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Stalactites Core Prospect as Environmental "Microbial Ark": The Actinomycetota Diversity Paradigm, First Reported from a Greek Cave

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Abstract

Speleothems found in caves worldwide are considered the natural libraries of paleontology. Bacteria found in these ecosystems are generally limited to Proteobacteria and Actinomycetota, but rare microbiome and "Dark Matter" is generally under-investigated and often neglected. This research article discusses, for the first time to our knowledge, the diachronic diversity of Actinomycetota entrapped inside a cave stalactite. The planet's environmental microbial community profile of different eras can be stored in these refugia (speleothems). These speleothems could be an environmental "Microbial Ark" storing rare microbiome and "Dark Matter" bacterial communities evermore.

K e y w o r d s: Actinobacteria, biogeography, rare microbiome, Microbial Dark Matter, refugium model, extreme environment, paleomicrobiology

Introduction

Extreme ecosystems, like those encountered in saltwater environments, are frequent reservoirs of rock salt or "rock salt", especially ancient microorganisms that are caught inside saltwater droplets. In addition, deep-sea sediments provide a cold, oxygen-free, UV-free environment that can facilitate the preservation of virus-containing microorganisms. Isolating pathogenic bacteria from human and animal samples can provide data on pathogenicity, source, systematic geography, and development of targeted microorganisms (Komnenou et al. 2021, Koitsanou et al. 2022). This process is well-known as retrospective disease diagnosis (Rivera-Perez et al. 2016). The epidemiology of old infectious diseases, particularly in people, may provide important information on nutrition, people density, and health dispersal status. Most established ancient pathogens are the etiologic pathogen of leprosy (*Mycobacterium leprae*) and tuberculosis (*Mycobacterium tuberculosis*), diseases that have plagued humanity for hundreds of years. DNA associated with these agents has been found in ancient cadavers discovered in different geographic areas of the globe (Rivera-Perez et al. 2016).

Analyzing the spread of these ancient pathogens and the co-evolution of host microorganisms is important for understanding the mechanisms of these bacteria association with animals and humans. Furthermore, it helps in finding new therapies. For instance, data gathered from both animal and environmental and aDNA research may yield insights including the composition and evolution of the genome before the extensive use of antibiotics, in addition to the virulence components and other mobile factors found in modern analogous available (Lemma et al. 2008).

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Speleothems in caves worldwide (stalagmites, stalactites, river rocks, etc.) are natural libraries of paleontology formed by groundwater, seawater, and downfall, respectively (Michail et al. 2021). They contain small volumes of water trapped within and between the calcite crystals of commonly named liquid inclusions, like the bubbles in ice cores. Rainwater from the cave surrounding area penetrates the ground and epicarst; consequently, it is carried through microcracks and eventually drips from the soda straws suspended from the roof of the cave down on the stalagmites (Michail et al. 2021). Based on the above, it is difficult to find instant, unchanged water, dispersed by previous rainfall on land and in caves around the world, able to construct stalactites and stalagmites that incorporate fossil water and trap well-preserved bacteria over millennia, a natural environment (Affolter et al. 2019).

Bacteria that grow in these environments are generally restricted to Proteobacteria, which are more abundant in air and water samples, and Actinomycetota, which are dominant in rock and sediment samples (Zhu et al. 2019). Recent data have shown that cave sediments, as oligotrophic environments, host a highly heterogeneous phylogenetic pluralism dominated by Actinomycetota and Proteobacteria. The prevalence of Proteobacteria species in the stalactite core structure may be related to their fast development rates in rich nutrient habitats (uppermost ground cave surroundings), establishing a substitute for the ground carbon input (Michail et al. 2021). De Mandal et al. (2017) proposed that bacteria correspond to a significant part of a cave's ecosystem and play an essential role in preserving a cave's biodiversity. Several scientists reported that subterranean stalactites could function as natural traps for bacteria in the fissure water (Coolen et al. 2011; Borgonie et al. 2015). Furthermore, learning about the rare microbial biosphere and its capabilities avail to the approach of screening microbial ecology at different levels: from host-microbiome relationships (Dawson et al. 2017; Troussellier et al. 2017; Tierney et al. 2020) to microbial reactions due to climate deviations (Fernández-Gómez et al. 2018; Liang et al. 2020).

Although caves have been investigated for many years, their rare microbiome is generally under-investigated and often neglected. Caves have been engaging the attention of microbiologists, in particular their microbial divergence, over the last 20 years (Laiz et al. 1999; Barton et al. 2004; Barton 2006; Michail et al. 2021). With the blending of exceptional conditions, including high humidity, moderate low but steady temperature, and scarce nutrients, caves are predicted to sanctum new microorganisms with biological activities like the γ-Proteobacteria, *Serratia proteamaculans*, and *Serratia liquefaciens* isolated from bat guano (Rangseekaew and Pathom-Aree 2019; Michail et al. 2021). The harmonization of Actinomycetota to unfriendly habitats and the unveiled related synergies have led to the development of unique biotechnological capabilities (Farda et al. 2022). Members of Actinomycetes are described to be the prevailing microbial community in many subterranean environments (Groth and Saiz-Jimenez 1999; Cheeptham et al. 2013; Tomczyk-Zak et al. 2016; Ghosh et al. 2017; Michail et al. 2021).

Until recently, most of the classification of the Actinomycetota species has been established based on biochemical and cultural procedures, acknowledging a substantial proportion of divergence present. Along with the dawn of modern sequencing techniques, it will be plausible to acquire considerable significant information concerning the cave's rare microbiome (Farda et al. 2022). Most scientific research is based on Actinomycetota isolated inside the cave environment and on superficial growth and evolution (Rangseekaew and Pathom-Aree. 2019; Farda et al. 2022).

This research presents and discusses the taxonomic outcome of a stalactite's Krona pie. We thoroughly believe that our approach offers the best perspective of the up-to-date Actinomycetota important biodiversity. Our detailed research goal goes beyond our previous study (Michail et al. 2021) of classification and illustrates for the first time the Actinomycetota entrapped over time inside a cave stalactite core.

Experimental

Materials and Methods

Research site. The sample site was Agios Athanasios cave. The cave is sited in central Greece, region of Thessaly (39°28'43.9'N, 22°52'53.9'E, 400 m above sea level, Fig. 1) and formed about a 200 m long horizontal labyrinth of passages. The cave is between 20- and 30-meters underground, and there no light is present. Water from Karla's Lake covers the area around the cave, and the dominating tree species on the peninsula is *Quercus coccifera*. Archaeologists have discovered evidence that the cave was utilized during the Mycenaean and Neolithic eras. It is regarded as one of the most historically prominent caves of Magnesia and Thessaly in general due to the findings unearthed there and its size.

Stalactite collection. For this investigation, we handled symmetrically sized, 7.5–8 cm diameter subsamples from a stalactite taken with sterile gloves and stored in sterile plastic containers. Inside Agios Athanasios cave, the stalactite sample was acquired in 2021 within a depth of more than 50 meters (Fig. 2). As presented by Durazzi et al. (2021), there are no statistically significant differences depicted among phyla, families, and genera, so in the present work, we firstly reported taxonomic results

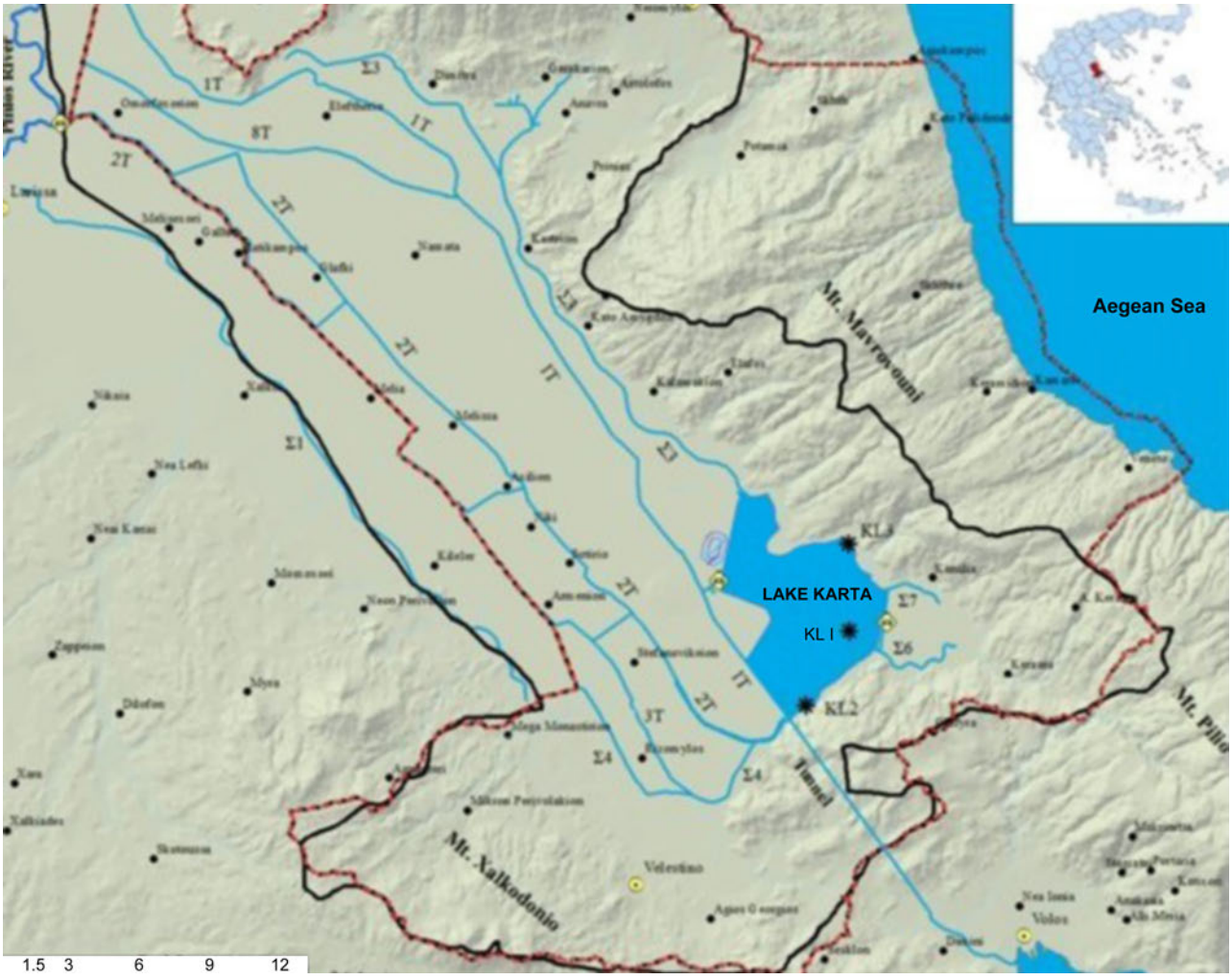


Fig. 1. Lake Karla location in Central Greece.

acquired by metataxonomics (16S rRNA gene sequencing) from a stalactite core sample. Noteworthy is that strict regulations, which Greek National Environmental and Archeological Authorities apply for all subterranean areas, restricted us from acquiring more stalactites as core samples. Nevertheless, the material was adequate to double-check our methodological approach and validate aliquots as tetra-plicate iterations.

DNA extraction and DNA sequence analysis. Following the manufacturer's recommendations, a NucleoSpin Soil DNA isolation kit (Macherey-Nagel, Germany) was used to extract the entire metataxonomic DNA from our stalactite sample rapidly. The same core sample in aliquots was replicated four times, according to Michail et al. (2021); however, DNA was extracted using different settings. The Quawell UV-Vis Spectrophotometer (Quawell Ltd., EU) was used to assess the DNA's quality and concentration (Q5000). Before the libraries were prepared, the DNA samples were kept at 20°C.

Samples taken from the stalactite core were sequenced at the Institute for Applied Biosciences, Thessaloniki, Greece. Illumina's 16S rRNA metataxonomics protocol (part number 15044223 rev. B; Illumina, Inc., USA) was used to amplify the V3 and V4 regions of the bacterial 16S rRNA gene utilizing the following primers containing Illumina overhang adaptors. Universal forward and reverse primer sequences used were those described by Klindworth et al. (2013), 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG-TATCTAATCC-3', respectively, yielding an amplicon of approximately 460 bp. 2x Kapa HiFi Hot-Start ReadyMix (Roche, Switzerland) was used for PCR amplification, and PCR products were purified with Beckman Coulter Agecourt AMPure XP Beads (Beckman Coulter, USA) according to the protocol.

Illumina indexes and sequencing adapters were attached using the Nextera® XT Index Kit (Illumina, USA), followed by secondary purification using Beckman Coulter Agecourt AMPure XP Beads. Library quantification was performed using a Qubit™ 3.0 fluorometer (Life Technology Ltd., UK). Additionally, 2 μl of the final library was run on the Fragment Analyzer



Fig. 2. Development of a speleothem at 50 m depth inside Agios Athanasios cave, Thessaly, Greece.

using the DNF-473-33-SS NGS Fragment 1–6,000 bp method to confirm library quality and size. After library quantification, normalization, and pooling, the concentration was adjusted to 125 pM and prepared for loading onto the Illumina MiSeq following Illumina's 16S rRNA metataxonomics protocol (part number 15044223 rev. B; Illumina, Inc., USA). Library pools were denatured and loaded onto the Illumina MiSeq at 12.5 pM, and paired-ends (2,300) were sequenced using the MiSeq Reagent Kit v3 (600 cycles) (Illumina, Inc., USA).

Moreover, one more significant issue that should be given attention to is that inside the stalactite core, the abundance of the bacteria entrapped is scarce, with a lower number of reads (less than 500,000) in contrast to other biological samples (i.e., alimentary tract with up to 2^{19} reads). Clearly, for this situation, the utilization of metataxonomics (16S rRNA) is ideal and functions in much more the same way, or even as far superior to other metagenomics (i.e., entire shotgun metagenomic sequencing) (Klindworth et al. 2013; Michail et al. 2021).

Bioinformatics and statistical analysis. The raw sequence data were processed using the Mothur pipeline (Schloss et al. 2009) and performed standard operating procedures (SOPs) for MiSeq data developed by the creators of Schloss Lab, a Mothur software package within Galaxy (Kozich et al. 2013; Afgan et al. 2018).

Firstly, we organized the 300 bp paired-end reads into paired collections and combined the forward and reverse reads for each sample into a single FASTA file. We then performed quality control and data cleaning to remove ambiguous bases and sequences over 460 bp. The files for computation are optimized to remove duplicate sequences. Thirdly, the sequence reads were compared to the Silva database reference alignment (silva.seed_v138_1.align) using Needleman alignment and k-mer search methods.

Further denoising of the sequences was performed by pre-grouping and sorting them by frequency. Finally, sequences artifacts known as chimeras and non-bacterial sequences such as archaeal, eukaryotic, and unknown were removed. Operational taxonomic units (OTUs) were identified at a DNA similarity level of 97% and an order control level. A total of 5,516 OTUs associated with bacteria were considered.

All of the above datasets are available as supplementary material such as order Micrococcales (Fig. S1–S6); order Propionibacteriales (Fig. S7–S11); order Pseudonocardiales (Fig. S12); order Mycobacteriales (Fig. S13–S16); order Rubrobacterales (Fig. S17); order Geodermatophilales (Fig. S18 and S19); order Micromonosporales (Fig. S20–S22); order Jiangellales (Fig. S23 and S24); order Streptosporangiales (Fig. S25 and S27); order Sporichthyales (Fig. S28); order Kineosporiales (Fig. S29); order Bifidobacteriales (Fig. S30); order Acidothermales (Fig. S31); order Gaiellales (Fig. S32); order Solirubrobacterales (Fig. S33–S36); order Acidimicrobiales (Fig. S37 and S38).

Results

Fig. 3–6 represent the bacterial communities and reads in the taxonomic rank of phylum (Fig. 3), class (Fig. 4), order (Fig. 5 and 6), and family (Fig. 6), recorded from our cave stalactite core in Agios Athanasios cave from central Greece, Thessaly.

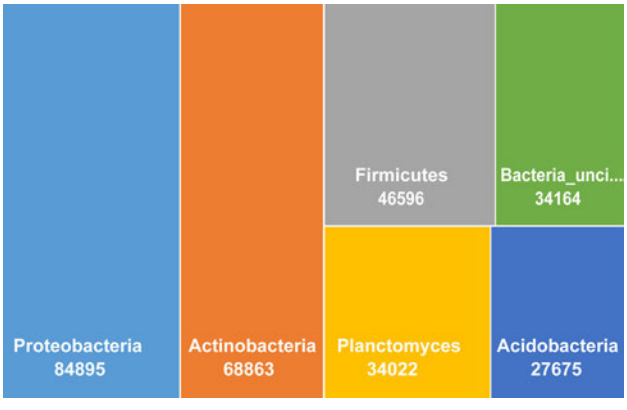


Fig. 3. Reads, bacteria phylum classification, and quantitation (e.g. Proteobacteria – 84,895 reads of Bacteria). Phylum Actinobacteria (Goodfellow et al. 2012) syn = Actinomycetota (Oren and Garrity 2022).

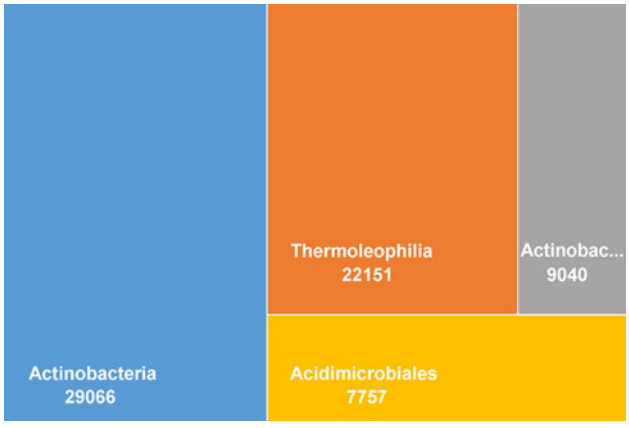


Fig. 4. Reads of the phylum Actinobacteria (syn = Actinomycetota) in the classification and quantitation of class (e.g. Thermoleophilia – 22,151 reads of Bacteria).

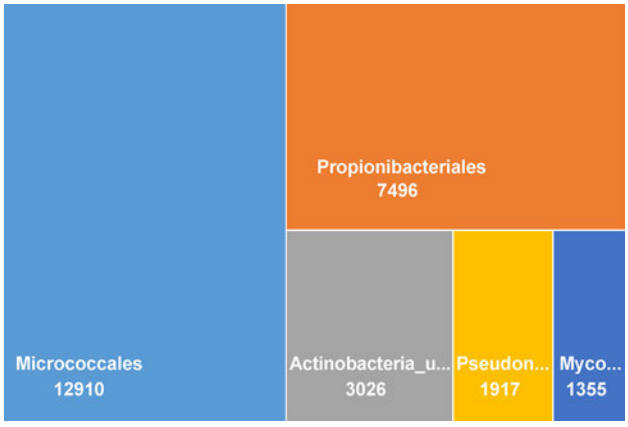


Fig. 5. Reads of the phylum Actinobacteria (syn = Actinomycetota) in the classification and quantitation of order (e.g. Micrococcales – 12,910 reads of Bacteria).

Data presentation. Tables SI to SXVI, shown in the supplementary materials' section illustrate the bacterial communities and reads found in our stalactite core in the taxonomic rank of orders, such as Micrococcales, Propionibacteriales, Pseudonocardiales, Mycobacteriales, Rubrobacterales, Geodermatophilales, Micromonosporales, Jiangellales, Streptosporangiales, Sporichthyales, Kineosporiales, Bifidobacteriales, Acidothermales, Gaiellales, Solirubrobacterales, and Acidimicrobiales, respectively.

In each Table, within each bacteria type order, the validly published families were recorded as presented by the LPSN.dsmz.de database. Moreover, the family and genus lists of bacteria found in our stalactite core are also given.

Furthermore, in each bacteria order of the Actinomycetes class, we solely discussed the abundance of the bacteria genera found in our stalactite's core from central Greece.

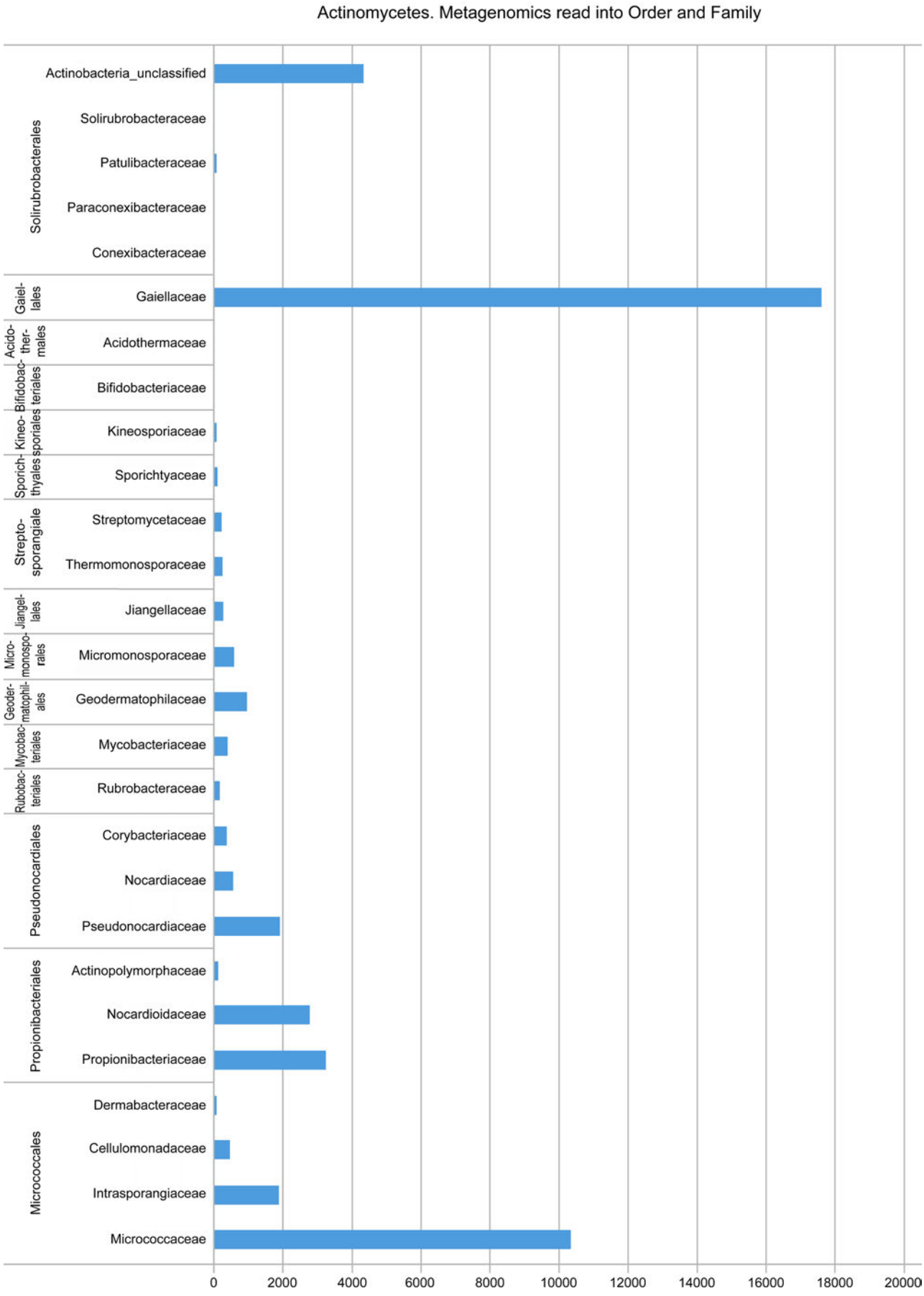


Fig. 6. Reads of the class Actinomycetes into order and family taxonomy (e.g. family taxonomy *Micrococcaceae* – 10,309 reads of Bacteria).

Order Micrococcales (Table SI)

Pseudarthrobacter (7,880 reads of bacteria or 80% of *Micrococcaceae* (family), 61% of Micrococcales (order), 11% Actinobacteria (phylum), and 2% of total Bacteria), (Fig. S1). A study on the isolation and characterization of *Pseudarthrobacter* species from Antarctic soil has shown that they are crucial for our understanding of Antarctic soil-related bacterial communities and for developing commercially beneficial cold-active enzymes (Shin et al. 2020).

Kocuria (479 reads of bacteria or 5% of *Micrococcaceae* (family), 4% of Micrococcales (order), 0.7% Actinobacteria (phylum) and 0.1% of total Bacteria), (Fig. S2). *Kocuria* species are skin and oropharynx rare bacterium in mammals (including man), and environmental organisms inhabiting the soil and several other ecological niches. Studies on species of the *Kocuria* genus showed that they were mainly located in Asia, and from Egyptian desert to Antarctica and to Siberian Sea (Savini et al. 2010).

Paeniglutamicibacter (3 reads of bacteria or 0.03% of *Micrococcaceae* (family), 0.02% of Micrococcales (order), 0.004% Actinobacteria (phylum), and 0.0009% of total Bacteria) (Fig. S3). Studies on *Paeniglutamicibacter* species demonstrated that they were frequently reported from Antarctic and alpine environments (Sakdapetsiri et al. 2019).

Oryzihumus (1,299 reads of bacteria or 70% of *Intrasporangiaceae* (family), 10% of Micrococcales (order), 2% of Actinobacteria (phylum), and 0.4% of total Bacteria) (Fig. S4). All species of *Oryzihumus* genus were primarily isolated in Asia (Kim et al. 2017).

Cellulomonas (458 reads of bacteria or 100% of *Cellulomonadaceae* (family), 4% of Micrococcales (order), 0.7% Actinobacteria (phylum) and 0.1% of total Bacteria) (Fig. S5). *Cellulomonas* species are mostly isolated from environmental samples (e.g., stored paddy rice, soil, air, seawater, and decayed tree), some from human clinical samples, rarely from wild animals, and the vast majority of environmental isolations of *Cellulomonas* spp. derive from Asia (Han et al. 2022).

Brachybacterium (65 reads of bacteria or 100% of *Dermabacteraceae* (family), 0.5% of Micrococcales (order), 0.09% Actinobacteria (phylum) and 0.02% of total Bacteria), (Fig. S6). Habitats range of *Brachybacterium* spp. are remarkably broader, ranging from sediment, soil, and poultry waste to steep corn liquor, medieval paints, and the rhizosphere (Stackebrandt 2014).

Order Propionibacteriales (Table SII)

Microlunatus (2,665 reads of bacteria or 83% of *Propionibacteriaceae* (family), 36% of Propionibacteriales (order), 4% of Actinobacteria (phylum) and 0.8% of Bacteria), (Fig. S7). And in this occasion, the majority of species of the *Microlunatus* genus were isolated in Asia (Xie et al. 2020).

Cutibacterium (551 reads of bacteria or 17% of *Propionibacteriaceae* (family), 7% of Propionibacteriales (order), 0.8% of Actinobacteria (phylum) and 0.2% of Bacteria), (Fig. S8). Species of that genus exist in different environments reflecting their uniqueness in their genetic makeup. The pathogenicity of these species includes inflamed acne, progressive macular hypomelanosis (PMH), infections in the heart, bone, breast, and lumbar discs, prostate cancer, liver abscesses, and sarcoidosis (Dekio et al. 2021).

Nocardioides (1,276 reads of bacteria or 49% of *Nocardioidaceae* (family), 17% of Propionibacteriales (order), 2% of Actinobacteria (phylum) and 0.4% of total Bacteria), (Fig. S9). The genus contains species with strains isolated from different environmental niches (Roh et al. 2020).

Marmoricola (1,269 reads of bacteria or 48% of *Nocardioidaceae* (family), 17% of Propionibacteriales (order), 2% of Actinobacteria (phylum), and 0.4% of total Bacteria), (Fig. S10). Species of the genus were isolated from various environments such as marble, marine sediment, soil, volcanic ash, marine sponge, and mangrove plant (Habib et al. 2020).

Aeromicrobium (21 reads of bacteria or 0.8% of *Nocardioidaceae* (family), 0.3% of Propionibacteriales (order), 0.03% of Actinobacteria (phylum), and 0.006% of total Bacteria), (Fig. S11). Species of the genus were isolated from various environments (Siddiqi et al. 2018).

Order Pseudonocardiales (Table SIII)

Pseudonocardia (1,492 reads of bacteria or 78% of *Pseudonocardiaceae* (family), 78% of Pseudonocardiales (order), 2% of Actinobacteria (phylum), and 0.46% of total Bacteria), (Fig. S12). They were discovered from different environmental samples, including soil, marine sediments, activated sludge, termite nests, gold mine core, and plant tissues (Parte et al. 2020).

Order Mycobacteriales (Table SIV)

Rhodococcus (517 reads of bacteria or 94% of *Nocardiaceae* (family), 38% of Mycobacteriales (order), 0.8% of Actinobacteria (phylum) and 0.1% of total Bacteria), (Fig. S13). Studies on members of the genus *Rhodococcus* demonstrated that they were isolated from different habitats, including soil, bug, wastewater, bat-dried guano of a cave, marine coral reef, from the coldest, desiccating, and oligotrophic permafrost soils of the high-elevation McMurdo Dry Valleys in Antarctica, and from Comau fjord of a pristine Marine Protected Area in the Chilean Patagonia (Undabarrena et al. 2018).

Corynebacterium (312 reads of bacteria or 100% of *Corynebacteriaceae* (family), 23% of Mycobacteriales (order), 0.5% of Actinobacteria (phylum) and 0.09% of

total Bacteria), (Fig. S14). They are widespread and can be isolated from the skin and the upper respiratory and gastrointestinal tracts. The strains of medical and veterinary interest are generally divided into two subgroups: diphtheria and non-diphtheria (Araújo et al. 2018).

Smaragdicoccus (27 reads of bacteria or 5% of *Nocardiaceae* (family), 2% of Mycobacteriales (order), 0.04% of Actinobacteria (phylum) and 0.008% of total Bacteria), (Fig. S15). A study on *Smaragdicoccus niigatensis* has shown that it is the only species member of the *Smaragdicoccus* genus isolated from petroleum-contaminated soil, obtained from a spurt of petroleum at Nishiyama-cho in Niigata, Japan (Adachi et al. 2007).

Mycobacterium (382 reads of bacteria or 100% of *Mycobacteriaceae* (family), 28% of Mycobacteriales (order), 0.58% of Actinobacteria (phylum) and 0.11% of total Bacteria), (Fig. S16). Studies on several significant human and animal pathogens including members of the *M. tuberculosis* complex and *Mycobacterium avium* subspecies *paratuberculosis*, the etiological agent of Johne's disease, a contagious disease listed by the World Organization for Animal Health can be enumerated (Brites et al. 2018).

Order Rubrobacterales (Table SV)

Rubrobacter (148 reads of bacteria or 100% of *Rubrobacteraceae* (family), 0.2% of Actinobacteria (phylum), and 0.04% of total Bacteria), (Fig. S17). Many studies on *Rubrobacter* species reveled that they were isolated from marine environments; thus, the ocean is a unique habitat for *Rubrobacter* organisms (Chen et al. 2020).

Order Geodermatophilales (Table SVI)

Blastococcus (591 reads of bacteria or 63% of *Geodermatophilaceae* (family), 0.9% of Actinobacteria (phylum), and 0.2% of total Bacteria), (Fig. S18). At the time of writing this manuscript, species were isolated from the Baltic Sea, from a Roman archaeological pool located in Tunisia, sand sediment of a beach in Jeju, Korea, an extreme hyper-arid soil sample from the Atacama Desert, sea-tidal sediment sampled at Republic of Korea, from sand samples collected from Gurbantunggut desert located in China (Yang et al. 2019).

Modestobacter (272 reads of bacteria or 29% of *Geodermatophilaceae* (family), 0.4% of Actinobacteria (phylum), and 0.08% of total Bacteria), (Fig. S19). Studies on members of this genus showed that they were recovered from diverse habitats,. They are associated with extreme biomes, and *Modestobacter* strains are a common feature of the Atacama Desert biome and they adapted to harsh environmental conditions (Bull et al. 2017).

Order Micromonosporales (Table SVII)

Allocatelliglobosispora (168 reads of bacteria or 30% of *Micromonosporaceae* (family), 0.2% of Actinobacteria (phylum), and 0.05% of total Bacteria), (Fig. S20). A study on *Allocatelliglobosispora,* a genus of bacteria from the family of *Micromonosporaceae*, with one known species, the *Allocatelliglobosispora scoriae* demonstrated that it was isolated from volcanic ash collected near Darangshi Oreum (a parasitic volcano or satellite cone) in Jeju, Republic of Korea (Lee and Lee 2011).

Plantactinospora (23 reads of bacteria or 4% of *Micromonosporaceae* (family), 0.03% of Actinobacteria (phylum) and 0.007% of total Bacteria), (Fig. S21). Currently, seven species of the genus *Plantactinospora* have been described and they were isolated from plants in China (Li et al. 2018).

Catellatospora (7 reads of bacteria or 1% of *Micromonosporaceae* (family), 0.01% of Actinobacteria (phylum) and 0.002% of total Bacteria), (Fig. S22). A study on *Plantactinospora* species revealed that they were first isolated from soil and volcanic sediment samples in Asia (Liu et al. 2020).

Order Jiangellales (Table SVIII)

Jiangella (222 reads of bacteria or 79% of *Jiangellaceae* (family), 0.03% of Actinobacteria (phylum), and 0.06% of total Bacteria), (Fig. S23). Despite a ange of different habitats from which species of the genus *Jiangella* can be isolated, a study has shown that arid and desert habitats attract special attention (Saygin et al. 2020).

Haloactinopolyspora (3 reads of bacteria or 1% of *Jiangellaceae* (family), 0.004% of Actinobacteria (phylum), and 0.0009% of total Bacteria), (Fig. S24). At the time of writing, the genus *Haloactinopolyspora* comprises only two species isolated from soil samples collected from northwest China (Zhang et al. 2014).

Order Streptosporangiales (Table SIX)

Actinocorallia (151 reads of bacteria or 63% of *Thermomonosporaceae* (family), 0.2% of Actinobacteria (phylum), and 0.04% of total Bacteria), (Fig. S25). The genus comprises nine species, some isolated from soil samples, a natural cave, and from plant roots in southwest China (Li et al. 2018).

Actinomadura (62 reads of bacteria or 27% of *Thermomonosporaceae* (family), 0.09% of Actinobacteria (phylum), and 0.02% of total Bacteria), (Fig. S26). Studies on *Actinomadura* strains have shown that are primarily distributed in terrestrial soils, in plant tissues, and marine sources (Abagana et al. 2016).

Streptomyces (156 reads of bacteria or 75% of *Streptomycetaceae* (family), 0.2% of Actinobacteria (phylum) and 0.04% of total Bacteria), (Fig. S27). In recent years, much of the attention has been focused on more extreme environment habitats such as deep-sea, desert, cryo, and volcanic environments for the isolation of potential *Streptomyces* species and the

discovery of important bioactive secondary metabolites including antibiotics, immunosuppressive drugs, anticancer drugs, and other biologically active compounds (Abdelkader et al. 2018).

Order Sporichthyales (Table SX)

Sporichthya (80 reads of bacteria or 100% of *Sporichthyaceae* (family), 0.1% of Actinobacteria (phylum) and 0.02% of total Bacteria), (Fig. S28). A study on these species demonstrated that they were isolated from greenhouse soil in the USA, and soil samples in Japan (Tamura et al. 1999).

Order Kineosporiales (Table SXI)

Thalassiella (50 reads of bacteria or 100% of *Kineosporiaceae* (family), 0.07% of Actinobacteria (phylum), and 0.01% of total Bacteria), (Fig. S29). A study on *Thalassiella azotovora*, the only family member *Kineosporiaceae*, described that it was collected from a seawater sample from Sadong Wharf, Ulleung-Island, South Korea (Lee et al. 2016).

Order Bifidobacteriales (Table SXII)

Bifidobacterium (15 reads of bacteria or 100% of *Bifidobacteriaceae* (family), 0.02% of Actinobacteria (phylum), and 0.004% of total Bacteria), (Fig. S30). Interestingly, most of the most recently discovered Bifidobacteria species have been isolated from mammals (Lugli et al. 2021).

Order Acidothermales (Table SXIII)

Acidothermus (1 read of bacteria or 100% of *Acidothermaceae* (family), 0.001% of Actinobacteria (phylum), and 0.0003% of total Bacteria), (Fig. S31). A study on *Acidothermus cellulolyticus* has presented that it is the only genus of the *Acidothermaceae* family, isolated from acidic hot springs in Wyoming, USA (temperatures of 45 to 65°C and a pH range of 4 to 5.5) (Sen et al. 2014).

Order Gaiellales (Table SXIV)

Gaiella (17,605 reads of bacteria or 100% of *Gaiellaceae* (family), 79% Thermoleophilia, and 5% of total Bacteria), (Fig. S32). At the time of writing, only one genus and one species, strain *Gaiella occulta* F2-233T isolated from a deep mineral water aquifer in Portugal at a temperature of 28.0°C with a pH of 5.9, is validly published in the family *Gaiellaceae* of the order Gaiellales (Albuquerque et al. 2011).

Order Solirubrobacterales (Table SXV)

Conexibacter (1 read of bacteria or 100% of *Conexibacteraceae* (family), 0.03% Solirubrobacterales (order) and 0.0003% of total Bacteria), (Fig. S33). A study on the species of the genus demonstrated that they were isolated from soil and lava cave stalactite samples (Lee 2017).

Paraconexibacter (1 read of bacteria or 100% of *Paraconexibacteraceae* (family), 0.03% Solirubrobacterales (order), and 0.0003% of total Bacteria), (Fig. S34). A study on the *Paraconexibacteraceae* family, with one validly published species, the *Paraconexibacter algicola*, showed that it was isolated from a eutrophic lake during the end of cyanobacterial harmful algal blooms from the surface water (0–10 cm depth) sampled at Lake Seo, Republic of Korea, (Chun et al. 2020).

Patulibacter (29 reads of bacteria or 100% of *Patulibacteraceae* (family), 1% Solirubrobacterales (order), and 0.0008% of total Bacteria), (Fig. S35). A study on these species revealed that they were isolated from rhizosphere samples (Jin et al. 2016).

Solirubrobacter (6 reads of bacteria or 100% of *Solirubrobacteraceae* (family), 0.2% Solirubrobacterales (order), and 0.0002% of total Bacteria), (Fig. S36). They were isolated from soil and plant samples (Zhang et al. 2014).

Order Acidimicrobiales, Synonyms: Lamiales (Table SXVI)

Actinomarinicola (784 reads of bacteria or 100 of *Lamiaceae* (Family), 6.5% of Acidimicrobiales (order), and 0.21% of total Bacteria), (Fig. S37). The *Actinomarinicola* genus comprises from a single species, the *Actinomarinicola tropica* and it was isolated from a sediment sample collected from the South China Sea (depth of 460 m) (He et al. 2020).

Desertimonas (182 reads of bacteria or 2% of Acidimicrobiales (order), and 0.05% of total Bacteria), (Fig. S38). The genus *Desertimonas* consists of one species, the *Desertimonas flava* and it was isolated from a soil sample of Gurbantunggut desert, China (Asem et al. 2018).

Discussion

The goal of the current study was to characterize further the specific differences in Actinomycetota genera reported by our previous study (Michail et al. 2021). The results above depict that the stalactites Actinomycetota diversity comprises several families, as presented in Fig. 3–6. The detailed presentation of the Actinomycetota genera incorporated in these families' reveals the remarkable microbial world entrapped diachronically within these speleothems. The retrieval and identification of this globally distributed microbial world into a considerably smaller but solidly condensed area of a Greek stalactite core make us characterize these speleothems a superior paradigm of environmental biodiversity, "Microbial Arks". A similar to our research idea was presented by Bello et al. (2016) in world human population "microbiota vault" pilot project

(https://www.microbiotavault.org/project). Moreover Long et al. (2019), concluded that Karst caves are important "Arks" of speciation and biodiversity, so the microbial strain isolated from it might be a novel source of new antimicrobial agents.

Specifically, the presence inside the core of our stalactite, that of genera with only two or three species discovered, worldwide supports our first reported findings. The *Oryzihumus* genus (1,299 reads) with only three species isolated only in Asia (Kim et al. 2017), the *Smaragdicoccus* genus (27 reads) with only one species isolated in petroleum-contaminated soil in Japan (Adachi et al. 2007), the *Allocatelliglobosispora* genus (168 reads) with only one species isolated from volcanic ash in Korea (Lee and Lee 2011), the *Haloactinopolyspora* genus (3 reads) with only two species isolated from soil samples from North-West China (Zhang et al. 2014), the *Sporichthya* genus (80 reads) with only two species isolated from USA and Japan (Zhi et al. 2009), the *Thalassiella* genus (50 reads) with only one member isolated from seawater wharf sample from South Korea (Lee et al. 2016), the *Acidothermus* genus (1 read) with only one species isolated from acidic hot spring in USA (Sen et al. 2014), the *Gaiella* genus (17,605 reads) with one species isolated from deep mineral water aquifer in Portugal (Albuquerque et al. 2011), the *Paraconexibacter* genus (1 read) with one species isolated from Lake Seo, Republic of Korea (Chun et al. 2020), and the *Desertimonas* genus with one species isolated from soil sample of Gurbantunggut desert, China (Asem et al. 2018), clearly verify that Actinomycetota genera are distributed in harsh and challenging environments displaying outstanding abilities and versatility all over the world, which are mirrored vividly and distinctively inside our stalactite's core.

Finally, yet importantly, the rest of the Actinomycetota families and genera analysis strongly answers the crucial clue and reinforces our theory that probably a stalactite core can be considered unique environmental biodiversity "Microbial Ark". Actinomycetota families and genera that exhibit a vast diversity-plasticity in numerous of environmental traits like moisture, optimal pH, thermophilic ubiquity, acidophilic and alkaliphilic species, and other genera isolated from remote, pristine habitats such as the Egyptian desert, Antarctic soil, Asian volcanic ash and Atlantic and Siberian Sea deep-sea sediments. The *Pseudarthrobacter*, *Kocuria*, *Paeniglutamicibacter*, *Cellulomonas*, *Brachybacterium*, *Microlunatus*, *Marmoricola*, *Rubrobacter*, *Blastococcus*, *Modestobacter*, *Plantactinospora*, *Catellatospora*, *Jiangella*, *Actinocorallia*, *Acidothermus*, *Actinomarinicola*, and *Desertimonas* genera found concurrently in the stalactite's "Microbial Ark" unveils the tremendous Actinomycetota (and not only) DNA archive information clustered safeguarded inside.

Although paleomicrobiology is not a recently evolving scientific area, the use of modern techniques, such as DNA deep-sequencing, has proven crucial and has supplied detailed data for antibiotic resistance, saprophytes, viruses, pathogens evolution, and ancient diseases and health status; indeed, new information about historical events, ethnic diets and the cultural customs of humans is yet to be revealed shortly (Rivera-Perez et al. 2016). Permanently frozen soil layers, underground and marine substrates, fecal masses, and fecaliths have been used to be screened by DNA sequencing, unveiling the complete ancient microbiome, along with ancient microbes, viruses, archaea, and eukaryotes (Coolen 2011; García-Descalzo et al. 2013; Appelt et al. 2014a; Appelt et al. 2014b).

Paleomicrobiology reveals essential features of microbial evolution and resistance. This scientific area is primarily related to the research of human and plant pathobiome, unfolding opportunities to study the development of ancient illnesses and their impact on us. However, this area is limited to the pathogenic microbiome. The normal microbial flora from various ancient ecosystems, such as bones, halites, permafrost, dental pulp, fecal masses, amber, coprolites, speleothems, and aquatic organisms, helps us to understand the microbial environment and microbial-host interactions (Rivera-Perez et al. 2016). The results of this research showing genetic information entrapped in the stalactite core from bacteria that were globally distributed in different ecological environments and continents aids in the understanding of microbial biogeography, the discipline of biology that rebuilds the patterns of allocation of biological diversity and to distinguish the procedures that have molded those distributions over time (Sanmartín 2012) and that under analogous environmental surroundings, microbial assemblages from different regions might function in different ways (Martiny et al. 2006).

Recently in November 2019, the "Mars Extant Life: What's Next?" conference attendants concluded that there is a real likelihood that Mars hosts an autochthonous living microbiome. The conference had as its overarching topic that finding refugia (or "oases") where the environment is either consistent or occasionally noticeably more friendly than normal is critical to the quest for the Martian existing life. Participants in the meeting identified four settings on Mars that could serve as refugia based on current information (not written in predominance order): caves, ices, salts, and deep below (Mann 2001). If Martian life had formerly been more common, it might have withdrawn to one or more of these safe havens, where it might have been able to live for a long time (Carrier et al. 2020). Additionally, mineral collection suites and distinctive characteristics such as pool fingers and "u"-loop speleothems are frequently

found in terrestrial caves as traces of previous life (Boston et al. 2001; Northup et al. 2011; Melim et al. 2016; Uckert et al. 2017; Jones et al. 2019; Northup et al. 2019).

The results verify that Actinomycetota represents a considerable and predominant "microbial component" of any stalactite's core, including our first reported sample. Remarkable, however, is that 15.6% of the recorded diversity is not ascribable to validly known published families (4,329 reads, Fig. 5). This remarkably high proportion of unidentified reads provides or composes a sound base of unclassified taxa, which probably could not yet be recovered using culture-dependent methods.

Moreover, the literature showed that in desert soils metagenomic analyses revealed an astounding Actinobacterial "Dark Matter" (Idris et al. 2017; Goodfellow et al. 2018; Jian-Yu et al. 2021). Microbial Dark Matter (MDM) is the vast variety of yet-uncultivated bacteria that scientists can only investigate using cultivation-independent methods. Lately, several worldwide schemes have surprisingly expanded our knowledge of the spread and allocation of microbiome, including the Genomic Encyclopedia of Bacteria and Archaea-Microbial Dark Matter (GEBA-MDM), the Earth Microbiome Project (EMP), the Genomic Encyclopedia of Bacteria and Archaea (GEBA), and Global Catalogue of Microorganisms (GCM), and some more primate microbial diversity schemes (Jian-Yu et al. 2021). Taking into consideration our findings and based on this remarkable finding of 4,329 unclassified reads (Fig. 5), we believe that this is strong evidence of a yet-uncultivated biosphere (Dance 2020), confirming that speleothems could also be a vast reservoir of "Microbial Dark Matter" (Idris et al. 2017, Goodfellow et al. 2018), (MDM) as reported by Jian-Yu et al. (2021).

Conclusions

In a continuously changing world where the loss of biodiversity is a significant challenge, our present findings illustrate that stalactites could serve as an environmental "Microbial Ark". Their stored rare microbiome and "Dark Matter" bacterial communities throughout their lifetime, from the upper or around cavern terrain, including soil, flora, fauna, and aquatic environment, needs to be thoroughly screened in the near future by utilizing deep sequencing techniques. Modern metagenomics has revealed the bacterial make-up of diverse extreme niches, but it does not allow researchers to answer fundamental questions about microbes, such as what they consume. What primary and secondary metabolites do they produce? Moreover, how do they interact with others in their extreme environment? To answer those questions, scientists must first isolate and then culture the organisms in the lab.

Based on the above findings, our study supports the hypothesis that the planet's microbial community profile can be successfully stored in these refugia. Stalactites could serve as novel biological approach samples recommending an environmental "Microbial Ark". They could further provide scientific explanatory links or proposals of the microbial interspecies interactions in our planet's ecosystem. Also, these results have the potential to be projected to other parts of the ecosystem microbiome, along with, but not restricted to, the eukaryotic and archaeal organisms. Other multi-omic systems (e.g., metatranscriptomics, metabolomics, and metaproteomics) could be used to discover the manifestation of genes and biochemical pathways and research metabolites' dynamics.

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Author contributions

G.M., A.R., A.E. designed and performed the experiments. P.M, L.K. analyzed the samples. P.M., A.R., L.K., analyzed the data. I.V., G.M , A.E., P.M. wrote manuscript. All authors have read and agreed to the published version of the manuscript. The first two authors I.V. and A.R., contributed equally to the manuscript.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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