ROMANIAN ACADEMY
School of Advanced Studies of the Romanian Academy
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PhD THESIS - SUMMARY

DIVERSITY, ECOLOGICAL ROLE AND APPLICATIVE POTENTIAL OF MICROORGANISMS FROM GLACIAL HABITATS

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1. INTRODUCTION

In the current context of rapid climate change, advancing the knowledge on the diversity, metabolic activity and ecological role of microbiomes from cold environments is of high importance given that the majority of the biosphere is constantly characterized by low temperatures, with 20% of the Earth's surface being covered by ice *(Margesin & Miteva, 2011*; *Yadav et al., 2017*). More so, the molecular components of cold-loving microorganisms have an important applicative potential in bio-nanotechnologies and various industries, because of their stability and activity at low temperatures. In view of understanding the cold environments microbial community's role in the biogeochemical cycle, and to uncover novel bacteria with specific functional characteristics as a source of improved solutions in biotechnologies and medicine, the investigation of their diversity, cold-adaptation mechanisms, climate impact and functional characteristics with applicative potential constitute a corroborative research priority.

To date, studies on ice caves microbiomes were scarce, the majority of data focusing on uncultured bacteria and fungi from recently formed ice and accumulated during the last 1,500 years (*Hillebrand-Voiculescu et al., 2013; Tebo et al., 2015; Itcus et al., 2018; Brad et al., 2018a*), with little data regarding the cultured cave microorganisms (*Popa et al., 2012*; *Hillebrand-Voiculescu et al., 2014*; *Itcus et al., 2016*). Thus, it is highly important to perform in depth studies of the active cave microbiomes, in order to unravel their biodiversity and functional characteristics in response to geochemical and climate variations, and to provide a putative new source of microbial strains and biomolecules with applicative potential in various industries and medicine.

According to the World Health Organization (WHO), antibiotics resistance considered one of the top ten global threats (*Meng et al., 2022*), is usually an old natural phenomenon, which predates the modern selective pressure given by the almost uncontrollable use of antibiotics in both clinical and industrial settings (*Allen et al., 2010*). The antibiotic resistance mechanisms have been intensely studied (*Kohanski et al., 2010*; *Thi et al., 2011*), with most studies regarding the antibiotic resistance of cold-adapted microorganisms, focused mostly on well-known low-temperature habitats: Antarctic samples, Arctic, high-altitude glaciers, cave environments, permafrost (*Byl et al., 2013*; *Tam et al., 2015*; *Ambrozic Avgustin et al., 2019*; *Belov et al., 2020*; *Mogrovejo et al., 2020*; *Ali et al., 2021*), but almost no information is known regarding the antibiotic resistance of microorganisms isolated from ice caves, so far. Thus, it is now known that non-pathogenic environmental microorganisms have the potential of being a reservoir of resistance genes transferable to pathogenic bacteria.

Bacterial strains originating from natural environments presenting a high resistance to known antimicrobial compounds also constitute a good source for biomolecules with antimicrobial activity against other bacteria or pathogens *(Zada et al., 2021; Kochhar et al., 2022*). Cold-adapted microbes able to produce antimicrobial compounds have been isolated mostly from soil of low temperature regions (*Belov et al., 2020*; *Kralova et al., 2021*). Meanwhile, the antimicrobial activity of

microorganisms from Polar aquatic samples has been poorly investigated (*Lo Giudice & Fani, 2016*). Moreover, extremophilic microorganisms with unique and versatile metabolic properties have possible biotechnological applications in several industrial areas, which is why cold-loving bacteria from various extreme environments (which are able to develop biomolecules for thriving in harsh conditions) have been viewed as sources of novel bioproducts, including antimicrobial agents *(Siddiqui, 2015; Dasila et al., 2022*).

Studies on cultured microbes originating from ice caves – unique, secluded, light-deprived and low content nutrients icy habitats (*Purcarea, 2018*) – in order to obtain cultivable bacteria from perennial ice accumulated in caves, and determine their metabolic and phenotypical characteristics, in particular their susceptibility to known antibiotics and antimicrobial, are also important in the present context of climate change, where old microorganisms trapped inside various ice deposits could be transferred to soil and water habitats after ice melting, with potential harmful impact on the human population.

Taking into consideration these priorities, the study making the object of this thesis constitute the first characterization of the diversity, community structure and function of the total and potentially active bacterial communities and cultured bacterial strains entrapped in a 13,000-years old perennial ice core chronosequence from Scarisoara Ice Cave, Romania.

The main objectives of this study were:

(**1**) to unravel the diversity and community structure of the total and potentially active bacteria along the 13,000-years old ice core from Scarisoara Ice Cave, the oldest cave ice microbiome characterized so far;

(**2**) to isolate and identify bacterial strains from the 13,000-years old cave ice chronosequence;

(**3**) to characterize the antibiotic resistance profile, the antimicrobial activity potential and the biochemical profile of these ancient cold-loving bacterial strains;

(**4**) to determine and analyze the whole genome sequence of the multi-drug resistant psychrophilic bacterial strain *Psychrobacter sp.* SC65A.3 isolated from the 5000-years old ice.

This study based on both culture independent- and dependent methods and biochemical analyses consisted of (**i**) sampling of an ice core of 25.33 meters corresponding to a chronosequence of up to 13,000-years old ice extracted from the perennial ice block of Scarisoara Cave, Romania, (**ii**) 16S rRNA gene Illumina sequencing of the total and potentially active ice prokaryotic communities preserved since the Late Glacial Period and correlation analyses of their diversity and taxa composition with the geochemical and climate variations, (**iii**) bacterial isolation, genetic identification, and functional characterization of the cave ice bacterial isolates, and (**iv**) whole genome sequencing and analysis.

The present thesis is divided in two major parts. The first part is dedicated to the current state of knowledge regarding extremophiles, particularly cold-loving microorganisms; being divided in three subchapters: (*i*) the biology of microorganisms from extreme environments; (*ii*) the functional

characteristics of cold-active microorganisms; and (*iii*) description of *Psychrobacter* genus. The second part comprises of the personal contributions, starting with the aims and objectives, followed by a chapter dedicated to the methodology. The next three chapters represent the original research experiments. The PhD thesis ends with the general conclusions and perspectives, the supplementary tables used in this thesis, a list of publications relevant to the thesis subject and the list of references.

2. CURRENT STATE OF KNOWLEDGE

2.1 Biology of microorganisms from extreme environments

2.1.1 Extremophiles

Extremophilic microorganisms can be classified based on the primary parameter influencing the environment: acidophiles, alkaliphiles, endoliths, hypoliths, halophiles, metallotolerant, oligotrophic, piezophiles, radioresistant, xerophiles, toxicotolerant, thermophiles, hyperthermophiles, psychrophilic *(Horikoshi & Bull, 2011; Rampelotto, 2013; Zgonik et al., 2021*). Extremophilic microorganisms are usually found in habitats associated with extreme conditions (North and South Pole, the tropics, deserts, deep sea, volcanoes, high-salt concentration lakes etc.), but also in "not so extreme" environments, such as caves, ice caves, glaciers, natural springs (thermal and mineral) (*Zgonik et al., 2021*).

2.1.2 Cold-environment microorganisms

The psychrophiles were defined as "organisms having an optimal temperature for growth at about 15°C or lower, a maximal temperature for growth at about 20°C and a minimal temperature for growth at 0°C or below" (*Morita, 1975*), while "psychrophile" is generally used to describe any microorganism with activity in low-temperature habitats (*Mikucki et al., 2011*). Psychrophiles have a large distribution, from frozen habitats (permafrost, glaciers, perennial frozen lakes, sea-ice, ocean deep water and polar ice-caps) (*Priscu & Christner, 2004*; *Margesin & Miteva, 2011*; *Anesio & Laybourn-Parry, 2012*; *Yadav et al., 2017*) to the surface and inside the intestines of marine fish, springs, lakes, rivers and soil from temperate regions (*Zarnea & Popescu, 2011*).

2.1.3 Scarisoara Ice Cave

Scarisoara Ice Cave is located in the Bihor Mountains, Eastern Carpathians, in the North-West part of Romania, situated at an altitude of 1,165 m.a.s.l. The 700 m long and 105 m deep limestone cave holds one of the largest (100,000 m^3) and oldest cave glacier in the world, and also one of the oldest glaciers found in a temperate climate zone (*Holmlund et al., 2005*; *Persoiu & Pazdur, 2011*; *Persoiu et al., 2017*). Scarisoara Ice Cave can be accessed through a 60 x 48 meters deep shaft, at the bottom of which perennial snow gives way to the large entrance (17 x 24 meters) into the 3,000 m^2 of the Great Hall of the cave which is occupied by the underground ice deposit, representing the floor of the room, surrounded by three passages that are partly ice free (*Racovita & Onac, 2000*; *Brad et al., 2018*).

Scarisoara Cave is one of the most-explored ice caves in the world (Persoiu et al., 2017), being investigated over the last century in order to unravel the associated climatic and glaciological processes (*Racovita & Onac, 2000*; *Persoiu et al., 2011*), and to reconstruct the climatic and environmental changes in the region (*Onac et al., 2007*; *Feurdean et al., 2011*; *Persoiu & Pazdur, 2011*; *Persoiu et al., 2017*).

2.2 Functional characteristics of cold-active microorganisms

2.2.1 Antibiotic resistance of bacteria from cold-habitats

Antimicrobial resistance concerns all aspects of medicine and health (human and veterinarian), the food security and societal development, making the discovery of novel antibiotics crucial for controlling infectious diseases and avoiding huge economic loses (*Lewis, 2020*; *Meng et al., 2022*). Prospecting the microbiome of extreme habitats could represent a promising approach for the discovery of new antimicrobial compounds (*Nunez-Montero & Barrientos, 2018*), and for understanding the occurrence and evolution of antibiotic resistance.

2.2.2 The antimicrobial activity of cold-environment bacteria

The antimicrobial potential of cold-adapted bacteria has been previously investigated, mostly because of their capacity to produce biomolecules with unique properties including antimicrobial compounds that possess specific structure and biological activity (*Hemala et al., 2014*). Antimicrobial activity screening studies focused on bacteria from Polar and Antarctic soils (*Nedialkova & Naidenova, 2005*; *Shekh et al., 2011*; *Lee et al., 2012a*; *Pan et al., 2013*), Polar lakes and ponds (*Lo Giudice & Fani, 2016*), and Antarctic and Arctic marine environments (*Lo Giudice et al., 2007a*; *Yuan et al., 2014*). It is believed that the cave environments unique characteristics (complete darkness, high humidity, constant low temperature, oligotrophy) could be a potential source of microorganisms capable of producing antimicrobial and anticancer compounds (*Zada et al., 2021*), but have not been studied in much detail as potential new sources of antimicrobials with pharmaceutical applications.

2.2.3 Cold-active enzymes with applicative potential

For the last decades, a large variety of industrial biotechnological processes started using microbial-originating biocatalysts, due to their availability and fast growth rate (*Keshwani et al., 2015*; *Khan & Selamoglu, 2020*). Mesophiles-originating biomolecules are the most common, but have limited stability and catalytic efficiency in response to different conditions of industrial catalytic processes, which is why, recently an interest was shown for extremophilic microorganisms, able to overcome the stressful conditions of extreme environments due to the unique characteristics of their enzymes (*Lavin et al., 2016a*; *Vila et al., 2019*). Given that low temperatures reduce unwanted chemical side-reactions in industrial processes (*Siddiqui, 2015*), the utilization of cold-active enzymes (psychrophilic microorganisms) is recently expanding in various industries: food and animal feed, pharmaceutical, medical, biofuels and energy production and so on (*Cavicchioli et al., 2011*).

2.3 Description of the bacterial genus *Psychrobacter*

The bacterial genus *Psychrobacter* (family Moraxellaceae, class Gammaproteobacteria) was reported for the first time in 1986 when describing the species *Psychrobacter immobilis* (*Juni &*

Heym, 1986). *Psychrobacter* species have cream/off-white-colored colonies, smooth, circular and convex, with an entire margin and a butter-like consistency, while some strains have a pale pink, almost orange coloring (*Bowman et al., 1997*; *Bowman, 2006*). In general, cells are Gram-negative, with the ability to retain the crystal violet dye, which causes them to stain Gram-positive, thus making the genus *Psychrobacter* Gram-variable. Strictly aerobic genus, growing on most common and complex media, mostly psychrotrophic (thriving at low temperatures), and also tolerating a wide range of salt concentration (*Juni, 2005*; *Bowman, 2006*; *Welter et al., 2021*).

The genus *Psychrobacter* has a widespread distribution, the different species being isolated from a variety of habitats, most of them originating from cold and saline environments (*Rodrigues et al., 2009*; *Lasa & Romalde, 2017*). To date, the antibiotic resistance of *Psychrobacter* species is not well known, with only a handful of studies done (*Romanenko et al., 2002*; *Bowman, 2006*; *Petrova et al., 2009*; *Dziewit et al., 2013*; *Abd-Elnaby et al., 2016*). Almost all described *Psychrobacter* strains have a cold-loving characteristic, which explains the industrial and biotechnological interest focused on the existence of novel cold-active proteins and enzymes with broad applicability of this genus (*Rothschild & Mancinelli, 2001*; *Bowman, 2006*).

PERSONAL CONTRIBUTIONS

3. THESIS AIMS AND OBJECTIVES

Despite the constantly revealed new data regarding the microbiome from various cold-environments, knowledge on the total and active microbes found in perennial cave ice is still limited (*Purcarea, 2018*). The studies making the object of this thesis represent a pioneering structural and functional characterization of the of the bacterial community entrapped along a 13,000-years old ice core from Scarisoara Ice Cave, a unique, secluded, light-deprived and low nutrient content perennial ice habitat. Gaining knowledge on the structural and functional characteristics of microorganisms from such frozen environments, on their adaptation mechanisms to harsh environments and temperature shifts, on their contribution to the ecosystem biogeochemistry, and their biotechnological applicative potential is of high importance for both fundamental and applicative research.

Study aims: investigating the bacterial communities from a 13,000-years old ice chronosequence of Scarisoara Ice Cave using culture-dependent and independent microbiological and biochemical methods, to reveal the **structural and functional characteristics of the total and potentially active communities and of isolated bacterial strains**, **and their putative applicative potential**.

Study objectives:

1. To determine the diversity, community structure, climate and geochemical impact on the total and potentially active bacteria found in the 0-13,000-years old ice chronosequence from Scarisoara ice cave.
2. To isolate bacterial strains from the 13,000-years old cave ice chronosequence.
3. To establish the antibiotic resistance profile of the isolated bacterial strains from this old secluded icy environment in response to climate variations, their antimicrobial activity, and biochemical profile.
4. To determine and characterize the whole genome sequence of a multi-drug resistant psychrophilic bacterial Scarisoara strain (*Psychrobacter sp.* SC65A.3).

4. MATERIALS AND METHODS

4.1 Ice sampling

A 25.33-meters ice core was collected from the Great Hall area of Scarisoara Ice Cave by vertically drilling into the perennial ice block using a modified PICO electric drill (*Koci & Kuivinen, 1984*). Aseptic collecting conditions were assured by the use of laboratory grade alcohol and flaming. The ice core fragments were recovered and transferred to sterile plastic bags, and were transported to the laboratory in special containers, under permanent frozen conditions and stored at -20°C until processed. The ice age of each sample was determined based on ^{14}C AMS radiocarbon analysis (*Persoiu et al., 2017*) and linear extrapolation.

4.2 Sample preparation

Ice samples (250-mL) were thawed at 4°C and the microbial biomass was collected by filtration on 0.22-µm sterile MF-membranes (Merck Millipore, Germany) under aseptic conditions using a vacuum driven stainless steel filtering system (Merck Millipore, Germany).

4.3 Uncultured techniques

4.3.1 Flow cytometry

Flow cytometry was used to measure the microbial cell abundance of the cave ice samples by utilizing a BD Accuri C6 Plus system (BD Biosciences, USA). The total microbial community cell density: 1 ml of cellular suspension from thawed ice samples was incubated with 1x SYBR Green I (Lonza Group, Switzerland). The potentially active community: 100-µL of freshly thawed ice was incubated with 1-µg mL^{-1} propidium iodide (PI) (ThermoFisher Scientific, Germany). The viable cells density was calculated by subtracting the number of PI-labeled cells (dead) from the number of SYBR Green I-labelled (total) ones.

4.3.2 Total DNA/RNA extraction

Total DNA and RNA were extracted from the selected ice core samples, following the manufacturers' protocol modified with an additional lysis step using specific molecular DNA/RNA extraction kit.

4.3.3 Illumina sequencing of 16S rRNA gene pool

The prokaryotic profile of Scarisoara microbiome was determined by 16S rRNA gene Illumina sequencing using the V3-V4 variable region, and the sequencing of barcoded amplicons was carried out using an Illumina MiSeq PE300 platform (McGill University, Génome Québec Innovation Centre, Canada).

4.3.4 Sequence analyses

The obtained Illumina sequences were analyzed using bioinformatical, statistical and phylogenetic analyses.

4.4 Cultured techniques

4.4.1 Cultured media

The following media were used in all culture-dependent methods: R2A, R2B, TSA, TSB, MHA, MHB, NA, LB agar and broth.

4.4.2 Isolation of bacterial strains

28 core ice samples were thawed, and 2-ml sample were inoculated in R2B media and cultivated at 4℃ and 15℃ for up to 120 days. A 10^{0} - 10^{-10} serial dilution of enriched cultures were used for plate inoculation, and morphologically different colonies were isolated and purified under the same growth conditions.

The growth temperature interval of the isolates was determined by cultivation on R2A at 4℃, 10℃, 15℃, 20℃, 25℃, 30℃ and 37℃ for up to 30 days. The growth salinity range of one cave isolated bacteria was tested in LB medium at 15℃ under agitation, in the presence of different NaCl and $MgCl_2$ concentrations.

4.4.3 Bacterial strain identification

Gene identification of bacterial isolates was performed by 16S rRNA gene sequencing of genomic DNA, at Macrogen, Netherlands.

4.4.4 Antimicrobial susceptibility test

The Kirby-Bauer method (disk diffusion) was used in order to establish the antimicrobial susceptibility of the cave isolates. 28 different types of antibiotic infused disks were used (OXOID, UK). The antibiotic susceptibility profile was determined based on the presence (S) or absence (R) of a growth inhibition zone (*Matuschek et al., 2014*).

4.4.5 Antimicrobial activity potential

The antimicrobial activity of the bacterial isolates was evaluated against two reference strains and 20 clinical isolates from the Research Institute of The University of Bucharest Microbial Collection. The cell free supernatant from the cave isolates was tested against the pathogenic strains, in a similar way as the Kirby-Bauer method (*Lavin et al., 2016*).

4.4.6 Biochemical characterization

Functional characterization of the cave isolates was performed at 15℃ using API ZYM and API 20NE systems (BioMérieux, France) according to the manufactures protocol.

5. RESULTS: UNCULTURED AND POTENTIALLY ACTIVE BACTERIA FROM 13,000-YEARS OLD CAVE ICE

5.1 Ice collection and analyzed chronosequence samples

The gDNA and cDNA samples selected from the 13,000-years old ice core chronology, used for sequencing, correspond to SC and SCR coded samples. The selected samples belong to an age interval of ~300 years for the first millennium, and ~1,000 years for the remaining samples.

The raw sequences of the 16S rRNA gene of both types of samples (gDNA and cDNA) were uploaded in the Sequence Read Archive (SRA) under accession number **SRP157726**.

5.2 Geochemistry of ice core samples from Scarisoara Ice Cave

The physicochemical and geochemical analyses of 15 melted ice samples were performed at the Laboratory of Hydrogeochemistry, "Emil Racovita" Institute of Speleology, Bucharest, Romania, by Dr. Constantin Marin and Dr. Alin Tudorache.

As expected for a limestone cave, calcium (Ca) was the predominant component, while dissolved organic (DOC) and inorganic (DIC) carbon, sodium (Na), potassium (K), magnesium (Mg), silicon (Si), sulphate (SO_4) and chloride (Cl) anions were also present as a major constituent in the ice cave samples. At the same time, manganese (Mn), iron (Fe), boron (B) and phosphorus (P) appeared in much lower concentrations.

The concentration profile of major chemical components found across the ice block showed a non-homogenous temporal distribution, with consistent fluctuations with the age of ice. Higher values occurred in ice layers deposited during the last millennium, in addition to a spike in all elements' content during the 4,500 - 5,000-years old period. Slightly increased concentrations of DOC, Si, Ca, P and Na were also observed in ice strata formed 7,000-years ago, followed by a slightly increasing concentration of these elements in older ice deposits (**Figure V.2**).

5.3 Microbial cell density in Scarisoara Ice chronosequence

The microbial abundance varied considerably across the ice core with both uncultured and potentially active communities showing a slight overall increase with the age of perennial ice deposits, showing a decline from 700-years old to 5,000-years old ice layer, a spike at 6,000-years old layer, and a prominent increase after 10,000-years old ice layer for the total and the viable communities (**Figure V.3**).

5.4 Metabolic rates of potentially active bacteria

The calculated metabolic rates of the cave ice microbiome based on the cell abundance of the potentially active community and the DOC content of each ice stratum (*Price & Sowers, 2004*), was variable at 0°C, Scarisoara perennial ice characteristic temperature (*Persoiu et al., 2011*). The variation corresponds to a maintenance metabolism in the majority strata of the ice block, with the exception of samples from 400-, 5,000- and 7,000-years old ice layers, where the higher metabolic rate values showed a growth/maintenance combination.

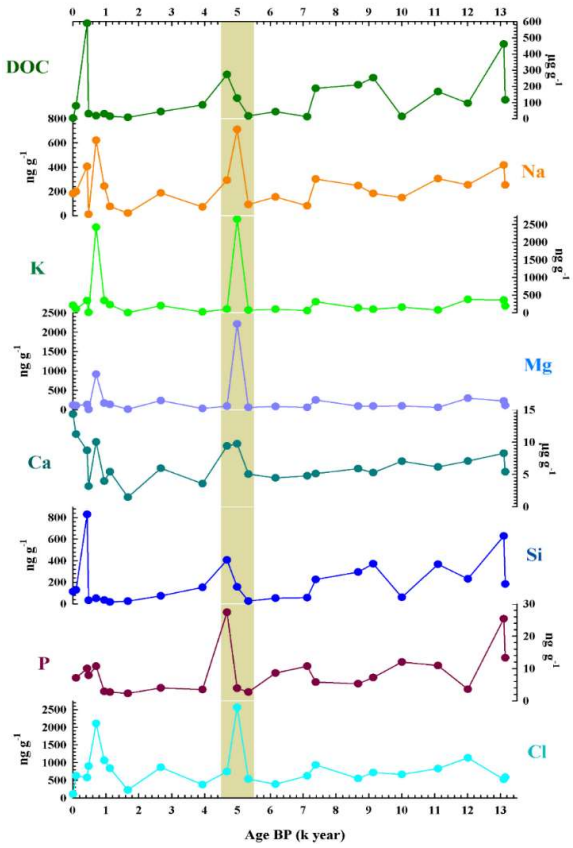

Figure V.2 Distribution profile of geochemical compounds across the 13,000-years old ice core of Scarisoara Cave. (*Paun et al., 2019*)

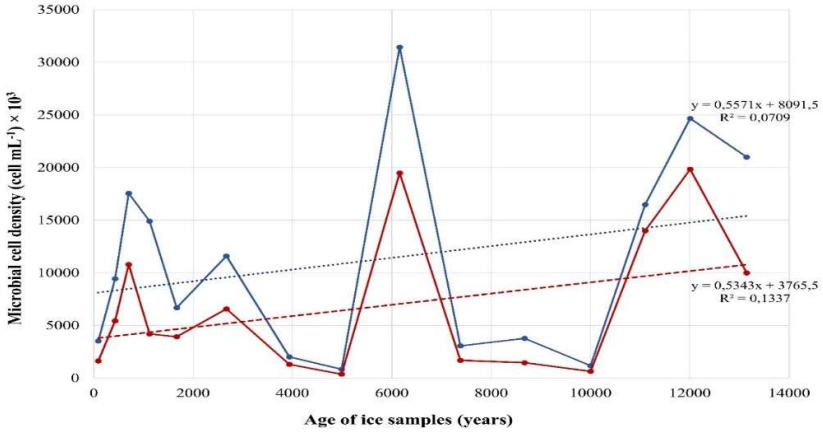

Figure V.3 Temporal variation profile of microbial cell density across the Scarisoara ice core for total (blue) and viable (red) communities (**Table V.3**). (*Paun et al., 2019*)

5.5 Diversity of Scarisoara ice samples

5.5.1 Illumina sequencing statistical indices

16S rRNA gene Illumina sequencing of the total bacterial community (gDNA samples) provided a total of 6,037,525 post quality control (QC) filtered reads and a median of 2,546 OTUs.

The potentially active bacterial community (cDNA samples) 16S rRNA gene Illumina sequencing led to 1,290,207 post QC filtered reads and a median of 585 OTUs.

5.5.2 Rarefaction curve

The rarefaction curves of the observed OTUs calculated for both gDNA and cDNA samples showed partial saturation for the 23,560 and 21,080 sequences per sample, respectively. This indicates an extensive but incomplete overview of the total and potentially active microbial diversity.

5.5.3 Alpha and beta diversity

Alpha diversity indices calculated for the sequenced gDNA samples revealed that the 100-years old ice microbiome (SC100) had the highest diversity, followed by the ice layers formed during the last 2 millennia (SC400, SC700, SC1K and SC2K samples), while the lowest diversity was observed in SC6K, SC4K and SC11K samples. There was no age-dependence of the observed OTUs and alpha diversity indices observed in the ice chronosequence. The variability of alpha diversity indices for cDNA samples was directly correlated with the number of observed OTUs, showing an uneven diversity of potentially active bacteria across the 13,000-years old Scarisoara ice core.

A principal component ordination analysis (PCoA) of OTU disparities across the 15 ice layers sequenced indicated a clear distinction between the total and potentially active communities' bacterial composition according to the two separate clusters of gDNA and cDNA OTUs. There was no age-dependant clustering found for either community (**Figure V.6**).

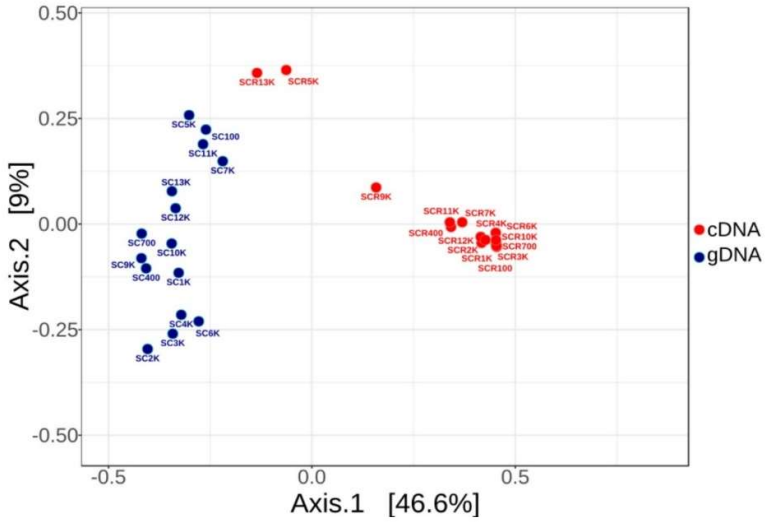

Figure V.6 PCoA of the bacterial OTUs distribution across the 13,000-years old cave ice core. Variation of the 16S rRNA amplicons from gDNA (triplicates of 15 SC samples) and cDNA (15 SCR samples) Illumina data was analyzed for the total (blue) and potentially active (red) communities, using average OTU values of the gDNA triplicate libraries. (*Paun et al., 2019*)

5.6 Prokaryotic community structure from Scarisoara ice core

The total ice core OTUs represent 99.18% bacterial phylotypes, while <0.45‰ belonged to Archaea and 0.819% were unassigned. Along the cave ice core, 38 phyla, 103 classes, 137 order, 274 family and 625 genera OTUs were identified in the total prokaryotic community (gDNA), while 31

phyla, 74 classes, 103 order, 215 family and 414 genera belonged to the potentially active bacterial community (cDNA).

5.6.1 Total bacterial community

The global distribution of bacterial phyla in the cave ice block showed Actinobacteria and Proteobacteria as the most dominant taxa (**Figure V.7**).

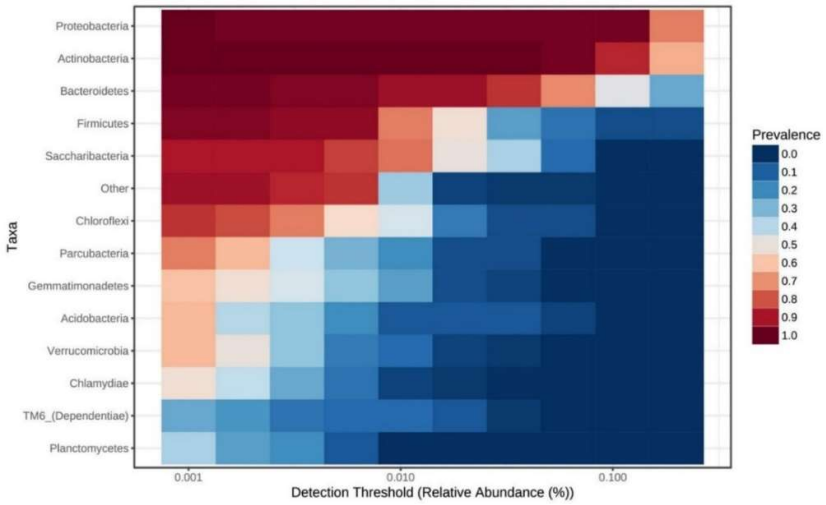

Figure V.7 Total bacterial community (gDNA) composition at phyla level in Scarisoara ice core. Y-axes represent the prevalence level of core features. (*Paun et al., 2019*)

Across the cave ice chronosequence, Proteobacteria and Actinobacteria phyla had the highest relative abundance in most of the ice layers, except for the SC7K sample where Firmicutes was dominant.

At class level, Actinobacteria showed a high relative abundance in most ice strata, while Alpha-, Beta-, Gamma- and Deltaproteobacteria were present in various proportions throughout the ice block (*Paun et al., 2019*).

Among the main taxa present in Scarisoara ice block, *Cryobacterium* was mostly found in SC6K, while *Pedobacter* OTUs showed the highest relative abundance in older ice strata (SC10K, SC13K, SC9K and SC5K). The SC3K ice layer was abundant in both *Aeromicrobium* and *Arthrobacter* OTUs, while *Escherichia_Shigella* taxa had a high presence in the 4,000-years old ice layer.

5.6.2 Potentially active bacterial community

The global distribution of the potentially active bacterial community (SCR samples) identified in the 13,000-years old Scarisoara cave ice core showed that Proteobacteria and Firmicutes had the highest relative abundances (**Figure V.13**).

The distribution profile of the potentially active bacterial in the cave ice core showed that Proteobacteria and Firmicutes were homogenously distributed across the ice chronosequence while, Actinobacteria and Bacteroidetes both had a rather low representation across the ice block. In the

potentially active community, at class level, Clostridia (Firmicutes), Beta- and Gammaproteobacteria had the highest representation throughout Scarisoara ice block.

The potentially active community dominant genera identified across the ice block were more uniformly spread throughout the ice layers, with *Pseudomonas*, *Clostridium sensu stricto 9* and *13*, *Janthinobacterium* and *Stenotrophomonas* genera observed in ice deposits formed during the last four millennia, while dominating the SCR10K sample.

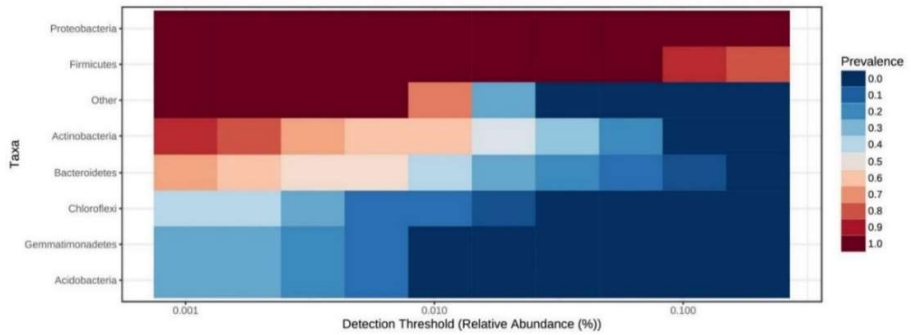

Figure V.13 Global distribution of the potentially active bacterial community (cDNA) composition at phyla level in Scarisoara ice core. Y-axes represent the prevalence level of core features. (*Paun et al., 2019*)

5.6.3 Archaeal community of Scarisoara ice chronosequence

The Archaeal community represents <1‰ of the Scarisoara ice cave core. Archaea in both communities is composed of the same 2 phyla, 2 classes, 3 order, 4 family and 4 archaea genera.

5.6.4 Environmental dependence

Redundancy analysis (RDA) of phyla distribution from both communities in relation with the geochemical parameters of ice core samples was used to explained the variance for the gDNA and cDNA libraries, respectively. The RDA canonical axes composed of several covarying geochemical parameters showed dissimilar correlation between phyla distribution of the total and potentially active prokaryotic communities with the ice chemistry, for Firmicutes, Proteobacteria and Actinobacteria. Changes in phyla distribution correlated with the DOC/DIC contents observed, suggested that the microbial composition was driven by the carbon type (organic and inorganic) (*Paun et al., 2019*) (**Figure V.23**).

Principal coordinate analysis (PCoA) of the OTUs variability of ice-contained prokaryotic communities based on the organic carbon content of ice core samples, indicated a very weak clustering of total microbial taxa from high and low carbon content strata, meaning that more complex environmental factors are involved in shaping the microbiome composition in old ice strata. In the case of the potentially active community, a stronger and significant correlation of these parameters occurred, suggesting an important contribution of post-depositional processes occurring in the potentially active microbiome from the SCR4K and SCR7K cave ice strata (*Paun et al., 2019*).

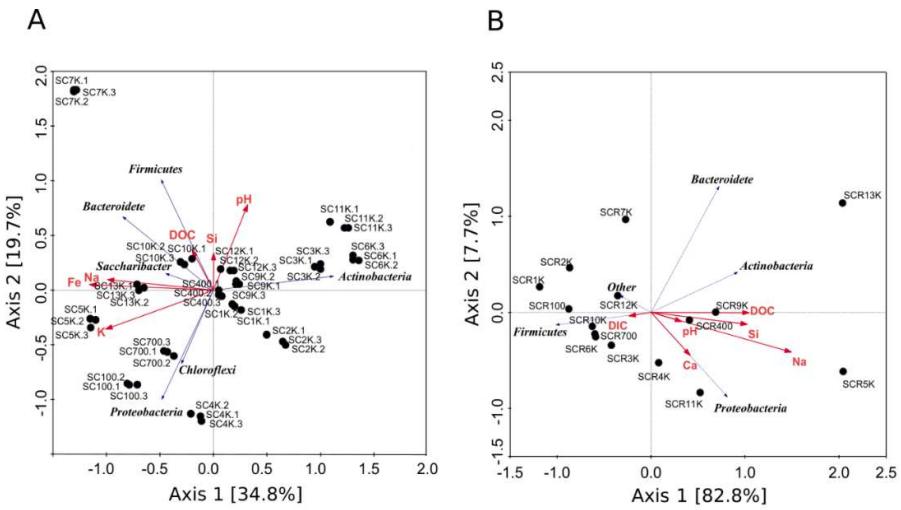

Figure V.23 RDA triplot of the geochemical dependence of Scarisoara bacterial phyla. The phyla distribution from **(A)** gDNA samples triplicates and **(B)** cDNA samples were analyzed in relation to relevant geochemical parameters pH, DIC, DOC, Ca, Na, K, Fe, Si. (*Paun et al., 2019*)

6. RESULTS: CULTURED BACTERIA FROM 13,000-YEARS OLD ICE FROM SCARISOARA CAVE

6.1 Bacterial isolation and identification

6.1.1 Isolation of bacterial strains from Scarisoara ice core

Inoculation from melted ice from the 28 core ice samples of Scarisoara cave on R2A medium at 4°C and 15°C led to the isolation and purification (**Figure VI.1**) of 146 bacterial colonies.

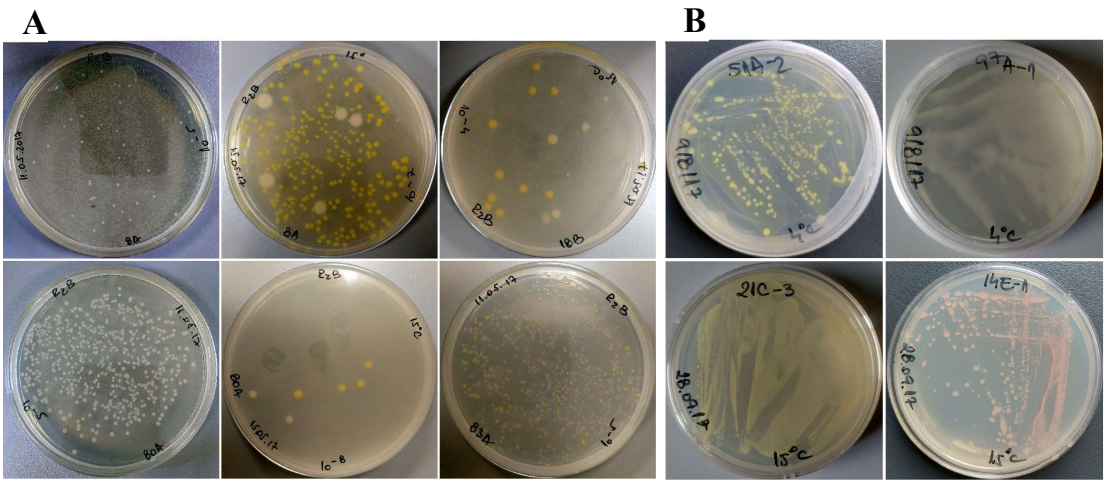

Figure VI.1 (A) Isolation of bacterial colonies and **(B)** purification of bacterial isolates obtained on R2A at 4°C and 15°C.

6.1.2 DNA isolation, 16S rRNA gene amplification and taxonomic assignment

Among the 146 strains, 97 were isolated at 15°C and 49 strains at 4°C. The total genomic DNA extracted from all cave ice strains for molecular identification, by PCR amplification of the 16S rRNA gene, and the nucleotide sequences were taxonomically assigned based on BLAST analysis, resulting in 4 phyla, 34 genera and 56 species. From the total identified bacterial strains, 70 isolates were selected for further characterization based on 16S rRNA gene sequence and age of ice layer. These strains represent isolates retrieved from 19 different cave ice layers of the ice chronosequence at an interval of ~300 years.

6.1.3 Taxonomic distribution of bacterial isolates along the 13,000-years old cave ice core

Phyla distribution across Scarisoara ice core indicated the presence of Actinobacteria in all ice layers except for 7,000- and 11,000-years old ice, Proteobacteria species in 12 of the 19 ice strata, while both Firmicutes and Bacteroidetes were observed in only 6 of the ice layers.

6.1.4 Growth temperature and bacterial morphology of Scarisoara bacterial strains

The isolated bacterial strains from Scarisoara ice were inoculated in 4 different media to investigate putative morphological changes based on growth substrate variations, and to determine the preference for a specific type of media of each of the isolates. Variation of growth media

provoked slight morphological differences for the majority of the isolates including the slight color change of the colonies, change of the shape of colony's edges and growth inhibition.

The growth temperature interval of the Scarisoara cave isolated bacteria varied between a minimum of 4°C (60 strains) or 10°C (10 strains), and a maximum of 15°C, 20°C, 25°C, 30°C or 37°C, with one isolated strain having the maximum growth temperature set at 28°C (**Figure VI.6)**. Based on the growth temperature range as described by Morita in 1975, five cave isolates could be classified as psychrophiles, while the remaining 65 isolated strains were characterized as psychrotrophs. Four psychrophilic and 9 psychrotrophic isolated cave strains were homologous to cold environments bacteria.

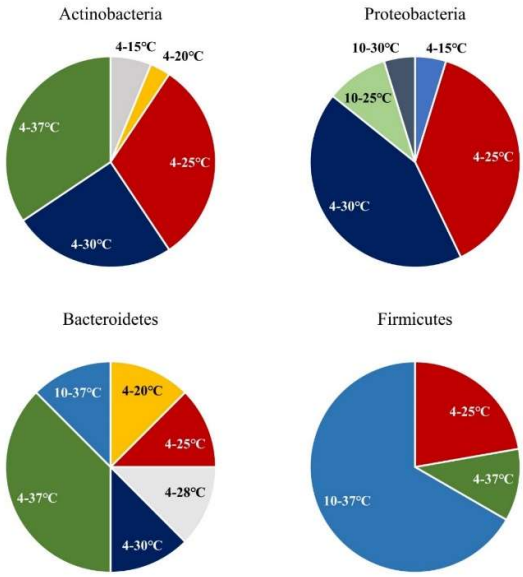

Figure VI.6 Growth temperature interval of Scarisoara cave bacterial phyla. (*Paun et al., 2021*)

6.2 Functional characterization of the isolated bacterial strains from Scarisoara ice

6.2.1 Antibiotic resistance

The antimicrobial resistance profile, tested against 28 antibiotics (17 classes), of the cave isolates showed 3 main phenotypes: multi-drug resistance MDR (for 25 strains), extensive-drug resistance XDR (8 strains), and pan-drug resistance PDR (2 strains). Gram-negative bacteria (Proteobacteria and Bacteroidetes) showed broader resistance to the great majority of antibiotic classes as compared to the Gram-positive (Actinobacteria, Firmicutes) isolates.

Three Gram-positive Actinobacteria cave strains showed an XDR phenotype, while 9 strains displayed a MDR phenotype, showing resistance to 20 – 25 out of 28 antibiotics tested (**Figure VI.7**). Ten Gram-negative Proteobacteria isolated strains displayed a MDR phenotype, 4 strains an XDR phenotype and 2 strains a PDR phenotype (**Figure VI.8**).

Gram-negative Bacteroidetes isolates were also characterized by a broad antimicrobial resistance, all 8 strains showing resistance to more than 50% of tested antibiotics. A lower

antimicrobial resistance can be observed for the Gram-positive Firmicutes, with only one cave strain characterized as a MDR phenotype (**Figure VI.9**).

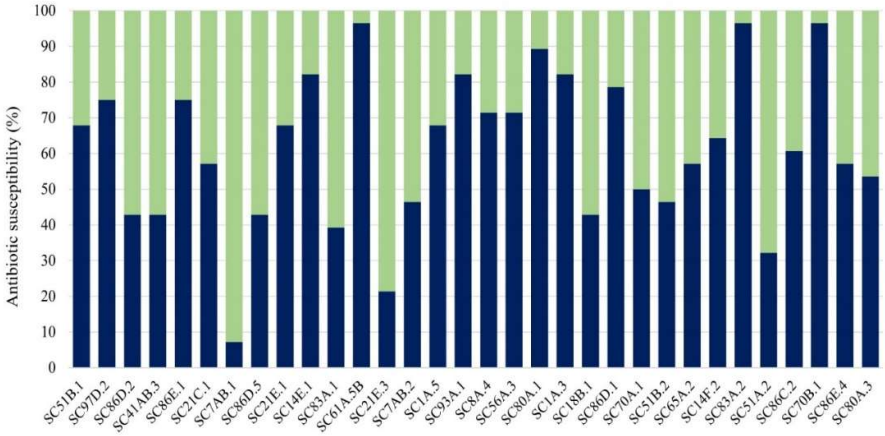

Figure VI.7 Antimicrobial susceptibility of cave ice Actinobacteria isolates – percentage of susceptible under standard dose (green) and resistant (blue) strains to the 28 tested antibiotics. (*Paun et al., 2021*)

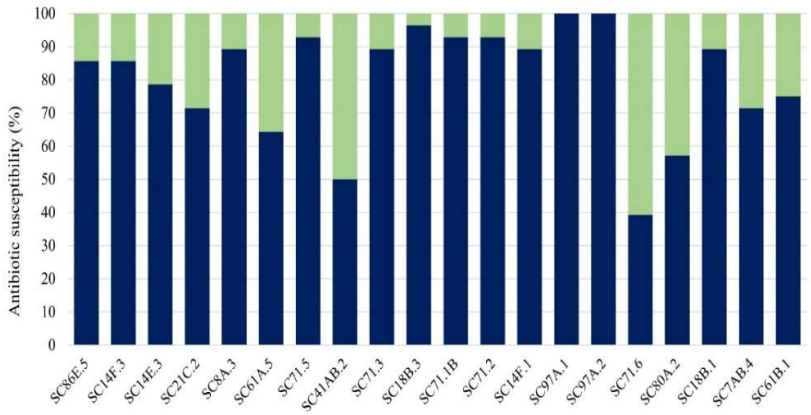

Figure VI.8 Antimicrobial susceptibility of cave ice Proteobacteria isolates – percentage of susceptible under standard dose (green) and resistant (blue) strains to the 28 tested antibiotics. (*Paun et al., 2021*)

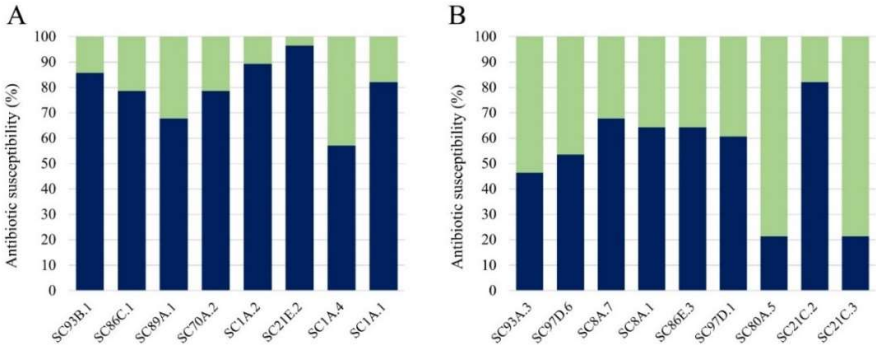

Figure VI.9 Antimicrobial susceptibility of cave ice bacterial isolates - percentage of susceptible under standard dose (green) and resistant (blue) strains to the 28 tested antibiotics was indicated for the strains belonging to **(A)** Bacteroidetes and **(B)** Firmicutes. (*Paun et al., 2021*)

6.2.2 Climate impact on the antimicrobial resistance reservoir of cave ice bacteria

Analysis of the cave strains' antimicrobial resistance in relation with the climate changes during ice deposition revealed a variable antibiotic resistance profile of cave isolates along the 13,000-years BP ice core, with MDR, XDR and PDR phenotypes predominant in the 100-years old ice, Medieval Warm Period (MWP; 953-1124 years old), Mid Holocene Warm Period (MHWP; 4751-5335 years old) and the 7,500-years old ice strata (**Figure VI.10**).

The isolated bacteria found in ice deposits accumulated during LIA, MWP and MHWP climate intervals showed a diverse resistance profile, with bacterial strains isolated from the warmer MWP and MHWP climate periods displaying a higher resistance to antibiotics as compared to those isolated from the colder LIA period (**Figure VI.10**).

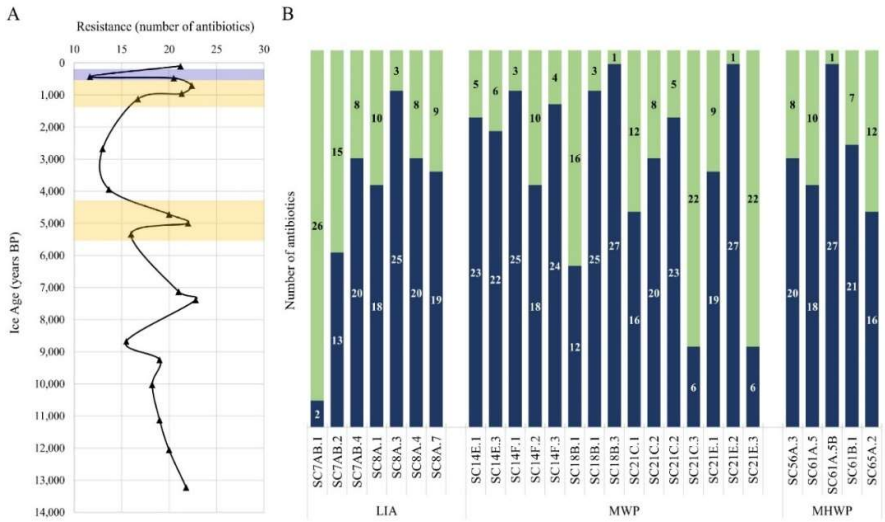

Figure VI.10 Substrate age and climate impact during ice deposition on the antimicrobial resistance of Scarisoara bacterial isolates. (*Paun et al., 2021*)

6.2.3 Antimicrobial activity

Eleven Scarisoara bacterial strains (5 Actinobacteria, 5 Proteobacteria, 1 Firmicutes) were selected based on optimal growth parameters and the diverse antimicrobial resistance profiles for further investigation of their antimicrobial activity. All isolates showed antimicrobial activity against 3 of the pathogenic strains: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* CN11 and MRSA 19081 F1.

Noticeably, the 13,000-years old *P. grimontii* SC97A.2 presented a noticeable spectrum of antimicrobial activity against 14 of the tested pathogenic strains, while the lowest antimicrobial activity was observed for two of the Scarisoara isolates, *B. toyonensis* SC86E.3 and *Pseudarthrobacter sp.* SC86E.4, showing an inhibitory effect against 6 pathogenic strain each. Furthermore, no antimicrobial activity was registered against 8 tested pathogens.

6.2.4 Biochemical characteristics

The selected 11 cave isolates were also characterized for their biochemical characteristics by screening enzymatic activities using the API (Analytical Profile Index) ZYM test system (*Gruner et al., 1992*) and for substrate assimilation using the API 20NE test system.

The functional profile obtained by API ZYM tests showed a distinct enzymatic profile of the tested cave bacteria, with none of the isolates able to metabolise the α-chymotrypsin and α-fucosidase substrates. All isolated strains showed a high enzymatic activity for Leucine arylamidase (4-5) and for Naphthol-AS-BI-phosphohydrolase (3-5).

Furthermore, API 20NE test system showed a distinct substrate utilization profile of the selected cave strains, with all 11 Scarisoara isolates able to hydrolyze β-glucosidase, while none of the strains could reduce nitrates to nitrogen and tested negative for indole production, glucose fermentation and arginine dihydrolysis. These ice cave isolates revealed a variable substrate assimilation pattern, with all tested strains able to use malate, and 10 strains could use glucose as nutritive substrates.

7. RESULTS: ISOLATION, FUNCTIONAL CHARACTERIZATION AND GENOME SEQUENCING OF THE *Psychrobacter sp.* SC65A.3 BACTERIAL STRAIN

7.1 Isolation and identification of *Psychrobacter sp.* SC65A.3

An orange/pink-pigmented colony was isolated at 4℃ from the 5,335 ± 54 years old ice sample. This bacterial strain (SC65A.3) could growth on both R2A and TSA media within a temperature interval of 4℃ - 15℃, being classified as psychrophilic according to Morita (1975). Taxonomic identification of SC65A.3 was done by PCR amplification of 16S rRNA gene and nucleotide sequencing, and BLAST analysis showed 97% identity with a homologous *Psychrobacter* strain isolated from cold environments.

7.2 Functional characterization of *Psychrobacter sp.* SC65A.3

7.2.1 Antimicrobial resistance profile

This bacterial strain exhibited a high resistance to all narrow spectrum antibiotics, metronidazole, and to 12 out of 21 of the broad-spectrum antibiotics used. Considering the observed resistance to 19 out of 28 antibiotics, *Psychrobacter sp.* SC65A.3 could exhibit a multi-drug resistance phenotype (**Figure VII.2**).

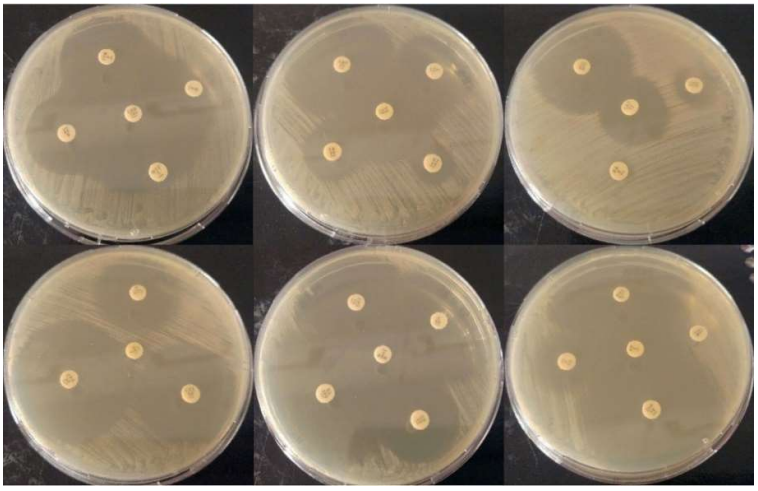

Figure VII.2 Antimicrobial resistance evaluation of Scarisoara strain SC65A.3 against 28 antibiotic disks.

7.2.2 Antimicrobial activity

The antimicrobial activity of SC65A.3 showed the inhibition against both *S. aureus* and *E. coli* ATCC collection strains, and 12 of the 20 clinical pathogens tested. Most notably, SC65A.3 exhibited an antimicrobial activity against Gram-negative pathogens belonging to genera *Enterobacter* (2 strains), and 3 each of *Pseudomonas* (3 strains) and *Klebsiella* (3 strains), and *E. coli* (**Table VII.2**).

Table VII.2 Antimicrobial activity of *Psychrobacter sp.* SC65A.3.

Test pathogen	Antimicrobial activity of *Psychrobacter sp.* SC65A.3 (+/-)
Staphylococcus aureus ATCC 25923	+
Escherichia coli ATCC 25922	+
Enterobacter asburiae 19069 ONE1	-
Enterobacter cloacae 19069 ONE2	+
Enterobacter cloacae 19069 ONE3	+
Pseudomonas CN11	+
Pseudomonas aeruginosa 19053 CNE5	+
Pseudomonas aeruginosa 19053 CNE6	+
MRSA 19081 F1	+
MRSA 19081 S1	-
MRSA 388	-
Klebsiella 8	-
Klebsiella 19094 CK1	+
Klebsiella 19094 CK2	+
Klebsiella 19094 CK3	+
Acinetobacter 19047 ENE4	-
Acinetobacter 19047 CNE5	-
Acinetobacter 19047 CNE3	-
Acinetobacter 18032 C3	-
Enterococcus falcium 19040 E1	+
Enterococcus falcium 19040 E2	+
Enterococcus falcium 19040 E3	+
Total	**14**

7.2.3 Biochemical characterization

Screening of the enzymatic activities of SC65A.3 using API ZYM test system evidenced a series of high enzymatic activities such as Lipase (C14), Alkaline phosphatase, Esterase (C 4), Esterase Lipase (C 8) and Naphthol-AS-BI-phosphohydrolase (**Table VII.3**).

The substrate utilization profile of the Scarisoara strain determined using the API 20NE test system, showed the ability to reduce nitrates to nitrites, to metabolize urea, and have a positive esculin hydrolysis and cytochrome oxidase activity (**Figure VII.5**).

Figure VII.5 API 20NE test system results for *Psychrobacter sp.* Scarisoara strain SC65A.3.

Table VII.3 API ZYM enzymatic activity evaluation of *Psychrobacter sp.* SC65A.3.

No.	Enzymatic activity	Applicative potential	Results
1	Control		**0**
2	Alkaline phosphatase	Clinical diagnostics; chromogenic assays; dairy industry	**3**
3	Esterase (C 4)	Food processing; beverage, perfume, pharmaceutical & chemical industries; agriculture & degradation of synthetic materials	**3**
4	Esterase Lipase (C 8)		**3**
5	Lipase (C 14)	Detergents industry; pharmaceutical industry; biofuels; food processing industry.	**4**
6	Leucine arylamidase	Food industry	**3**
7	Valine arylamidase		**2**
8	Cystine arylamidase		**2**
9	Trypsin	Food processing industry	**1**
10	α-chymotrypsin		**1**
11	Acid phosphatase		**0**
12	Naphthol-AS-BI-phosphohydrolase	Clinical markers for disease	**3**
13	α-galactosidase		**0**
14	β-galactosidase		**0**
15	β-glucuronidase		**0**
16	α-glucosidase	Producing IMOs with prebiotic activity; biofuel industry; medical biosensors.	**1**
17	β-glucosidase		**0**
18	N-acetyl-β-glucosaminidase	Clinical applications	**1**
19	α-mannosidase		**0**
20	α-fucosidase		**0**

The salinity range for the growth of SC65A.3 was of 0 M – 1.9 M NaCl, and 0M – 0.9 M MgCl2, when cultivated at 15°C in LB medium for 7 days.

7.3 Whole genome sequencing and analysis

The whole genome of SC65A.3 cave strain was sequenced for identifying structural elements associated with the natural antibiotic resistance of this cave strain entrapped in ice for the last 5 millennia and for future studies investigating novel cold-active enzymes and bioactive molecules.

De novo genome assembly generated a unique circular contig of 3,046,103 bases length, with a GC content of 42.52%. BLAST analysis showed the highest homology score with *Psychrobacter cryohalolentis* [CP022043.2]. Analysis of the assembled SC65A.3 genome revealed the presence of 2,602 genes among (**Table VII.7**). The functional annotation using EggNOG database revealed a number of 2,536 coding regions (CDS), from which 29 loci/genes were associated with antibiotic resistance, most belonging to the defense mechanism category with the EggNOG "V" notation.

Table VII.7 Genome annotation for Scarisoara strain SC65A.3.

Contig name	Length	Number of genes	CDS	tRNA	rRNA
SC65A.3	3,046,103	2,602	2,536	50	15

A maximum-likelihood phylogenetic analysis (16S rRNA gene sequence) was performed in order to show the relationship between *Psychrobacter sp.* strain SC65A.3 and the 17 most closely related reference species (**Figure VII.9**). The analysis indicated a close affiliation with psychrotolerant and psychrophilic *Psychrobacter* strains, while clustering with 5 psychrophilic *Psychrobacter* species: *P. adeliensis*, *P. okhotskensis*, *P. urativorans*, *P. glacincola* and *P. cryohalolentis*. (**Figure VII.9**).

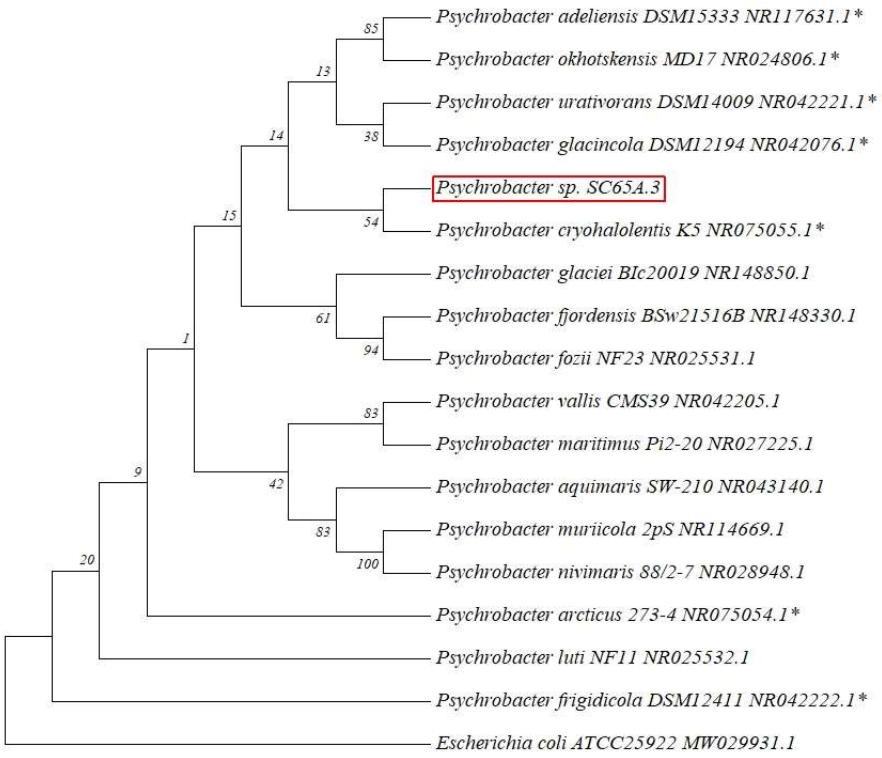

Figure VII.9 Maximum-likelihood phylogenetic tree between strain SC65A.3 (red box) and the 17 most closely related reference species (* = psychrophilic *Psychrobacter species*).

8. GENERAL CONCLUSIONS AND PERSPECTIVES

This study reports the investigation of Scarisoara microbiome from the oldest ice accumulated in caves and focused on identification and characterization of the bacterial community structure and function starting from the sampling of a 13,000-years old ice core chronosequence from Scarisoara ice cave, Romania.

Both the total and potentially active bacterial communities, and isolated bacterial strains were investigated in order to extend the knowledge on this particular cold-environment microbiome and highlight its applicative potential. In addition, the whole genome of a Scarisoara isolated strain from 5,335 ±54 years old cave ice was obtained and characterized for the first time.

Different bacterial taxa common to cold environments dominated both types of communities, revealing a distinct relative abundance pattern of total and potentially active bacteria across the ice chronosequence.

The uncultured bacterial community (gDNA samples) was dominated by Actinobacteria and Proteobacteria in most ice age layers, except for the 7,000-years old ice layer, where Firmicutes was the most dominant phylum.

In the potentially active community (cDNA) Proteobacteria and Firmicutes dominated the chronosequence, while being homogenously distributed. Bacteroidetes OTUs were found in all ice layers, from both types of communities, but less represented than the other three most prominent phyla.

This data also correlates with the low number of Bacteroidetes isolated strains found in Scarisoara ice. Even if Firmicutes was one of the dominant phyla of the potentially active community, a rather low number of bacterial strains were isolated and characterized from Scarisoara ice.

The overall microbial cell density determined in Scarisoara Ice block is rather low, highlighting a particular spike in the 6,000-years old layer. Correlation of microbial cell density with the dissolved organic carbon (DOC) content and silica concentrations revealed the major contribution of depositional processes in modeling the ice microbiome abundance throughout the cave ice block.

A more active microbiome capable of growth in ice strata associated to a more abundant microbiome and higher DOC content was observed, making the organic carbon contents of the Scarisoara ice core samples potentially responsible for shaping the bacterial community composition from this perennial ice block.

70 distinct cold-active strains were isolated, identified by 16S rRNA gene sequencing, and characterized regarding their growth temperature, antibiotic resistance, enzymatic and antimicrobial activity profiles. These strains represent the first bacteria isolated from perennial cave ice accumulated in the last 13,000-years, providing also the first culture-based evidence of a resistome from this type of pristine environment.

The bacterial isolates from Scarisoara ice belonged to 4 phyla, 34 genera and 56 species, with most of them belonging to Actinobacteria, followed by Proteobacteria, Firmicutes and Bacteroidetes. The low 16S rRNA gene identity percentage (<97%) with homologous database counterparts in the case of 18 of the strains could correspond to putative new ice cave species.

Based on growth temperature profile, 5 psychrophiles were identified, while the rest were psychrotolerant bacteria. 13 of the Scarisoara isolates were homologous to strains reported in other cold environments, among which 11 were characterized as psychrotrophs.

The antibiotic susceptibility profile of all the Scarisoara bacterial isolates revealed that Gram-negative strains were more resistant to the majority of tested antibiotics as compared to the Gram-positive ones. 35 Scarisoara strains exhibited MDR, XDR and PDR phenotypes.

The multi-drug resistance phenotypes prevalence in the perennial Scarisoara ice corroborated with the multiple resistance from old ice samples from permafrost, Arctic soil and samples from a cave isolated from the surface for over 4 Myr (*D'Costa et al., 2011*; *Perron et al., 2015*), shows the existence of a diverse resistome, where resistance is correlated with cold-adaptation mechanisms, and also with HGT of integron-associated antimicrobial resistance genes between local bacterial communities (*Miller et al., 2014*).

Due to the extended resistance profile and the antimicrobial activity of all isolates, particularly against the Gram-negative pathogens, these cave bacterial strains retrieved from the 13,000-years old ice chronosequence could provide important responses that might help unravel the evolution of natural and clinical antibiotic resistance (*Paun et al., 2021*).

The enzymatic activities profile of selected cold-loving Scarisoara isolates, indicated their potential as a valuable source of new catalysts for various industries including clinical biomarkers and diagnostics, food and dairy industries, biofuel production, prebiotic industry and medical biosensing, pharmaceutical, cosmetics and detergent production.

The whole genome sequence of a Scarisoara isolated strain was determined and characterized for the first time. The 16S rRNA gene sequence of the psychrophilic SC65A.3 Scarisoara strain was 97% homologous with an Arctic *Psychrobacter glaciei* strain *(Zeng et al., 2016*), showing resistance to 19 out of 28 antibiotics, and having antimicrobial activity against 14 tested pathogens. Based on the biochemical profile of SC65A.3, this bacterial strain has the potential to be further explored in order to obtain performant cold-active biocatalysts for various industries (Gheorghita et al., 2021).

The reported data represent the first characterization of antibiotic resistance and antimicrobial activity of a bacterial strain isolated from the perennial ice of Scarisoara Cave, in addition to promising enzymatic activities with industrial applicative potential.

The whole genome sequence of this strain will be further explored to unravel the molecular basis of SC65A.3 strain's natural resistome and to identify new molecules for fighting the current antimicrobial broad resistance and putative new cold-active high-stability biocatalysts.

The SC65A.3 genome represent a unique circular contig of 3,046,103 bases length homologous (97% identity) to a Siberian permafrost *Psychrobacter cryohalolentis* strain (Bakermans et al., 2006) containing 2,602 genes, 2,536 CDS genes, 50 genes coding for tRNA and 15 genes coding for rRNA. Among these, a number of 29 putative genes involved in antibiotic resistance were highlighted.

Further studies will make the object of in-depth investigation of various metabolic pathways and functional genes of this Scarisoara cave isolated strain exploiting the genome sequence information by structural analysis as well as functional conformation of by RT-PCR and gene cloning of obtaining and characterizing new recombinant cold-active biocatalysts.

DISSEMINATION OF RESULTS

Published articles

1. **V.I. Paun**, G. Icaza, P. Lavin, C. Marin, A. Tudorache, A. Persoiu, C. Dorador, C. Purcarea (2019) *Total and potentially active bacterial communities from a Late Glacial through Holocene ice core of Scarisoara Ice Cave, Romania*. **Front. Microbiol**. 10:1193. doi: 10.3389/fmicb.2019.01193 (**Q1**)

2. **V.I. Paun**, P. Lavin, M.C. Chifiriuc, C. Purcarea (2021) *First report on antibiotic resistance and antimicrobial activity of bacterial isolates from 13,000-year old cave ice core.* **Sci. Rep.** 11:514. doi: 10.1038/s41598-020-79754-5 (**Q1**)

3. Gheorghita G.R., **Paun V.I.,** Neagu S., Maria G.-M., Enache M., Purcarea C., Parvulescu V.I., Tudorache M. (2021) *Cold-Active Lipase-Based Biocatalysts for Silymarin Valorization through Biocatalytic Acylation of Silybin*. **Catalysts.** 11:1390. doi: 10.3390/catal11111390 (**Q2**)

Other published articles

1. Iancu L., Angelescu I.R., **Paun V.I.**, Henríquez-Castillo C., Lavin P., Purcarea C. (2021) *Microbiome pattern of Lucilia sericata (Meigen) (Diptera: Calliphoridae) and feeding substrate in the presence of the foodborne pathogen Salmonella enterica.* **Sci. Rep.** 11:15296. doi: 10.1038/s41598-021-94761-w (**Q1**)

2. Necula-Petrareanu G, Lavin P, **Paun VI**, Gheorghita GR, Vasilescu A, Purcarea C. (2022) *Highly Stable, Cold-Active Aldehyde Dehydrogenase from the Marine Antarctic Flavobacterium sp. PL002*. **Fermentation**. 8(1):7. doi: 10.3390/fermentation8010007 (**Q1**)

3. Mondini A., **Paun V**., Necula-Petrareanu G., Iancu L., Purcarea C., 2019, *Cold adaptation mechanisms of aspartate transcarbamoylase from Glaciibacter superstes, an Arctic psychrophilic bacterium*, Romanian Journal of Biology, 64, 19 – 30. (BDI)

4. **Victoria I. Paun**, Georgiana Necula-Petrareanu, Antonio Mondini, Cristina Purcărea. 2018. "*Aspartate transcarbamoylase obtained from a psychrophilic bacterium (Rugamonas sp.) isolated from an Antarctic lake*". In: The novel results of the Institute of Biology Bucharest into fields of Ecology, Microbiology and Citobiology, Ed. Ars Docendi, Bucuresti. ISBN 978-606-998-044-6 (Book chapter)

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