1: *Rhodococcus jostii* strain IFO 16295| NR_118421.1.
Gallery III	CC172	WH	AIA	1409	99	99	*Sporosarcina globispora* strain NBRC 16082| NR_ 113837.1; *Sporosarcina psychrophila* strain NBRC 15381| NR_113775.2; *Sporosarcina globispora* strain 785| NR_029233.1.
Gallery III	CC181	MW	AIA	1393	100|	99	*Pseudomonas poae* P 527/13| NR_028986.1; *Pseudomonas trivialis* 513/19| NR_028987.1; *Pseudomonas marginalis* ICMP 3553| NR_117821.1.
Gallery III	CC195	NT	R2A	1374	100	99	*Streptomyces spororaveus* NBRC 15456| NR_112469.1; *Streptomyces nojiriensis* NBRC 13794| NR_112414.1; *Streptomyces avidinii* NBRC 13429| NR_041132.1.
Gallery III	CC196	MW	R2A	1406	100	99	[*Brevibacterium*] *frigoritolerans* strain DSM 8801| NR_117474.1; [*Brevibacterium*] *frigoritolerans* strain DSM 8801I NR_115064.1| *Bacillus simplex strain* LMG 11160| NR_114919.1.
Gallery III	CC205	MW	R2A	1352	100	99	*Ensifer adhaerens* NBRC 100388| NR_113893.1; *Ensifer adhaerens* LMG 20216| NR_042482.1; *Ensifer sesbaniae* CCBAU 65729| NR_133053.1.
Gallery III	CC254	WH	AIA	1414	100	98	*Bacillus pocheonensis* Gsoil 420| NR_041377.1; *Bacillus soli* NBRC 102451| NR_114095.1; *Bacillus ginsengisoli* DCY53| NR_109068.1.
Gallery IV	CC277	P	YM	1422	100	100	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821| NR_024697.1;

Gallery IV	CC282	NT	YM	1405	100	99	*Bacillus simplex* LMG 11160| NR_114919.1; *Bacillus simplex* NBRC 15720| NR_112726.1; *Bacillus simplex* DSM 1321| NR_042136.1.
Gallery IV	CC292	NT	AIA	1391	100	99	*Pseudomonas poae* P 527/13| NR_028986.1; *Pseudomonas trivialis* 513/19| NR_028987.1; *Pseudomonas marginalis* ICMP 3553| NR_117821.1.
Gallery IV	CC293	MW	AIA	1391	100	99	*Pseudomonas poae* P 527/13| NR_028986.1; *Pseudomonas trivialis* 513/19| NR_028987.1; *Pseudomonas marginalis* ICMP 3553| NR_117821.1.
Gallery IV	CC294	RC	AIA	1392	100	99	*Microbacterium oxydans* DSM 20578| NR_044831.1; *Microbacterium liquefaciens* DSM 20638| NR_026162.1; *Microbacterium maritypicum* DSM 12512| NR_042351.1.
Gallery IV	CC304	MW	R2A	1419	100	98	*Paenibacillus macquariensis* subsp. *defensor* M4-2| NR_041635.1; *Paenibacillus antarcticus* 20CM| NR_027213.1; *Paenibacillus glacialis* KFC91| NR_116453.1.
Gallery IV	CC316	WH	R2A	1355	100	99	*Paenibacillus pabuli* NBRC 13638| NR_113627.1; *Paenibacillus taichungensis* BCRC 17757| NR_044428.1; *Paenibacillus pabuli* HSCC 492| NR_040853.1.
Gallery IV	CC318	WH	R2A	1422	100	100	*Bacillus mycoides* NBRC 101228| NR_113990.1; *Bacillus mycoides* ATCC 6462| NR_115993.1; *Bacillus weihenstephanensis* DSM 11821 NR_024697.1.
Gallery IV	CC320	P	R2A	1390	100	99	*Streptomyces spororaveus* NBRC 15456| NR_112469.1; *Streptomyces nojiriensis* NBRC 13794| NR_112414.1; *Streptomyces avidinii* NBRC 13429| NR_041132.1.
Gallery IV	CC340	RC	AIA	1374	100	99	*Pseudomonas poae* P 527/13| NR_028986.1; *Pseudomonas trivialis* 513/19| NR_028987.1; *Pseudomonas marginalis* ICMP 3553| NR_117821.1.
Gallery IV	CC362	MW	R2A	1379	100	99	*Bacillus idriensis* strain SMC 4352-2| NR_043268.1; *Bacillus cibi* strain JG-30| NR_042974.1; *Bacillus indicus* strain Sd/3| NR_029022.1.
Gallery IV	CC365	NT	R2A	1374	100	99	*Streptomyces vinaceus* NBRC 13425| NR_041131.1; *Streptomyces cirratus* NBRC 13398| NR_112388.1; *Streptomyces cirratus* CSSP547| NR_043356.1.
Gallery IV	CC376	MW	R2A	1384	100	99	*Ensifer adhaerens* NBRC 100388| NR_113893.1; *Ensifer adhaerens* LMG 20216| NR_042482.1; *Ensifer sesbaniae* CCBAU 65729| NR_133053.1.
Gallery IV	CC391	NT	YM	1364	100	99	*Streptomyces spororaveus* NBRC 15456| NR_112469.1; *Streptomyces nojiriensis* NBRC 13794| NR_112414.1; *Streptomyces avidinii* NBRC 13429| NR_041132.1.
Gallery III	CC397	MW	AIA	1382	100	99	*Pseudomonas vancouverensis* strain DhA-51| NR_041953.1; *Pseudomonas reinekei* strain MT1|NR_042541.1I; *Pseudomonas koreens strain* Ps 9-14| NR_025228.1.

3.4.3. Antimicrobial activity from Cerâmica Cave isolates

The potential to produce compounds with antimicrobial activity effective against Gram-positive and Gram-negative bacteria was evaluated for each identified strain that grew within 24 hours on Mueller-Hinton agar via modified disk diffusion assay (**Table 4**). Overall, from the screened cave bacterial strains, 37.5% exhibited antimicrobial activity at least against one of the following pathogens, *A. salmonicida* ATCC 33658, *B. cereus* ATCC 14579, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25923. No screened isolate was able to inhibit the remaining test bacteria (*E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883 and *S. typhimurium* ATCC 14028) (**Table 4**). According to the sequencing results, isolates demonstrating bioactivity were affiliated to the genera of *Bacillus*, *Cupriavidus*, *Paenibacillus* and *Streptomyces*. In general, our study demonstrated a higher antimicrobial activity from isolates belonging to *Firmicutes* rather than actinobacteria groups. Although several representatives from actinobacteria including the genera *Arthrobacter, Microbacterium*, *Sporosarcina* and *Streptomyces* were screened, only two isolates belonging to *Streptomyces* genus were active against the pathogens. From this genus, the isolate CC138 (*Streptomyces exfoliatus*) was found to inhibit *A. salmonicida* ATCC 33658 and *P. aeruginosa* ATCC 27853, while isolate CC365 (*Streptomyces vinaceus*) was only able to inhibit the latter (**Table 4**). Interestingly, this is the first report of inhibition of *A. salmonicida* from cave bacterial strains, revealing the potential of cave microbiomes to be a source of metabolites with bioactive abilities against this pathogen.

Bacterial surveys in caves have shown a great diversity of *Actinobacteria* with antagonistic properties against human pathogens, mainly from the *Streptomyces* genus (Belyagoubi *et al*., 2018; Ghosh *et al*., 2017b; Herold *et al*., 2005; Jiang *et al*., 2015; Kay *et al*., 2013; Klusaite *et al*., 2016; Maciejewska *et al*., 2016; Nakaew *et al*., 2012; Ningthouja *et al*., 2009; Rule and Cheeptham, 2013; Stankovic *et al*., 2012; Tomova *et al*., 2013; Yücel and Yamaç, 2010). As known, members of this taxon are especially prolific in bioactive secondary metabolites with antifungals, antivirals, antitumoral, anti-hypertensives, antimicrobial and immunosuppressives activities (Lima *et al*., 2012). In caves, numerous *Streptomyces* spp. strains have been found to possess antimicrobial capacities against a broad range of human pathogens including several strains of *Acinetobacter baumannii, Micrococcus luteus, Bacillus subtilis, X. oryzae, Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium, Escherichia coli, P. putida, Enterococcus faecalis, E. faecium, S.*

*epidermidi*s (Axenov-Gribanov *et al*., 2016; Herold *et al*., 2005; Jiang *et al*., 2015; Rajput, Yogita Biswas and Rai, 2012; Rule and Cheeptham, 2013; Stankovic *et al*., 2012). Moreover, *Streptomyces tendae* (strain HKI 0179) isolated from Grotta dei Cervi cave system was found to produce Cervimycins A–D polyketide glycosides that exhibited antibiotic capacities against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* SG511, multidrug resistant *S. aureus* 134/93 (MRSA), efflux-resistant *S. aureus* EfS4 and vancomycin-resistant *Enterococcus faecalis* 1528 (Herold *et al*., 2005). Another study has also reported the production of a novel pyranonaphthoquinone antibiotic Xiakemycin A by *Streptomyces* sp. CC8-201 isolated from the soil of a remote karst cave in Chongqing city, China. Xiakemycin A was found to be bioactive against several strains of *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *E. faecium*, including vancomycin resistant strains (Herold *et al*., 2005). Regarding the *Bacillus* genus, in our study, five isolates (CC50, CC69, CC108, CC277 and CC318) have demonstrated antibacterial activity against at least one of the following pathogens: *A. salmonicida, B. cereus, P. aeruginosa* and *S. aureus*. According to the 16S rRNA gene sequence data, four of the isolates were affiliated to *Bacillus mycoides* NBRC 101228 while one isolate (CC69) was affiliated to *Bacillus paramycoides* MCCC 1A04098. Interestingly, a total of 9 isolates that were affiliated to *Bacillus mycoides* were screened for antibacterial activity, from which only 4 were bioactive. Moreover, the four strains exhibited different patterns of antimicrobial activity, for instance, CC108 and CC277 were able to inhibit *A. salmonicida*, *P. aeruginosa* and *S. aureus*, while CC318 presented inhibition against *B. cereus, P. aeruginosa* and *S. aureus*. Isolate CC50 only showed activity against *P. aeruginosa*. Curiously, all the five isolates (including *B. paramycoides)* presented antibacterial activity against *P. aeruginosa*, perhaps, suggesting the presence of a similar biosynthetic gene cluster that is responsible for the observed activity. In caves, different antimicrobial activity patterns between isolates of the same species have also been reported by Rajput and co-workers (2012), where 3 strains (KCA3, KCA8 and KCA22) of *Streptomyces prasinosporus* isolated from different zones of the cave (entrance zone, twilight zone and deep zone, respectively) exhibited dissimilar activity patterns. *Streptomyces prasinosporus* KCA3 and KCA8 were able to inhibit *E. coli* MTCC1667 and *P. aeruginosa* JNMC while *S. prasinosporus* KCA22 was able to inhbit *S. aureus* MTCC96, *E. coli* MTCC1667 and *P. aeruginosa* JNMC (Rajput, Yogita Biswas and Rai, 2012). These findings corroborate with the hypothesis that phylogenetically closely related bacteria can

demonstrate distinct abilities in the production of bioactive molecules. Therefore, when prospecting extreme environments such as caves, strains from common species may indeed exhibit different bioactive patterns (Ghosh *et al*., 2017a; Todorova and Kozhuharova, 2010; Yilmaz *et al*., 2006). In cave antagonist assays have revealed several species from the *Bacillus* genus to have bioactivities against *Bacillus megaterium, Corynebacterium glutamicum, Mycobacterium phlei, Bacillus thuringiensis, Micrococcus luteus, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella typhi* (Klusaite *et al*., 2016; Yasir, 2017; Zada *et al*., 2016).

Furthermore, in this study, isolate CC166 and CC316 (affiliated to *Cupriavidus necator* and *Paenibacillus pabuli*, respectively) exhibited antimicrobial activity against *P. aeruginosa* (**Table 4**). Additionally, isolate CC316 also demonstrated activity against *B. cereus* and *S. aureus* (**Table 4**). Both genera *Paenibacillus* and *Cupriavidus* have been found in several caves however only the first has been reported for its antimicrobial activity from cave representatives. Regarding the *Paenibacillus* genus, a study conducted by Yasir *et al* (2017) found two strains of *Paenibacillus lautus* (*Paenibacillus* sp. strain MY-CB14 and MY-CB145) which were able to inhibit *Salmonella typhi.* Moreover, isolate MY-CB14 also presented antagonistic properties against *S. aureus* (Yasir, 2017).

In Lechuguilla Cave, a remote and pristine system, the authors isolated a strain of *Paenibacillus* (*Paenibacillus* sp. LC231) that was found to be resistant to 26 of the 40 tested antibiotics (Pawlowski *et al*., 2016). The resistance diversity encompassed five new resistance mechanisms as well as 12 orthologues of known resistance gene families. This study not only demonstrated that resistance mechanism is ancient, as well as that strains from cave environments may contain interesting genomes worth exploring (Pawlowski *et al*., 2016). These studies emphasise the potential to find novel strains as well as metabolic pathways from the *Paenibacillus* genus in cave systems.

Regarding the *Cupriavidus* genus, as far as we are aware, no reports have been done regarding the antimicrobial potential of *Cupriavidus* species isolated from cave environments. Moreover, the genus *Cupriavidus* has been widely studied regarding their ability to tolerate a wide array of metals but few reports have been made regarding the bioactivity against human pathogens (Balkwill, 2015; Byloos *et al*., 2018; Casida, 1988; Chalia *et al*., 2017; Sun *et al*., 2016; Ismail *et al*., 2017;Nies, 2016; Punjee *et al*., 2018; Ramachandran *et al*., 2014; Seccareccia *et al*., 2016; Vicentin *et al*., 2018). Nonetheless,

Ramachandran and colleagues (2014) found a novel polymer-producing bacterium designated as *Cupriavidus* sp. (USMAHM13) to produce a yellow pigment. The crude yellow extract demonstrated bioactivity against *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 11303, *Pseudomonas aeruginosa* USM-AR2 and *Staphylococcus aureus* ATCC 12600 (Ramachandran *et al*., 2014). The study of the *Cupriavidus* genus regarding antimicrobial ability could be of beneficial interest since few studies have been conducted in this area.

This study represents the first report on the antimicrobial activity from bacterial isolates acquired in karstic caves from Portugal, revealing the potential of cave microbiomes to harbour bacterial strains from *Actinobacteria, Firmicutes* and *Proteobacteria* to be bioactive against several Gram-positive and Gram-negative pathogens.

Table 4. Antibacterial activity of cave isolates against Gram-positive and Gram-negative pathogenic bacteria.

Isolates Nº	*Aeromonas salmonicida* ATCC 33658	*Bacillus cereus* ATCC 14579	*Enterococcus faecalis* ATCC 29212	*Escherichia coli* ATCC 25922	*Klebsiella pneumoniae* ATCC 13883	*Pseudomonas aeruginosa* ATCC 27853	*Salmonella typhimurium* ATCC 14028	*Staphylococcus aureus* ATCC 25923
CC2	-	-	-	-	-	-	-	-
CC45	-	-	-	-	-	-	-	-
CC46	-	-	-	-	-	-	-	-
CC50	-	-	-	-	-	+	-	-
CC64	-	-	-	-	-	-	-	-
CC69	+	-	-	-	-	+	-	-
CC70	-	-	-	-	-	-	-	-
CC75	-	-	-	-	-	-	-	-
CC80	-	-	-	-	-	-	-	-
CC91	-	-	-	-	-	-	-	-
CC101	-	-	-	-	-	-	-	-
CC108	+	-	-	-	-	+	-	+
CC138	+	-	-	-	-	+	-	-
CC166	-	-	-	-	-	+	-	-
CC172	-	-	-	-	-	-	-	-
CC196	-	-	-	-	-	-	-	-
CC277	+	-	-	-	-	+	-	+
CC282	-	-	-	-	-	-	-	-
CC292	-	-	-	-	-	-	-	-
CC294	-	-	-	-	-	-	-	-
CC304	-	-	-	-	-	-	-	-
CC316	-	+	-	-	-	+	-	+
CC318	-	+	-	-	-	+	-	+
CC365	-	-	-	-	-	+	-	-

3.5. General conclusions

This is the first report on the culturable bacteria and its antimicrobial potential from a karstic cave in Portugal. This study was set at investigating the microbiome associated with Cerâmica Cave soils by culture-dependent method (employing three culture media allied with seven pre-treatments) together with the analysis of 16S rRNA gene sequences for the identification of the bacterial isolates revealing several genera such as *Agromyces, Arthrobacter, Bacillus, Cupriavidus, Ensifer, Methylobacterium, Microbacterium, Nocardia, Peaenibacillus, Pseudomonas, Rhodococcus, Sporosarcina* and *Streptomyces*. The use of pre-treatments allowed the acquisition of a higher diversity of genera, being microwave treatment the one with the most diversity observed. Moreover, the antagonistic activity of members belonging to *Actinomycetes*, *Proteobacteria* and *Firmicutes* from Cerâmica Cave (Portugal) were evaluated against Gram-positive and Gram-negative pathogenic microorganisms. Overall, 37.5% of the screened bacterial strains exhibited activity against at least one of the following pathogens: *A. salmonicida* ATCC 33658, *B. cereus* ATCC 14579, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25923. Furthermore, it was possible to observe strain dependent antimicrobial activity patterns by isolates affiliated to *Bacillus mycoides*. The study outcome provided preliminary data that confirmes the potential of bacterial strains from cave habitats as potential source for bioactive metabolites.

3.6. References

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<u>Chapter 4:</u> Modulatory effects on the antimicrobial and pigment production from *Cupriavidus* sp. CC166 isolated from Cerâmica Cave, Portugal

Chapter 4. Modulatory effects on the antimicrobial and pigment production from *Cupriavidus* sp. CC166 isolated from Cerâmica Cave, Portugal

4.1. Abstract

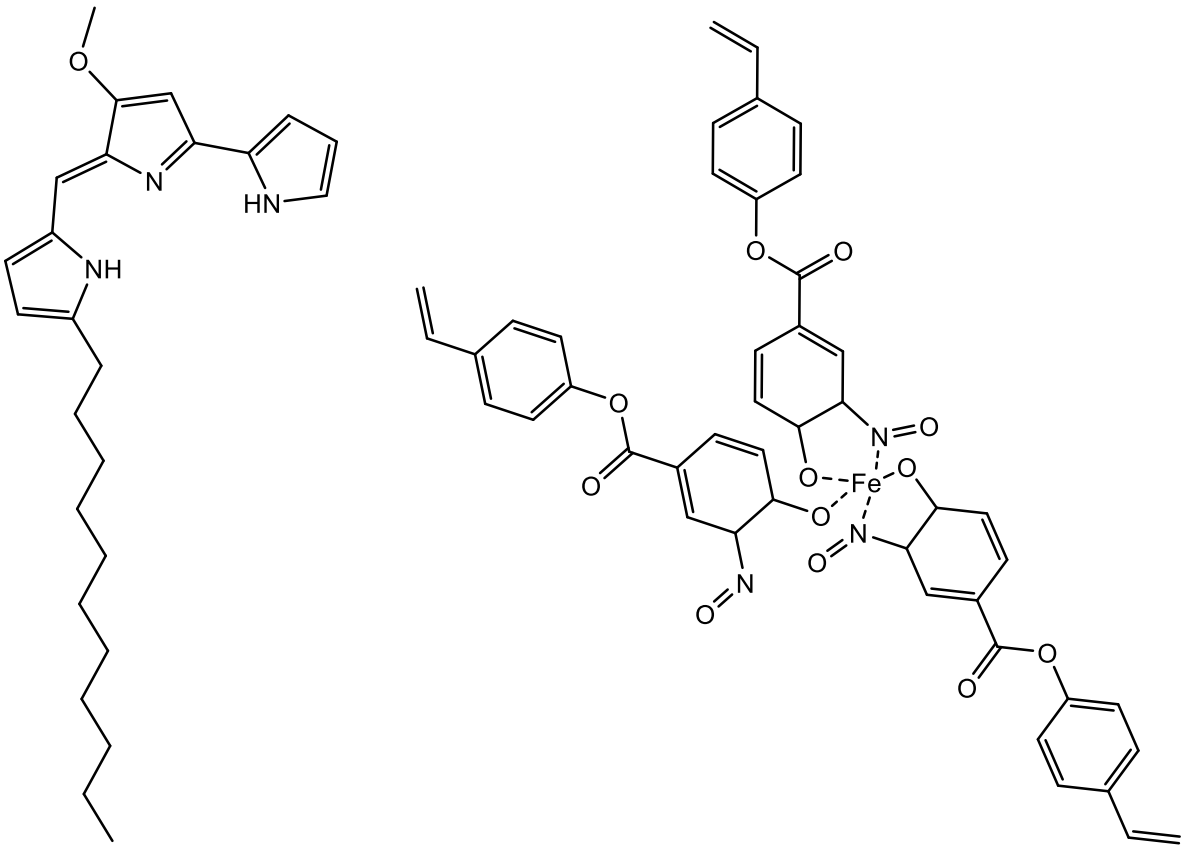
Presently, there is not only a high demand for new antibiotics but also for new natural products such as natural pigments, which have a wide range of applications including in food, textile, cosmetic and pharmaceutical industries. Due to health and environmental concerns, the search for new natural sources of pigments is continuously increasing and bacterial pigments have been gaining relevance since bacteria have the advantage of growing rapidly and being easily processed. In addition, bacterial pigments, besides colour, typically present other biological properties such as antimicrobial activity. In order to find new microbial sources, able of providing such natural pigments, the exploration of new niches such as extreme environments its highly advised. Cave habitats are considered extreme and an unexplored reservoir of microbial populations with potential bioactive metabolites. Herein, we report for the first time the modulation of pigment and antimicrobial activity from *Cupriavidus* sp. (isolate CC166) isolated from Cerâmica Cave, Portugal. Through the employment of 11 different nutritional media, *Cupriavidus* sp. CC166 was found to produce 5 different phenotypic pigmentation including white, pale brown, pale pink, intense pink and intense red phenotypes. Furthermore, when applying Bennet's agar as culture media and 14 days of incubation period, a purple pigmentation was observed. Moreover, using cross streak method for *in vitro* evaluation of antimicrobial activity, *Cupriavidus* sp. CC166 demonstrated different inhibitory patterns that could be linked to pigment production. Remarkably, in some of the employed media, *Cupriavidus* sp. CC166 was able to inhibit all screened pathogens (*Aeromonas salmonicida* ATCC, 33658 *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923). This study highlights the efficiency of the application of several culture media options to modulate antimicrobial and pigment production abilities from *Cupriavidus* sp. CC166 isolated from a Portuguese karstic cave. As far as we are aware, this is the first study on antimicrobial activity from *Cupriavidus* sp. isolated from karstic caves.

4.2. Introduction

The use of pigments is common in several economic sectors such as agricultural practices, textile industries as well as in food and pharmacological industries (Numan *et al*., 2018; Parmar and Singh, 2017). In the past decades, synthetic pigments have been the target of public and scientific concerns, namely related to environmental and health issues (Amchova *et al*., 2015). Due to the increase of awareness and implementation of safety laws concerning these products, several synthetic dyes and colorants have already been banned due to carcinogenic effects and environmental disposal hazards (Numan *et al*., 2018; Tuli *et al*., 2014). Presently, an evident shift towards natural sources is being observed, and natural pigments are widely employed in several industries (Cortez *et al*., 2016; Kulandaisamy *et al*., 2013). Although pigments derived from natural flora and fauna are an appellative source, bacterial pigments provide noteworthy advantages, for instance, ease to culture and further scale up for large-level production (Kulandaisamy *et al*., 2013; Numan *et al*., 2018; Parmar and Singh, 2017). Moreover, bacterial pigments are usually easier to extract and provide an eco-friendly synthesis, being overall cost-effective and safer (Numan *et al*., 2018). Pigments extracted from bacteria have also proven to encompass a diverse range of commercial applications, not only for food industry as dyes and colorants, but also in textile, cosmetic and pharmaceutical sectors being an emergent field for exploration (Kulandaisamy *et al*., 2013; Rao *et al*., 2017).

Currently, the search for novel metabolites and pigment producing strains is beginning to focus on the exploration of the microbial culturable diversity of extreme habitats (García-López *et al*., 2017). Caves are a barely unexplored niche regarding antimicrobial and pigment producer strains, and the prospection of its microbiome could provide a valuable source of novel metabolites as well as novel pigments (Ghosh *et al*., 2017). For instance, *Streptomyces* sp. JS520 isolated from a cavy area of Miroc mountain (eastern Serbia), was found to produce high quantities of undecylprodigiosin (**Figure 10**) with antibacterial, antioxidant, and UV-protective properties (Stankovic *et al*., 2012) while in the cave 'Grotte des Collemboles' (Belgium) a novel *Streptomyces* species designated *Streptomyces lunaelactis* sp. nov., was found to produce several intracellular and diffusible pigments including green-pigmented secondary metabolite identified as ferroverdin A (**Figure 10**) (Maciejewska *et al*., 2015). The only studies in our territory focusing on cave microbiota were carried out in the Azorean volcanic caves (Dalton *et al*., 2016; Northup *et al*., 2011).

Considering the versatility and the adaptative metabolism, one very interesting genus found in caves is the *Cupriavidus* genus (Jugder *et al*., 2015; Lal *et al*., 2013; Punjee *et al*., 2018; Sharma *et al*., 2016; Vicentin *et al*., 2018). This genus is composed by Gram-negative bacteria and it is known for presenting tolerance to a wide array of metals, as well as for surviving in a variety of harsh oligotrophic habitats including caves (Barton, 2015; Jugder *et al*., 2015; Lal *et al*., 2013; Punjee *et al*., 2018; Sharma *et al*., 2016; Vicentin *et al*., 2018). Regarding pigment production, one study found that *Cupriavidus* sp. USMAHM13 is able to produce a yellow pigmented extract with antimicrobial activity against *S. aureus* ATCC 12600, *B. thuringiensis* ATCC 10792, *B. subtilis* ATCC 6633, *P. aeruginosa* USM-AR2 and *E. coli* ATCC 11303 (Ramachandran *et al*., 2014). Although several species from this genus have been widely explored for its resistance to a wide array of metals, little is known about the antimicrobial potential and pigment-producing abilities of *Cupriavidus* genus.



Undecylprodigiosin Ferroverdin A

Figure 10. Structure of natural pigments uncovered in cave microorganisms (Maciejewska *et al*., 2015; Stankovic *et al*., 2012).

This work aimed at modulating the pigment production of *Cupriavidus* sp. CC166 isolated from Cerâmica Cave in Sicó massif, Portugal, by employing a vast array of nutritional conditions. Furthermore, we aimed to evaluate the antimicrobial activity of each pigment phenotype against Gram-positive and Gram-negative pathogenic bacteria, using the cross streak method.

4.3. Material and methods

4.3.1. Isolation of *Cupriavidus* sp. CC166 from cave systems

Strain CC166 (closest related match with 99% similarity according to 16S rRNA gene sequence to *Cupriavidus necator* N-1) was isolated from a soil sample of Cerâmica Cave, Sicó massif, Portugal. Isolation was achieved by letting the soil sample air dry for 30 days and further suspending in sterile solution (NaCl 0.9 %) prior to dilution plating on actinomycetes isolation agar (**AIA**) (Himedia, Mumbai, India) for 2 weeks. *Cupriavidus* sp. CC166 was further purified using **AIA** media and a 48h incubation period until pure colonies were achieved. All incubation steps were performed in the absence of light in order to mimic cave conditions. Stock cultures of *Cupriavidus* sp. CC166 were preserved at -20 ºC in tryptic soy broth (**TSB**) (**Table 5**) (Liofilchem, Roseto degli Abruzzi, Italy) media with 30% glycerol.

4.3.2. Modulation of pigment production from *Cupriavidus* sp. CC166

Cupriavidus sp. CC166 was inoculated in different media to modulate colony structure and coloration. With this aim, bacterial isolates were cultured onto eleven agar media: R2A agar (**R2A**) (Oxoid, Hampshire, United Kingdom), corn meal agar (**CMA**) (Oxoid, Hampshire, United Kingdom), Bennet's agar (**BA**) (Himedia, Mumbai, India), nutrient agar (**NA**) (Himedia, Mumbai, India), Mueller-Hinton agar (**MHA**) (Oxoid, Hampshire, United Kingdom), yeast-malt agar (**YMA**) (Himedia, Mumbai, India), blood agar base (**BAB**) (Liofilchem, Roseto degli Abruzzi, Italy), plate count agar (**PCA**) (Liofilchem, Roseto degli Abruzzi, Italy), tryptone soya agar (**TSA**) (Himedia, Mumbai, India), starch casein agar (**SCA**) (Himedia, Mumbai, India) and **AIA** (**Table 6**). For this, *Cupriavidus* sp. CC166 was previously grown in 2 mL of **TSB** for 48 h at room temperature in an orbital shaker at 200

rpm in absence of light. For each culture media petri dish, a streak was performed in its centre with the cave *Cupriavidus* sp. CC166. The inoculated plates were further incubated for 7 days at room temperature in the absence of light. The bioassays were performed in triplicate and 20 µL of sterile **TSB** was used as negative control. In order to evaluate if *Cupriavidus* sp. CC166 was able to produce pigmentation in broth cultures it was also inoculated in 5 different broth media: **TSB**, yeast malt broth (**YMB**) (VWR, Radnor, USA), Nutrient broth Nº2 (**N2B**) (Oxoid, Hampshire, United Kingdom), Mueller-Hinton broth (**MHB**) (Oxoid, Hampshire, United Kingdom) and Luria broth (**LB**) (NZY, Lisboa, Portugal) (**Table 5**). *Cupriavidus* sp. CC166 was grown in Bennet's agar for 7 days and pure colonies were resuspended in 5 mL of saline solution (NaCl 0.9 %) until a 0.5 McFarland inoculum density was achieved. Thereafter, 1 mL of the suspension was used to inoculate 9 mL of each screened broth. Each inoculated broth was further incubated for 7 days at room temperature in an orbital shaker at 200 rpm in the absence of light.

Table 5. Broth media constituents used in this study (**TSB, YMB, LB, MHB, N2B**). The final volume of the media is 1 Liter.

TSB	YMB	LB	MHB	N2B
Final pH (at 25°C) 7.3±0.2	Final pH (at 25°C) 6.2±0.2	Final pH (at 25°C) 7.0±0.2	Final pH (at 25°C) 7.3±0.1	Final pH (at 25°C) 7.5 ± 0.2
Pancreatic digest of casein 17.0g	Peptic digest of animal tissue 5.0g	Tryptone 10.0g	Beef, infusion from 300.000g	Lab-Lemco' powder 10.0g
Papaic digest of soya bean 3.0g	Yeast extract 3.0g	Yeast extract 5.0g	Casein acid hydrolysate 17.500g	Peptone 10.0g
Sodium chloride 5.0g	Malt extract 3.0g	Sodium chloride 5.0g	Starch 1.500g	Sodium chloride 5.0g
Dipotassium hydrogen phosphate 2.5g	Dextrose 10.0g			

Glucose Monohydrate 2.5g

4.3.3. Bacterial strains and culture conditions for *in vitro* evaluation of antimicrobial activity

Seed cultures from *Cupriavidus* sp. CC166 were obtained by cultivation in Bennet´s agar for 4 days at room temperature. Pure colonies were picked into 25 mL falcon tubes with 5 mL of saline solution (NaCl 0.9 %) and diluted until a final suspension equivalent to an inoculum density of 0.5 McFarland was reached to further use in antimicrobial susceptibility assays.

The following bacterial strains were used as test microorganisms in antagonistic assays: *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Enterococcus faecalis* ATCC 29212, *Aeromonas salmonicida* ATCC 33658 and *Bacillus cereus* ATCC 14579. Each test human pathogen was inoculated in **TSA** plates and incubated at 37ºC for 24 hours. Pure colonies were collected onto 25 mL falcon tubes with 5 mL of saline solution (NaCl 0.9 %) and diluted until reaching an absorbance equivalent to an inoculum density of 0.5 McFarland. The final suspensions were used as seed cultures of test bacteria for antimicrobial activity assays.

4.3.4. *Cupriavidus* sp. CC166 antibacterial activity screening

To assess whether culture media composition affects secondary metabolite production with antimicrobial activity of the isolate, two assays were conducted: i) modified disk diffusion method (with broth media) and ii) cross streak method (with agar media). The assays were performed in triplicate. The culture media used in this study are illustrated in **Table 6**.

To assess the release of any compounds to the broth media, which could be able to inhibit the growth of the test microorganisms, the disk diffusion method was employed. For this purpose, 1 mL of *Cupriavidus* sp. CC166 seed culture was used to inoculate 9 mL of

each screened broth (with a total of 10 mL) and incubated for 7 days at room temperature with orbital shaking at 250 rpm. Each fermentation broth was further centrifuged at 6.000 x g for 10 minutes and the supernatant was collected and filtered through a 0.22 µm membrane. The resulting filtrate was used as crude extract. In parallel, seed cultures of the test bacteria were independently inoculated in **MHA** plates (seed plates) and were left to dry for 15 minutes. Afterwards, 20 µL of each broth crude extract was spotted in the top of the agar plate (without the aid of any disc) and let dry for 15 minutes. The antimicrobial activity was determined after 48h of incubation by the presence or absence of a halo.

In the second assay, the cross streak method was employed to evaluate how *Cupriavidus* sp. CC166 interacted through its metabolites with the test bacterial strains, and how these interactions were affected by media composition. With this purpose, the cross streak assay was carried out by streaking *Cupriavidus* sp. CC166 seed cultures in a vertical line in each tested agar media and incubated for 4 and 8 days at room temperature. After the incubation period of *Cupriavidus* sp. CC166, the human pathogenic bacteria seed cultures were used by streaking a perpendicular line to the full-grown line of *Cupriavidus* sp. CC166. Plates were incubated for 48 hours at room temperature and positive inhibition was recorded in mm. The negative control used was saline solution (NaCl 0.9 %).

4.3.5. Fermentation process for pigment production by cave *Cupriavidus* sp. CC166

Considering that *Cupriavidus* sp. CC166 exhibited an intense red pigmentation on Bennet's agar, this culture medium was selected for proceeding with the culture for pigment extraction. With this intent, *Cupriavidus* sp. CC166 was grown as previously described, and five spots of 20 µL were inoculated in Bennet's agar. The inoculated plates were incubated for 7 days at room temperature and in the absence of light. After the incubation period, 54 bacterial colonies were used for proceeding with the extraction of red pigmentation.

4.3.6. Pigment extraction from *Cupriavidus* sp. CC166

To evaluate the best suitable solvent for extraction of the observed intracellular red pigmentation, seven different solvents were tested: acetone, ethyl acetate, methanol, ethanol, pure water, hexane and dimethyl sulfoxide (DMSO). The sampling and extraction procedure

for each solvent was performed as follows: one bacterial colony was aseptically scrapped into a 2 mL eppendorf tube with 1 mL of the solvent and sonicated for 30 minutes at 40 ºC (**Figure 11**). Following sonication (for disruption of cells) the mixture was centrifuged at 6.000 × g for 15 min and the supernatant was collected. The pellet was washed twice with the respective solvent to screen for any visual pigmentation.

After selection of the best solvent (based on visual pigmentation extraction), the final extraction was carried out as follows: *Cupriavidus* sp. CC166 colonies from 7 days cultures were aseptically scrapped into a 50 mL falcon tube containing 25 mL of methanol and sonicated for 30 minutes at 40 ºC. Following sonication, the mixture was centrifuged at 6.000 x g for 15 minutes and the supernatant was collected. The cell pellet was further washed four times consecutively according to the following cycles: two times with 25 mL of methanol (methanol extract) and two times with 25 mL of DMSO (DMSO extract). In each cycle the supernatant was collected and added to the respectively final extract. Obtained methanol extract was further evaporated using a rotary evaporator yielding 72 mg of dry extract (**Figure 11**). Every step of the extraction process and storage of the pellets was carried out in the dark.

Table 6. Agar media constituents used in this study (Actinomycetes Isolation agar; R2A Agar; Mueller-Hinton agar; Tryptone Soya agar; Corn Meal agar; Bennet's agar; Nutrient agar; Yeast Malt agar; Blood agar Base; Starch Casein agar and Plate Count agar).

Actinomycetes Isolation agar	**R2A agar**	**Corn Meal agar**	**Bennet's agar**	**Mueller-Hinton agar**	**Tryptone Soya agar**	**Nutrient agar**	**Yeast Malt agar**	**Blood agar Base**	**Starch Casein agar**	**Plate Count agar**
Final pH (25 °C) 8.1 ± 0.2	Final pH (25 °C) 7.2 ± 0.2	Final pH (25 °C) 6.0 ± 0.2	Final pH (25 °C) 7.3 ± 0.2	Final pH (25 °C) 7.3 ± 0.1	Final pH (25 °C) 7.3 ± 0.2	Final pH (25 °C) 7.4 ± 0.2	Final pH (25 °C) 6.2 ± 0.2	Final pH (25 °C) 7.3 ± 0.2	Final pH (25 °C) 7.2 ± 0.2	Final pH (25 °C) 7.0 ± 0.2
Sodium caseinate 2.00g	Yeast extract 0.5g	*Corn meal extract 2.0g	Yeast extract 1.0g	Beef, infusion from 300.0g	Pancreatic digest of casein 15.0g	Peptic digest of Animal tissue 5.0g	Peptone 5.0g	Meat extract 10.0g	Starch 10.0g	Tryptone 5.0g
L-Asparagine 0.10g	Proteose peptone 0.5g	Agar 15g	Beef extract 1.0g	Casein and Hydrolisate 17.5g	Papaic digest of soyabean meal 5.0g	Sodium Chloride 5.0g	Yeast extract 3.0g	Tryptose 10.0g	Casein Powder 1.0g	Glucose 1.0g
Sodium propionate 4.00g	Casein hydrolysate 0.5g		Casein enzymatic hydrolysate 1.0g	Starch 1.5g	Sodium chloride 5.0g	Beef extract 1.5g	Malt extract 3.0g	Sodium Chloride 5.0g	Sea water 37.0g	Yeast extract 2.5g
Dipotassium phosphate 0.50g	Glucose 0.5g		Dextrose 10.0g	Agar 17.0g	Agar 15.0g	Yeast extract 1.5g	Dextrose 10.0g	Agar 15.0g	Agar 15.0g	Agar 15.0g
Magnesium sulfate 0.10g	Starch 0.5g		Agar 15.0g			Agar 15.0g	Agar 20.0g			
Ferrous sulphate 0.001g	Di-potassium phosphate 0.3g									
Agar 15.0g	Magnesium sulphate 0.024g									
	Sodyum pyrante 0.3g									
	Agar 15.0g									

Note: *(from 50g whole maize)

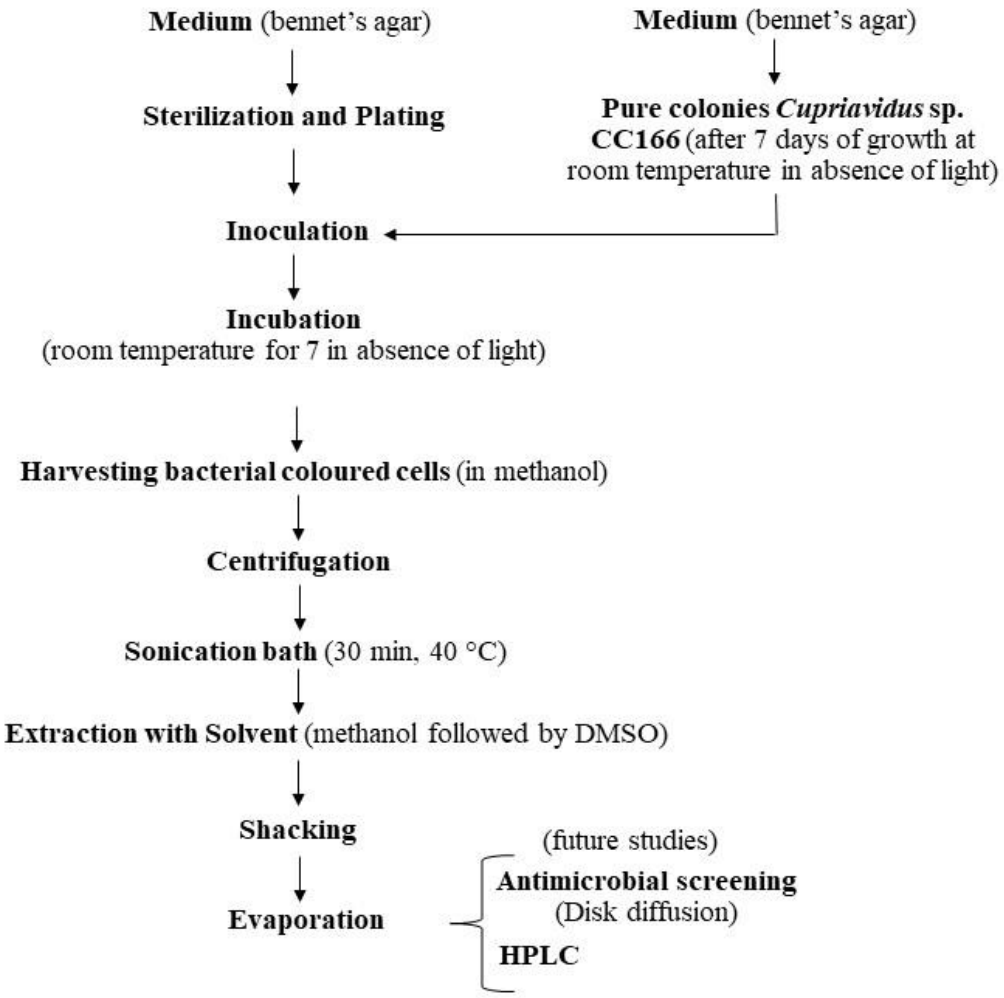


Figure 11- Flowsheet for the production and analysis of pigment producer *Cupriavidus* sp. CC166.

4.4. Results and discussion

4.4.1. Pigment modulation using different nutritional media

In laboratorial conditions, a commonly employed strategy to modulate pigment production from bacteria is to modify the physico-chemical parameters that sustain the bacterial growth and proliferation, such as the culture media composition (Ahmad *et al*., 2012). Therefore, the selection of a wide array of culture media is considered vital in order

to screen for pigment production, especially when studying unknown strains. With this objective, *Cupriavidus* sp. CC166 was cultured onto 11 different agar media (**Table 6**). This resulted in the production of different growth morphologies and colour patterns after 4 and 7 days of incubation, at room temperature in the absence of light. Contrarily, regarding the broth modulation (**Table 5**), no visual pigmentation was observed.

Overall, *Cupriavidus* sp. CC166 grew on all selected media, presenting different morphologies and colour patterns, ranging from white to pale light brown, light to intense pink and intense red. The red coloration was only observed in **BA** and **YMA** media, being more prominent in the first one. Regarding the pink coloration, this was visible in **SCA** and **PCA** media. In another way, in **AIA**, **NA** and **CMA** media different degrees of pale pink coloration were observed while in **R2A** medium a white coloration was present. At last, a pale brown pigmentation was observed in the case of **TSA**, **MHA** and **BAB** media (**Figure 12**). Furthermore, the appearance of a red and pink coloured ring at the junction of the bacterial colony on the **BA** media and **SCA** was visible, suggesting the ability from *Cupriavidus* sp. CC166 to release extracellular pigmented metabolites.

Media composition is known to affect in high degree the behaviour and morphology of bacterial colonies in pure cultures. More specifically, studies regarding the type of carbon source, nitrogen, carbon/nitrogen ratio, temperature and pH level have demonstrated how crucial these parameters are for pigment modulation (Ahmad *et al*., 2012; Das and Das, 2015; Gulani *et al*., 2012; Zengler, 2009). For instance, prodigiosin (**Figure 13**) is a red pigment with several reported activities such as antimycotic, immune-modulating, antibacterial, anti-tumor and anti-malarial (Darshan and Manonmani, 2015; Li *et al*., 2018). This compound is produced by several bacteria, such as *Vibrio psychroerythrus*, *Serratia marcescens*, *Hahellache juensis* and *Streptomyces griseoviridis* (Tuli *et al*., 2014). In a study conducted by Elkenawy *et al*. (2017), set to analyse the production of prodigiosin by *Serratia marcescens* MN5, the authors found that among the screened carbon sources, crude glycerol (waste from biodiesel industry) yielded the highest prodigiosin production. Regarding nitrogen sources, peptone lead to the highest production of this molecule when compared with other sources such as sodium nitrate (Elkenawy *et al*., 2017).

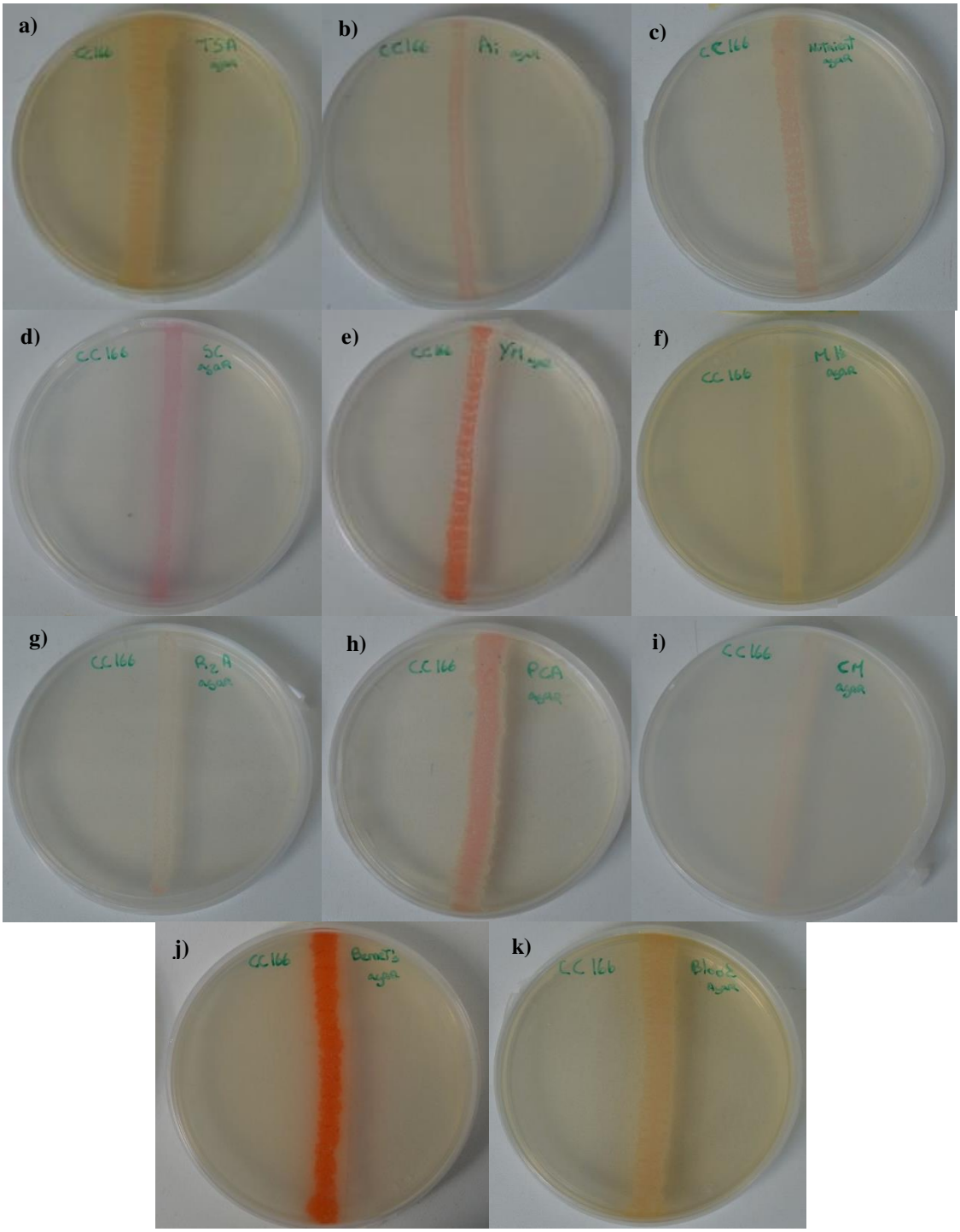


Figure 12 – *Cupriavidus* sp. CC166 culture colour and morphology after 7 days grown in the eleven media employed **a)** Tryptone Soya agar; **b)** Actinomycetes Isolation agar; **c)** Nutrient agar; **d)** Starch Casein agar; **e)** Yeast Malt agar; **f)** Mueller-Hinton agar; **g)** R2A agar; **h)** Plate Count agar; **i)** Corn Meal agar; **j)** Bennet's agar; **k)** Blood agar Base.

In our study, *Cupriavidus* sp. CC166 demonstrated red coloration only in the **BA** and **YMA** media. Interestingly, these are the only media employed that contain dextrose in its composition (both with 10 gL^{-1}), suggesting that this may be the factor for the observed red coloration. Moreover, in the case of **YMA**, it also contains peptone (5.0 g) reported to affect positively the pigment production by microorganism, as stated above (Elkenawy *et al*., 2017). It is important to note that even though a red pigmentation was observed in both media, the appearance of a red coloured ring junction of diffusible pigments was only visible in **BA**. This fact, allied with the higher intensity of coloration produced in this culture medium reveals the potential of Bennet's agar for exponentiation of pigment production by *Cupriavidus* sp. CC166.

Curiously, in **BA**, the coloration of *Cupriavidus* sp. CC166 suffered alterations over time. In day 1 and 2 the colonies showed a pale brown pigmentation that shifted to orange between day 3 and 4, and the red state was reach at day 6 to 7. Moreover, after 12 days of incubation in **BA** medium, the colonies started to shift their colour to an intense purple pigmentation. Purple pigments such as violacein (**Figure 13**) have been found in several bacteria, such as *Janthinobacterium lividum*, *Pseudoalteromonas tunicata* and *Chromobacterium violaceum* (Tuli *et al*., 2014). Moreover, violacein has been linked to several bioactivities such as anticancer and antimicrobial activity (Choi *et al*., 2015; Dodou, Hilania Valéria Batista *et al*., 2017).

As stated above, in cave systems two different *Streptomyces* strains have been found to produce pigment metabolites: *Streptomyces* sp. JS520 was able to yield high quantities of undecylprodigiosin while *Streptomyces lunaelactis* sp. nov. produced several pigments including ferroverdin A (green pigment) (Maciejewska *et al*., 2015; Stankovic *et al*., 2012). Nonetheless, as far as we are aware, this is the first report on a red coloration observed in *Cupriavidus* sp. CC166, highlighting the importance of the present study. Furthermore, through the achieved data, the importance of different culture media application is evident when screening for pigment production. These results highlights, the benefits of screening several culture media for further exploration of the isolated components of the agar formulation that allowed for pigment production. Moreover, the identification of the molecule responsible for the observed pigmentation may bring new insights for its application on several sectors (e.g. food, textile, cosmetic and pharmaceutical industries).

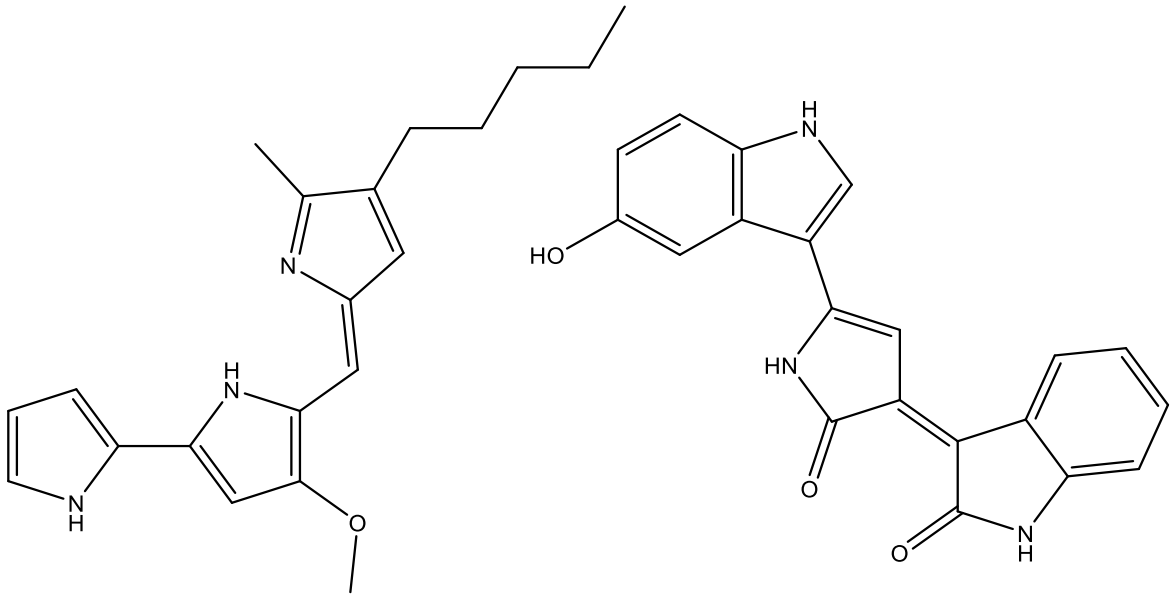


Figure 13. Structure of prodigiosin and violacein, two pigments produced by cave microorganisms.

4.4.2. Antimicrobial activity of cave *Cupriavidus* sp. CC166

Aiming to evaluate the potential antimicrobial activity of each pigment and non-pigment phenotype of *Cupriavidus* sp. CC166 after 4 and 8 days of incubation, cross streak was employed for each screened agar culture media with exception of **TSA** (**Table 7**), against Gram-positive and Gram-negative pathogenic bacteria. The results from the antibacterial cross streak are presented in **Table 7**, and selected representative examples of the cross streak results are illustrated in **Figure 14**.

Remarkably, *Cupriavidus* sp. CC166 was able to inhibit all tested pathogenic agents: *Aeromonas salmonicida* ATCC 33658*, Bacillus cereus* ATCC 14579*, Enterococcus faecalis* ATCC 29212*, Escherichia coli* ATCC 25922*, Klebsiella pneumoniae* ATCC 13883*, Pseudomonas aeruginosa* ATCC 27853*, Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923, when employing **SCA**, **BA** and **YMA** as the culture media. Interestingly, in all these cultures media, strong pigment coloration was observed in contrast to other phenotypes observed in the screened media. Moreover, *Cupriavidus* sp. CC166 displayed its highest activity against *A. salmonicida* (35mm), *E. faecalis* (37mm) and

P. aeruginosa (35mm) in **SCA**. Highest inhibition was observed against *S. aureus* (35mm) *S. typhimurium* (35mm) and *K. pneumoniae* (32mm) using **YMA**. In the case of *E. coli*, the highest inhibition (17mm) was achieved when employing **BA**. Furthermore, *S. typhimurium, K. pneumoniae* and *E. coli* were only inhibited in media where intense pigment phenotype was observed (intense pink and red). This may be a sign that the pigment compounds may be related with the antimicrobial activity observed by *Cupriavidus* sp. CC166. Another hypothesis consists on the expression of by-products with antimicrobial activity when pigment production is being exhibited (Liu *et al*., 2013). Curiously, in Mueller-Hinton media no inhibition was reported against any of the test pathogenic bacteria.

Determination of the optimal culture conditions is also relevant in order to achieve the maximal production of antimicrobial compounds (Banerjee *et al*., 2014). Therefore, two incubation periods were used to understand the relationship between antimicrobial metabolites production and the duration of incubation for each culture media. Overall, stronger activities were observed when employing 8 days of incubation in contrast to 4 days of culture, as observed in **Table 7**. For instance, in **SCA**, **BA**, **R2A**, **PCA**, **BAB** and **CMA** media the activities were always higher in the 8 days period in contrast to the 4 days against all test pathogens. Nevertheless, in some cases, a reduction or no alteration of activity was observed in the two incubation periods. For instance, in **AIA** when screening *B. cereus E. faecalis* and *S. aureus* this phenomenon is observed. Curiously, in **BA** the higher inhibition was observed against *K. pneumoniae* and *S. typhimurium* when employing 4 days of incubation while in **AIA** media, *B. cereus, E. faecalis* and *S. aureus,* the inhibition remained almost the same after 8 days.

As it is known, several biosynthetic gene clusters remain silent until the proper conditions are present enabling the metabolic pathway (Maciejewska *et al*., 2016). For instance, Maciejewska *et al*. (2016) analysed the PKS/NRPS profile and antimicrobial activity using cross streak assay of several cave *Streptomyces* sp. isolates and found different modulatory patterns when employing different culture conditions (media and supplements) (Maciejewska *et al*., 2016). Furthermore, numerous cave bacterial strains, mainly from the *Streptomyces* genus, such as the *Streptomyces tendae* (strain HKI 0179) from Grotta dei Cervi, have shown remarkable antimicrobial activities, being the responsible agent the complex of polyketide glycosides Cervimcycins A-D that shown strong antimicrobial capacities against efflux-resistant *S. aureus* EfS4 and *Enterococcus faecalis* 1528 (VRE)

within a MIC range of 1.6–12.5 µg mL^{-1}(Herold *et al*., 2005). Another remarkable example is the pyranonaphthoquinone antibiotic Xiakemycin A, produced by the *Streptomyces* sp. CC8-201 (isolated from a remote karst cave Chongqing city, China), found to be bioactive against type strains of *S. aureus*, *S. epidermidis*, *E. faecalis* and *E. faecium* including vancomycin resistant strains (Jiang *et al*., 2015). Interestingly, several studies have demonstrated the ability of cave bacterial strains to inhibit the same strains types used in the present study namely *E. faecalis* ATCC 29212*, E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883*, P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Klusaite *et al*., 2016; Lamprinou *et al*., 2015; Maciejewska *et al*., 2016; Stankovic *et al*., 2012). In Francthi' Cave (Greece), two cyanobacteria *Toxopsis calypsus* (type strain: ATHU-CY 3314) and *Phormidium melanochroun* (type strain: ATHU-CY 3315) were able to inhibit *E. faecalis* ATCC 29212 while in moonmilk deposits from Springtails' Cave (Belgium) numerous actinobacteria, mainly *Streptomyces*, were found to inhibit *K. pneumoniae* ATCC 13883 (Adam *et al*., 2018; Axenov-Gribanov *et al*., 2016; Lamprinou *et al*., 2015). Nakaew and co-workers (2012, 2009a and 2009b) have found several strains of *Actinobacteria*, *Nonomuraea monospora* strain PT708, *Actinocorallia aurantiaca* strain PT725, *Nonomuraea roseola* strain PT708 isolated from PhaTup Cave Forest Park (Thailand) and *Micromonospora chersina* strain PNK404 *Spirillospora albida* strain PNK470 and CMU-PNK470 isolated from Phanangkhoi cave (Thailand) that were able to inhibit *B. cereus* (Nakaew *et al*., 2012, 2009a, 2009b). Although there is a vast list of cave microorganisms capable of inhibit several photogenic strains, to the best of our knowledge, the present study is the first report in the inhibition of *A. salmonicida* ATCC 33658 and *S. typhimurium* ATCC 14028 from a cave bacterium.

Regarding the use of broth media as culture media of *Cupriavidus* sp. CC166 for antimicrobial metabolites production, the disk diffusion assay did not reveal any inhibition from all screened broth extracts. For the genus *Cupriavidus*, one report has found a strain of this *taxon* designated as *Cupriavidus* sp. USMAHM13, that produces a yellow pigment when cultivated in **NB** media culture. The crude yellow extract exhibited antimicrobial activity against both Gram-positive and Gram-negative pathogenic bacteria (Ramachandran *et al*., 2014).

This study highlights the efficiency in the application of several culture options to modulate antimicrobial and pigment production capacities from *Cupriavidus* sp. CC166 isolated from cave systems. Moreover, a relation between pigment production and antimicrobial activity was observed, highlighting the possibility that pigments might be a cause for the displayed activities. Most of Portugal karstic caves are yet to be explored and this study reveals the potential of this enormous natural reservoir to harbour unexpected bacterial strains with antimicrobial and pigment production.

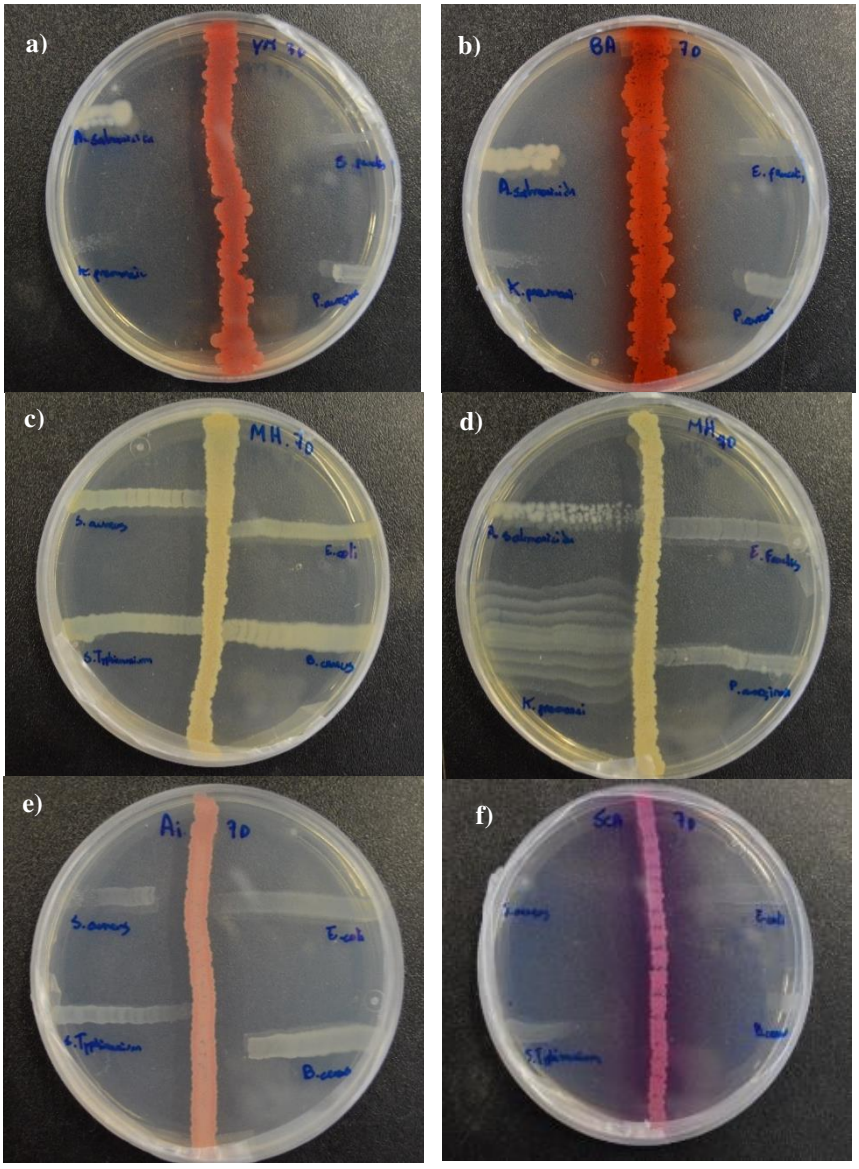


Figure 14. Selected representative examples of the cross streak results after 8 days of incubation period. **a)** Yeast malt agar; **b)** Bennet's agar; **c)** Mueller-Hinton agar; **d)** Mueller-Hinton agar; **e)** Actinomycetes Isolation agar; **f)** Starch Casein agar.

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Table 7. Antimicrobial activity *Cupriavidus* sp. CC166 using cross streak bioassay against both Gram-positive and Gram-negative bacteria by employing 10 different media using two different incubation periods: four days (**4d**) and eight days (**8d**).

	Incubation (days)	AIA	NA	SCA	YMA	MHA	R2A	PCA	CMA	BA	BAB
Aeromonas salmonicida ATCC 33658	4	**6.0±1.4**	**10.5±0.7**	**22.0±1.4**	**18.5±3.5**	**0**	**8.0±1.4**	**0**	**12.0±0.0**	**12.0±0.0**	N.D
	7	**10.3±1.5**	**15.0±3.0**	**36.7±1.5**	**22.5±3.5**	**0**	**13.7±1.2**	**0**	**21.7±0.6**	**16.5±2.1**	N.D
Bacillus cereus ATCC 14579	4	**6.5±0.7**	**8.0±2.8**	**24.5±0.7**	**24.0±1.4**	**0**	**6.0±1.4**	**2.0±0.0**	**15.0±0.0**	**15.5±0.7**	**5.0±0.0**
	7	**7.0±0.0**	**13.3±1.5**	**32.0±2.0**	**32.5±3.5**	**0**	**9.7±2.3**	**10.7±0.6**	**19.0±1.0**	**23.5±0.7**	**11.7±1.5**
Entereococcus faecalis ATCC 29212	4	**9.0±1.4**	**11.5±0.7**	**9.0±1.4**	**16.5±2.1**	**0**	**8.0±0.0**	**5.5±0.7**	**13.0±0.0**	**12.5±0.7**	**6.5±2.1**
	7	**8.3±1.5**	**7.7±1.5**	**33.7±2.9**	**22.0±1.4**	**0**	**11.0±1.0**	**6.5±0.7**	**21.0±1.0**	**16.0±1.4**	**7.3±2.5**
Escherichia coli ATCC 25922	4	**0**	**0**	**2.5±0.7**	**11.5±0.7**	**0**	**0**	**0**	**0**	**10.0±1.4**	**0**
	7	**0**	**0**	**5.0±2.0**	**14.0±1.4**	**0**	**0**	**0**	**0**	**16.5±2.1**	**0**
Klesbiella pneumoniae ATCC 13883	4	**0**	**0**	**17.5±0.7**	**22.0±0.0**	**0**	**0**	**0**	**0**	**21.5±0.7**	**0**
	7	**0**	**0**	**27.0±1.0**	**32.5±3.5**	**0**	**0**	**0**	**0**	**16.0±1.4**	**0**
Pseudomonas aureoginosa ATCC 27853	4	**5.5±0.7**	**15.0±1.4**	**20.5±0.7**	**20.5±0.7**	**0**	**7.5±2.1**	**5.5±0.7**	**18.0±0.0**	**12.5±0.7**	**11.0±1.4**
	7	**10.0±1.0**	**14.7±2.1**	**34.7±2.5**	**22.5±0.7**	**0**	**13.0±1.0**	**6.5±0.7**	**24.0±2.0**	**20.0±0.0**	**13.0±2.0**
Salmonella typhimurium ATCC 14028	4	**0**	**0**	**12.0±1.4**	**24.5±0.7**	**0**	**0**	**0**	**0**	**20.0±0.0**	**0**
	7	**0**	**0**	**22.0±2.0**	**35.0±0.0**	**0**	**0**	**0**	**0**	**12.0±0.0**	**0**
Staphylococcus aureus ATCC 25923	4	**6.0±1.4**	**4.5±2.1**	**23.0±4.2**	**23.0±1.4**	**0**	**5.0±0.0**	**0**	**11.0±0.0**	**20.0±0.0**	**5.0±0.0**
	7	**7.0±1.0**	**10.3±2.5**	**33.0±1.0**	**35.0±0.0**	**0**	**9.0±3.5**	**8.3±3.1**	**16.3±0.6**	**27.5±0.7**	**9.0±1.0**

Note: Culture media abbreviations: **AIA** (Actinomycetes Isolation Agar), **NA** (Nutrient Agar), **SCA** (Starch Casein Agar), **YMA** (Yeast Malt Agar), **MHA** (Mueller-Hinton Agar), **R2A** (R2A agar), **PCA** (Plate Count Agar), **CMA** (Corn Meal Agar), **BA** (Bennet's Agar), **BAB** (Blood Agar Base). Inhibition zones were measured in millimetres (mm).

4.4.3. Extraction of pigments from *Cupriavidus* sp. CC166

Pigment extraction from bacterial cells is influenced by a variety of factors such as the type of solvent, mechanical disruption, and duration of extraction. Furthermore, bacterial cells walls present diverse chemical and mechanical properties adding complexity to pigment extraction processes (Ruivo *et al*., 2014). Since no studies were performed regarding pigment extraction on *Cupriavidus* spp. from cave environments, several solvents were tested (methanol, chloroform, acetyl acetate and acetone, DMSO and hexane). In the preliminary results, partial intracellular pigment metabolites were recovered when employing methanol (red crude extract) and DMSO (blue crude extract) (**Figure 15**).

Methanol is a widely employed solvent for the extraction of several metabolites including pigments (Stafsnes and Bruheim, 2017). Other extraction solvents also applied are acetone, dichloromethane, chloroform and DMSO (Stafsnes and Bruheim, 2017). DMSO has a high extraction capacity that encompasses a large number of compounds, being recommended in the screening of unknown bioactive metabolites including pigments (Stafsnes and Bruheim, 2017). Nevertheless, its high boiling point affects negatively its removal in further concentrations steps (Stafsnes and Bruheim, 2017). Therefore, in our study only the methanol extract was further evaporated yielding 72 mg of dry extract. Further studies will be conducted in order to identify the mixture from the methanol red crude extract and the molecules that are responsible for the observed pigmentation. Moreover, the red crude extract will be further screened for its antimicrobial activity against Gram-positive and Gram-negative pathogens

Figure 15. Recovered extracts from intracellular red pigmentation of *Cupriavidus* sp. CC166 using 2 cycles of methanol (red extract) and DMSO (blue extract) as solvent.

4.5. Conclusions and future perspectives

Caves are a reservoir of novel strains and several studies (Axenov-Gribanov *et al*., 2016; Herold *et al*., 2005; Jiang *et al*., 2015; Maciejewska *et al*., 2016) have proven the ability of the cave microbiome to be bioactive against several pathogenic bacteria, even multidrug resistant ones. Nevertheless, target screening of actinobacteria or their members is usually employed and there are few studies with more detailed analysis from members of other phyla. Herein, we reported for the first time the successful application of different culture condition, exclusively by changing media culture, to modulate both antimicrobial activity and pigment production from *Cupriavidus sp.* CC166 isolated from Cerâmica Cave, Portugal. Remarkably, *Cupriavidus* sp. CC166 exhibited 6 different colour patterns including white, pale brown, pink, intense pink and red (in several media and 7 days incubation) along with purple coloration in **BA** media after 14 days culture. Moreover, it was able to inhibit all pathogenic bacteria screened in this work which included *Aeromonas salmonicida* ATCC, 33658 *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 29212, *Echerichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. At last a possible link between pigment production phenotypes and antimicrobial activity was made. Nevertheless, future studies will be focused in the identification and characterization of both the compound responsible for antimicrobial activity and also for the pigments observed from *Cupriavidus* sp. CC166.

4.6. References

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Chapter 5: Final remarks and future perspectives

Chapter 5. Final remarks and future perspectives

Throughout this dissertation, the importance of the screening of cave microbiomes and it's potential to harbour drug producing strains has been extensively explored. On a global scale, the lack of studies in these ecosystems is evident revealing the untapped reservoir of bacterial strains that are yet to be studied. From the literature available it was possible to conclude that caves harbour a unique microbial diversity with several members belonging to *Proteobacteria*, *Actinobacteria* and *Firmicutes* with reported antimicrobial activity. The isolation of cave bacterial strains remains challenging, nevertheless, several efforts have been made by applying different settings as culture conditions, such as pre-treatments and a broad array of media cultures with different supplements. Screening methodologies rely on agar well method and disk diffusion assays with no standardization. Nevertheless, promising techniques and the use of molecular approaches, such as genome mining, may further elucidate the incredible potential of cave microbial populations.

To further explore this topic, the search for novel microbial diversity with antimicrobial activity in karstic caves from Portugal was conducted for the first time. In general, it was possible to isolate and identify several genera from the Cerâmica Cave system with antimicrobial activity against *Aeromonas salmonicida* ATCC, 33658 *Bacillus cereus* ATCC 14579 *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923. Moreover, novel strains/species may be present belonging to the genera *Bacillus*, *Arthrobacter* and *Paenibacillus*, revealing that these ecosystems encompass several still unknown species for science.

Further exploration using isolate *Cupriavidus* sp. CC166 acquired from Cerâmica Cave soil samples revealed the potential for being modulated through culture media, to promote different patterns of antimicrobial capacities as well as pigment production. *Cupriavidus* sp. CC166 was found to exhibit variation in its pigmentation ranging from white colour to pale light brown colour, light and intense pink and intense red coloration when employing different culture media as culture option along with purple coloration after 12 days of growth when employing **BA** culture medium. Moreover, each pigment phenotype was evaluated regarding its antimicrobial activity revealing that the pigmented morphologies (red and pink) exhibited strong antimicrobial capacities in contrast to less pigmented phenotypes. Overall, *Cupriavidus* sp. CC166 was able to inhibit all screened test bacteria (*Aeromonas salmonicida* ATCC 33658, *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 29212,

Escherichia coli ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923).

This dissertation unveiled the potential to find novel drug producing strains from cave systems and more specifically from Portugal karst caves. Remarkably, it was possible to demonstrate the metabolic versatility on the production of pigment and antimicrobial metabolites from cave bacterial strain using *Cupriavidus* sp. CC166 as case of study. The mentioned findings are novel and critical to the understanding of microbial communities, and present additional information on bacterial diversity and potential from cave systems, highlighting the vast reservoir of cave that are yet to be explored.

In the future, further exploration of the pigmented phenotypes with antimicrobial activity will be conducted in order to identify and characterize the molecules present that exhibit pigmentation as well as the ones with antimicrobial activity. The assessment of the true agent responsible for the pronounced activities is vital to understand the present biochemistry from bacterial strains belonging to cave ecosystems.