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Unearthing microbial diversity, environmental and human impacts in cave ecosystems

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ABSTRACT

Caves are spatially confined, subsurface environments widespread on Earth, where peculiar abiotic factors and microclimatic conditions impose special adaptations to microbial life-forms. Yet, the growing touristic interest and transformation of natural hypogean sites into authentic tourist attractions, coupled with global climate instability, dramatically challenges the preservation of these fascinating underground ecosystems and their cultural and natural heritage. This thesis aims to investigate cave microbial communities to provide new insights about changes in compositional and diversity patterns as a response to the impact of human activities and external environmental drivers still lacking to date. Advanced sequencing technologies led to the assembly of an extensive sediment microbiomes dataset from 4 tourist and 1 natural Italian caves for the first time. Overall, our findings indicated how showcaves share common microbial traits in contrast to the natural one and some evidence of potential direct microbiome cave contamination by human-related bacteria and commensal/opportunistic human associated fungi were reported. Then, by adopting an advanced ecological framework approach, we highlighted how bacteria was the most sensitive microbial assemblage to tourism pressure and that the stochastic forces (Drift) were the dominant selective processes in driving composition of microbial communities across anthropized habitats . Finally, by using a meta-analysis approach, we investigated, for the first time, cave microbiomes at global scale and we found that climatic variables were the stronger predictors of compositional and diversity patterns of top dominant phylotypes. This knowledge is critical to further develop long-term approaches and strategies aimed at preserving the astounding natural and cultural heritage of caves worldwide.

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CHAPTER 1. Introduction

1.1 Caves: fragile and extreme environments

Caves are widespread underground environments on Earth, located in many different geological landscapes, climatic regions and in all continents including Antarctica. The spread and variety of cave environments has a significant extent on Earth;according to recent reports, between 45,000 and 50,000 karstic caves have been reported in the USA, around 100,000 in Europe, 12,000 in Slovenia, and up to 35,000 in Italy (Pipan & Culver, 2013). The most common caves in terrestrial environments are i) karst caves, formed by chemical dissolution of rock substrates (e.g. sedimentary, metamorphic and igneous rocks); ii) lava tubes, as remnants of the internal drainage conduits generated by the differential flow and cooling of lava; iii) glacial caves, formed by the melting of ice as a result of hydro-geothermal forces (De Waele & Gutiérrez, 2022).

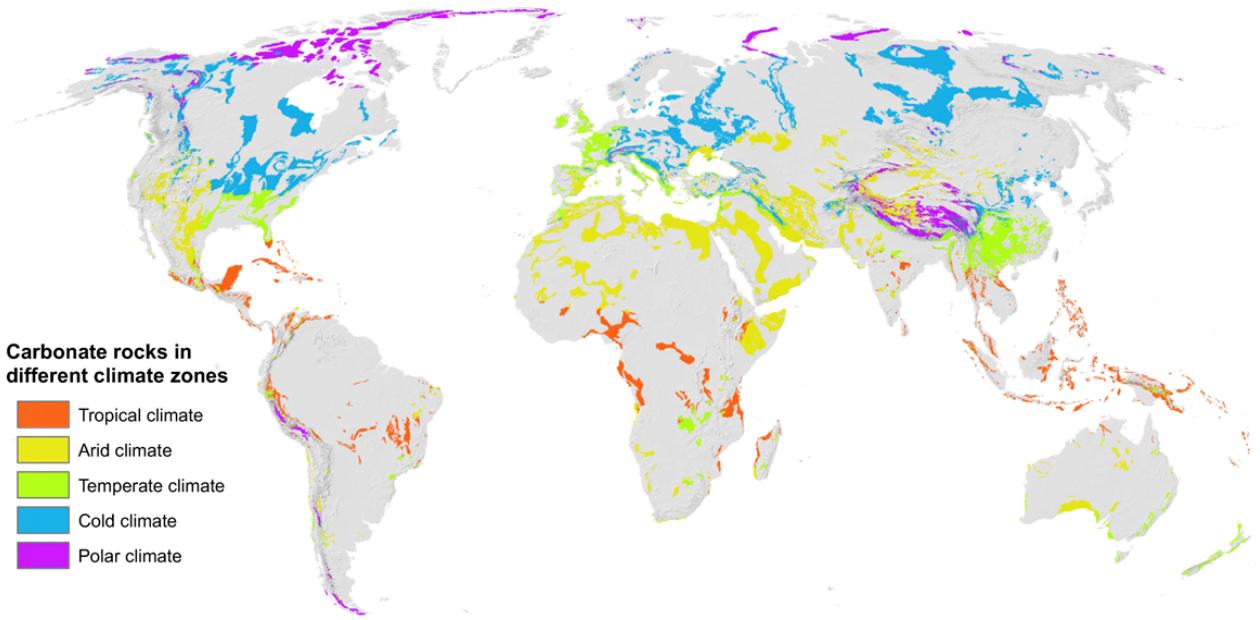

Fig.1: Worldwide distribution of karst cave-forming landscapes, underlining the wide coverage across different climatic regions (credits to: Goldscheider et al., 2020).

Although geomorphological, geochemical and structural differences, we can describe caves as multifaceted ecosystems, where the definition of stable and extreme environments can coexist. The cave structure is extremely zonal, especially for karstic caves, and based on light penetration can be classified in four main areas: 1) the “Entrance” is the first transition zone, caught between epigean and hypogean cave systems, is characterized by gradients of structural, biological or physical

changes and often described as ecotone; (2) the “Twilight” or “Transition-zone”zone, where light gradually disappears, the photosynthetic activities halt, no plants survive in this zone and variations of abiotic factors and (humidity and evapotranspiration potential) and airflows are weaker; 4) the “Deep” zone where air is stationary and saturated by water vapor, the bedrock is kept humid and the evapotranspiration potential is constant over time; iv) The “Air-stagnant zone”, not always present in caves, shows little or no air exchange with high CO_2 concentration (Gabriel & Northup, 2013). The spatial confinement and zonality of underground environments ensure a general microclimatic stability, in particular of their temperature and humidity (Bourges et al., 2014).

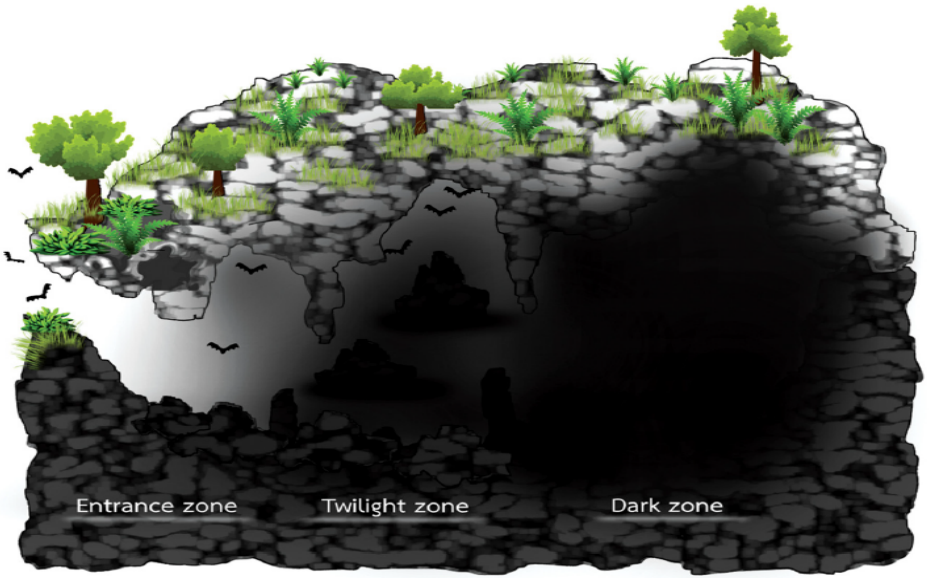

Fig.2: The sectoralised structure of the subterranean environments, according to light penetration and intensity three main zones are defined 1) Entrance 2) Twilight and 3) Dark zone. (credits to Wiseschart & Pootanakit, 2020).

The geophysical and geochemical conditions of underground settings are driven by multiple factors, such as the climate, the surrounding lithological context, altitude, depth and caves structure morphology. For instance, cave ventilation is driven by the difference in air density and temperature between cave and surface, whose direction (inhalation or exhalation) change cyclically according to daily and seasonal external temperature fluctuations. This phenomenon called "cave breathing" plays a key role in regulating natural CO_2 fluxes derived directly from soils/epicarists or indirectly by outgassing drip water. Cave breath-derived CO_2 levels are independent of whether the cave is karstic or not (Lang et al., 2017), while climatic (temperature and precipitation seasonality) and geographic (altitude, sun exposure of entrance) factors have a critical impact in driving cave

ventilation and gas exchange (De Freitas et al., 1982). Furthermore, in carbonate rock settings due to carbon dioxide balance and large dissolution of $[Ca_2^+]$ + $[Mg_2^+]$, the karstic water chemical medium is buffered at pH 7-9, while in non-carbonate bedrock (silicates, gypsum, halite), or in the presence of additional minerals capable of oxidizing and forming acids (e.g. sulphuric acid), the pH of dry and water cave can reach extreme values (pH 1-2) (Lauritzen, 2018). It becomes clear how percolating water and gaseous exchanges combined with lithological and structural properties produce a peculiar physical-chemical balance ruling cave environments.

Furthermore, hydrological and atmospheric dynamics established with surface ecosystems play a key role in managing energy flows in underground environments. Organic matter enters caves in different ways and forms, through underground waterways, percolating water, wind or animals, passing through pores, fractures, cavities and entrances in rock structures (Barton and Jurado 2007). Mainly carried by water flows, dissolved organic matter (DOM) changes in complexity and amounts during transport across soil horizons, where transit speed and microbial activity inversely influence its concentration and assimilability (Venarsky & Huntsman, 2018). The particulate organic matter (POM) includes plant material (leaves, wood, fruit, seeds), guano and animal carrion reaching caves mainly by large water flows or flooding, falling from openings and fractures, or carried by animals and ventilation. Its quality ratios (C/N/P) can vary greatly across POM types, following the general pattern: plant POM < guano < animal carrion. Yet, in most cave ecosystems, POM represents a small portion of the total organic matter budget. Like in surface ecosystems, microbial communities support the entire food web (from small crustaceans, insects, to vertebrate predators) in cave environments. Bacteria and fungi dominate and regulate the organic matter decomposition and nutrient recycling, with fungi generally dominating the microbial biomass in the early stages of complex organic matter decomposition (POM in particular), followed by bacterial dominance in the latter stages. In contrast, bacteria appear to dominate DOM uptake (Culver & Pipan, 2019; Ravn et al., 2020). However, where energy support from external ecosystems is limited by degraded environmental conditions, like across drylands, a non-photosynthetic base of primary production is provided by specialized archaeal and bacterial chemolithoautotrophic

microorganisms. These microbes can derive energy from oxidation of inorganic electron donors such as hydrogen, carbon monoxide, sulfur and nitrogen compounds, or divalent cations (e.g., Fe2+ and Mn2+) for fixing inorganic carbon into organic carbon. Despite energy flows in different ways and forms in caves, the limited nutrient loads, fluctuating supply patterns and non-uniform nutrient distributions, lead to establishment of a general oligotrophic conditions across these ecosystems (Engel, 2015;Venarsky & Huntsman, 2018).

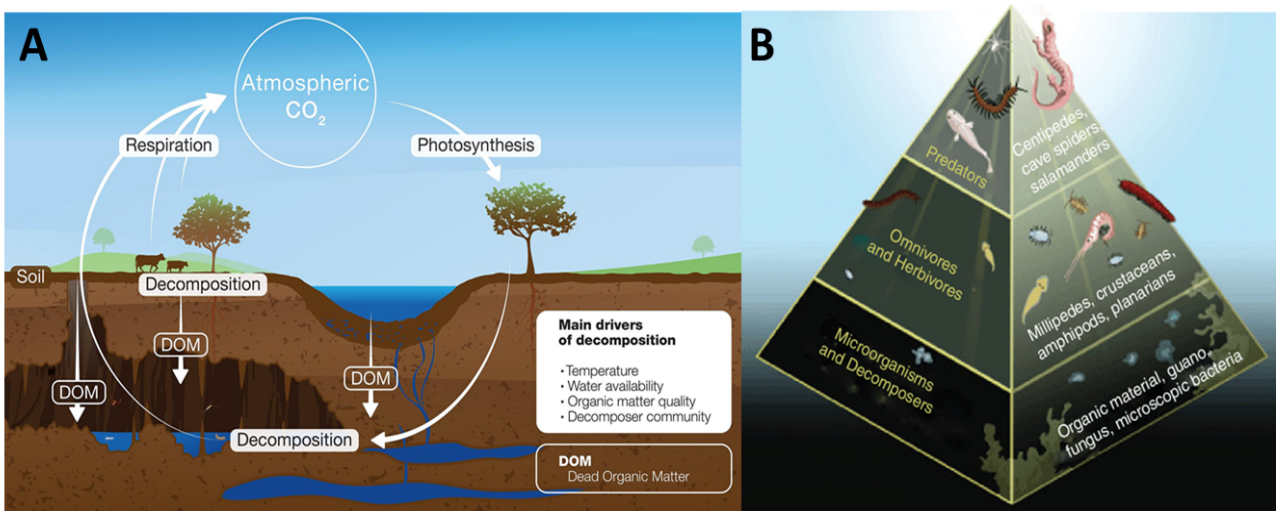

Fig.3: Schematic model of flow and decomposition cycles of organic matter (A) and trophic network (B) in subterranean environments. Important point is the fundamental contribution of microbial communities in sustaining the whole cave ecosystem (credits to: Fig.3A, Ravn et al., 2020; Fig.3B, Lee et al., 2012).

From a biological point of view, caves are considered extreme environments, as the result of unfavorable conditions that limit the development of life. In general, proceeding to the deepest areas, cave ecosystems appear completely dark, with high humidity, pH fluctuations, low temperature, a high concentration of minerals and a marked lack of nutrients. Taken together, these environmental factors act as a selective pressure and impose special physiological and metabolic adaptations to life-forms in caves (Howarth & Moldovan 2018). Microorganisms in particular are the most adapted and specialized in colonizing and exploiting cave environments, due to their broad metabolic and physiological plasticity enabling them to cope with challenging environmental constraints (Bontemps et al., 2022). Besides supporting cave trophic networks, microorganisms play a key role in fundamental biogeochemical processes (Venarsky et al, 2018; Wiseschart & Pootanakit, 2020) and supply several important activities for caves development (e.g. rock dissolution and mineral precipitation), contributing to the formation of cave structures and

speleothems (e.g. stalactites and stalagmites, moonmilk, pool fingers) (Martin-Pozas et al., 2020). Being acquainted with very stable, besides extreme, conditions, these highly adapted microbial communities are very prone to external perturbations. Changes in cave microbiomes could affect the functionality and sustainability of the entire cave ecosystems, leading to loss of native biodiversity and endangering the conservation of their natural and cultural heritage.

1.2 Microbiome caves and anthropic impact

In the last decades, interest in underground environments has grown considerably, not only in scientific terms, but also in economic aspects, attracting around 500,000 tourists per year in caves, leading to a constant transformation of natural hypogeal sites into authentic tourist attractions; this dramatically affected the preservation of these fascinating underground ecosystems and their cultural and natural heritage (Chiarini et. al, 2022), even influencing the integrity of the peculiar biodiversity hosted in these fragile and sensitive ecosystems (Cigna, 2016). Tourist caves, contrary to wild ones, experienced extensive logistical and architectural modifications for tourist exploitation. Tourist reception spaces, walkways, barriers and artificial lighting have been established to permit visiting of caves (Cigna & Burri, 2000; Fernández-Cortés et al., 2011; International Show Caves Association, 2018). Tourism exploitation impacts cave environments stability in multiple ways, including: increasing air temperature and relative humidity leads to condensation-corrosion phenomena. The increase in CO_2 concentration directly influences air quality and indirectly the geochemical balance of rock structures such as speleothems (Fernández-Cortés et al., 2006, 2011) and the preservation of possible archaeological artifacts such as cave paintings. Of crucial importance is the role that visitors play in transporting organic matter and microorganisms into the caves, with direct effects on the balance of ecosystems and microbiomes (Northup et al., 2000; Chelius et al., 2009). The coupled effect of visitors and artificial lighting has relevant impacts in spreading and proliferation of allochthonous microorganisms such as bacteria, fungi and photosynthetic organisms (such as algae, diatoms, ferns and mosses) commonly known as Lampenflora (Falasco et al., 2014; Vanderwolf et al., 2014). Proliferation of

exogenous organisms and growth of photosynthetic biofilms on the wall surfaces and speleothems causes aesthetic, physical and chemical damage, representing a serious threat to cultural and naturalistic heritage sites with great socio-economic consequences. Furthermore, increased cave openings and tourist flows cause extra inputs of organic matter and microbial airborne spores, affecting cave microbiomes and promoting blooms of microbes, threatening the functionality of cave ecosystems and visitor safety (Mulec et al., 2017; Alonso et al., 2019). Although ecological consequences of human impact on these underground ecosystems are so pronounced, to date this phenomenon has been mainly investigated at local scale, taking into account just a few components of the ecosystem, limiting a multidimensional understanding. Therefore, a multidisciplinary knowledge advance on cave ecology and the impact of tourist exploitation is of paramount importance today (Cigna & Burri, 2000; Cigna, 2016)**.**

1.3 Microbiome caves and climate change, a new challenge?

Climate change is a key driver of biodiversity loss across global terrestrial and aquatic ecosystems. Given the critical role of microbial communities in sustaining the functionality and trophic networks of entire ecosystems, biodiversity losses pose a serious challenge to their long-term future conservation (Pettorelli et al., 2021). Understanding patterns of microbial ecological responses to climatic fluctuations at a global scale is crucial for developing predictive models of compositional and functional changes, with the purpose of establishing protection and conservation policies. Hence, terrestrial caves represent optimal natural laboratories for investigating the impacts of climate change in its biotic and abiotic components and assessing effects on biodiversity *in situ* since: (i) caves are spatially confined geosystems widespread on Earth and located in different climatic regions; (ii) are buffered from external fluctuations and generally characterized by remarkable thermal stability; (iii) temperatures within caves are highly correlated with mean annual surface temperatures; (iv) microbiome has evolved and adapted to cope with the particular cave environmental conditions, thus particularly sensitive and responsive to biotic and abiotic

perturbations (Mammola et al., 2018, 2019). Yet, knowledge of climate change effects on microbiome caves at a global scale are still largely unexplored to date.

However, we can speculate how large fluctuations in seasonal temperature and precipitation may directly and indirectly affect the stability of cave microbial communities. For instance, due to the fundamental hydrogeological and atmospheric dynamics between caves and surface ecosystems, increased aridity conditions could further limit organic matter supply with severe consequences in maintaining trophic webs and the functionality of the entire ecosystem (Venarsky et al., 2018). It has been highlighted how growing arid conditions lead to a reduction in plant coverage and primary production of soils with a consequent decrease in microbial diversity and C-organic content globally (Maestre et al., 2015; Delgado‐Baquerizo et al., 2016). Moreover, less productive donor ecosystems and scarce water flows could drastically affect the broad heterotrophic microbial assemblage of subterranean ecosystems. Indeed, caves situated in drylands present highly specialized microbial communities in coping with high organic matter deficiency by exploiting the large amounts of inorganic compounds to fix organic carbon through chemolithoautotrophy (Ikner et al., 2007; Venarsky et al., 2018). On the other hand as indirect effect, large daily external thermal fluctuations due to increasing temperatures would enhance caves ventilation patterns and support the input of airborne microbial spores with consequent alteration of the cave microbiome (Mulec et al., 2017; Alonso et al., 2019). Finally, given the impact of tourist exploitation on cave structures and microbiomes, it is crucial to gain more insight on exposure of anthropized sites to the impacts of climate change to protect and preserve these largely impacted geo-ecosystems.

1.4 Aim of the thesis and chapters description

This thesis aims to investigate cave microbial communities to provide new insights about changes in compositional and diversity patterns as a response to the impact of human activities in caves. Furthermore, we aim to unravel the ecological responses of cave fungal and bacterial communities to environmental and climatic drivers at global scale, to gain more insight on the exposure of cave ecosystems (anthropized and natural) to environmental variations driven by climate change. Tourism exploitation impacts on cave ecosystems have been assessed in the context of project

"Showcave" funded as Research Project of National Relevance (PRIN). By investigating the microbiomes of e 4 show-caves and 1 natural cave, along Italian peninsula we aimed to: i) unravel the microbial diversity of caves and highlight possible compositional differences between anthropised and natural habitats; ii) Understand the processes driving the composition and diversity of subterranean microbial communities in relation to possible tourism-induced disturbances. These tasks are discussed in detail across Chapters 2 and 3. Finally, as a part of international joint research activities, we assembled a global dataset of cave fungal and bacterial communities, derived from amplicon-sequencing analysis of 1,058 microbiome samples from different substrates, collected across 85 distinct caves, located in different climatic regions and lithological features. From a global ecological perspective, we aim for the first time to i) explore the contribution of sample types and different geographical, climatic lithological conditions in driving responses of whole fungal and bacterial cave communities diversity; (ii) elucidate fungal and bacterial taxa dominant caves worldwide and (iii) unravel the relative contribution of climatic variables in predicting structural and diversity variability patterns, in relation to environmental alteration phenomena driven by climate change. These tasks are discussed in detail across Chapter 4.

After the first introductory chapter, the second chapter provides an extensive description and taxonomic comparison of principal microbial traits found in anthropized and pristine hypogean habitats, starting to shed light on potential microbial taxa that may be considered as indicative of human impact. We used an amplicon-sequencing analysis approach to investigate, for the first time, the whole microbiota (including fungi, algae, bacteria and archaea) of four Italian show-caves and recently discovered natural cave, applied an extensive sampling of sediments along the whole extension of each cave. The chapter concludes highlighting how show-caves share common microbial traits in contrast to the natural one related to outdoor environment and provides new insights into the potential microbiota cave contamination by human-related bacteria and commensal/opportunistic human associated fungi.

The third chapter proposes an extensive NGS sequencing analysis on sediment samples to disentangle the tourism-induced effects on the diversity of Fungi, Bacteria and Archaea in four

Italian showcaves. Samples were collected by adopting a replicated factorial sampling design at progressive lateral distance from the tourist path, which was intended as proxy of tourism pressure. By adopting an advanced ecological framework approach we highlighted how tourism in caves has a direct effect on Bacteria and an indirect influence on Fungi and Archaea in driving community composition of the three microbial groups.

The fourth chapter represents a first draft and preliminary results of a meta-analysis of cave fungal and bacterial communities worldwide, aiming at exploring their ecological responses in relation to biological, geographic and climatic factors. Results seem to indicate a different ecological preference of fungal and bacterial diversity for cave environments placed in temperate-tropical and temperate-continental climatic regions respectively, while arid climate leads to a global reduction in cave microbial diversity. Furthermore, for the first time, we provided evidence of a shared microbiome core among caves worldwide, including 50 fungal and 52 bacterial dominant phylotypes. Finally, climatic variables appear as stronger predictors of compositional and diversity patterns of microbial dominant phylotypes, selecting specific dominant taxa across gradients of growing aridity conditions.

General conclusions and summary are presented in Chapter 5.

1.5 References

Alonso, L., Pommier, T., Kaufmann, B., Dubost, A., Chapulliot, D., Doré, J., ... & Moënne‐Loccoz, Y. (2019). Anthropization level of Lascaux Cave microbiome shown by regional‐scale comparisons of pristine and anthropized caves. Molecular ecology, 28(14), 3383-3394.

Barton, H. A., & Jurado, V. (2007). What's up down there? Microbial diversity in caves.

Bontemps, Z., Alonso, L., Pommier, T., Hugoni, M., & Moënne-Loccoz, Y. (2022). Microbial ecology of tourist Paleolithic caves. Science of the Total Environment, 816, 151492.

Bourges, F., Genthon, P., Genty, D., Lorblanchet, M., Mauduit, E., & d'Hulst, D. (2014). Conservation of prehistoric caves and stability of their inner climate: Lessons from Chauvet and other French caves. Science of the Total Environment, 493, 79-91.

Cigna, A. A., & Burri, E. (2000). Development, management and economy of show caves.

Cigna, A. A. (2016). Tourism and show caves. Zeitschrift für Geomorphologie, 60(2), 217-233.

Chiarini, V., Duckeck, J., & De Waele, J. (2022). A global perspective on sustainable show cave tourism. Geoheritage, 14(3), 82.

Chelius, M. K., Beresford, G., Horton, H., Quirk, M., Selby, G., Simpson, R. T., ... & Moore, J. C. (2009). Impacts of alterations of organic inputs on the bacterial community within the sediments of Wind Cave, South Dakota, USA. International Journal of Speleology, 38(1), 1.

Culver, D. C., & Pipan, T. (2019). The biology of caves and other subterranean habitats. Oxford University Press.

De Freitas, C. R., Littlbjohn, R. N., Clarkson, T. S., & Kristament, I. S. (1982). Cave climate: assessment of airflow and ventilation. Journal of Climatology, 2(4), 383-397.

De Waele, J., & Gutiérrez, F. (2022). Karst hydrogeology, geomorphology and caves. John Wiley & Sons.

Delgado‐Baquerizo, M., Maestre, F. T., Reich, P. B., Trivedi, P., Osanai, Y., Liu, Y. R., ... & Singh, B. K. (2016). Carbon content and climate variability drive global soil bacterial diversity patterns. Ecological Monographs, 86(3), 373-390.

Engel, A. S. (Ed.). (2015). Microbial life of cave systems. De Gruyter.

Falasco, E., Ector, L., Isaia, M., Wetzel, C. E., Hoffmann, L., & Bona, F. (2014). Diatom flora in subterranean ecosystems: a review. International Journal of Speleology, 43(3), 1.

Fernandez-Cortes, A., Calaforra, J. M., & Sanchez-Martos, F. (2006). Spatiotemporal analysis of air conditions as a tool for the environmental management of a show cave (Cueva del Agua, Spain). Atmospheric Environment, 40(38), 7378-7394.

Fernandez-Cortes, A., Cuezva, S., Sanchez-Moral, S., Cañaveras, J. C., Porca, E., Jurado, V., ... & Saiz-Jimenez, C. (2011). Detection of human-induced environmental disturbances in a show cave. Environmental Science and Pollution Research, 18, 1037-1045.

Gabriel, C. R., & Northup, D. E. (2013). Microbial ecology: caves as an extreme habitat. Cave microbiomes: a novel resource for drug discovery, 85-108.

Ghosh, S., Kuisiene, N., & Cheeptham, N. (2017). The cave microbiome as a source for drug discovery: reality or pipe dream?. Biochemical pharmacology, 134, 18-34.

Howarth, F. G., & Moldovan, O. T. (2018). The ecological classification of cave animals and their adaptations. Cave ecology, 41-67.

Ikner, L. A., Toomey, R. S., Nolan, G., Neilson, J. W., Pryor, B. M., & Maier, R. M. (2007). Culturable microbial diversity and the impact of tourism in Kartchner Caverns, Arizona. Microbial ecology, 53, 30-42.

International Show Caves Association. (2018). www.i-s-c-a.org, Genga, Ancona

Lang, M., Faimon, J., Godissart, J., & Ek, C. (2017). Carbon dioxide seasonality in dynamically ventilated caves: the role of advective fluxes. Theoretical and Applied Climatology, 129, 1355-1372.

Lauritzen, S. E. (2018). Physiography of the Caves. Cave ecology, 7-21.

Maestre, F. T., Delgado-Baquerizo, M., Jeffries, T. C., Eldridge, D. J., Ochoa, V., Gozalo, B., ... & Singh, B. K. (2015). Increasing aridity reduces soil microbial diversity and abundance in global drylands. Proceedings of the National Academy of Sciences, 112(51), 15684-15689.

Mammola, S., Goodacre, S. L., & Isaia, M. (2018). Climate change may drive cave spiders to extinction. Ecography, 41(1), 233-243.

Mammola, S., Piano, E., Cardoso, P., Vernon, P., Domínguez-Villar, D., Culver, D. C., ... & Isaia, M. (2019). Climate change going deep: The effects of global climatic alterations on cave ecosystems. The Anthropocene Review, 6(1-2), 98-116.

Martin-Pozas, T., Gonzalez-Pimentel, J. L., Jurado, V., Cuezva, S., Dominguez-Moñino, I., Fernandez-Cortes, A., ... & Saiz-Jimenez, C. (2020). Microbial activity in subterranean ecosystems: Recent advances. Applied Sciences, 10(22), 8130.ù

Mulec, J., Oarga-Mulec, A., Šturm, S., Tomazin, R., & Matos, T. (2017). Spacio-temporal distribution and tourist impact on airborne bacteria in a cave (Škocjan Caves, Slovenia). Diversity, 9(3), 28.

Northup, D. E., Dahm, C. N., Melim, L. A., Spilde, M. N., Crossey, L. J., Lavoie, K. H., ... & Barns, S. M. (2000). Evidence for geomicrobiological interactions in Guadalupe caves. Journal of Cave and Karst Studies, 62(2), 80-90.

Pettorelli, N., Graham, N. A., Seddon, N., Maria da Cunha Bustamante, M., Lowton, M. J., Sutherland, W. J., ... & Barlow, J. (2021). Time to integrate global climate change and biodiversity science‐policy agendas. Journal of Applied Ecology, 58(11), 2384-2393.

Ravn, N. R., Michelsen, A., & Reboleira, A. S. P. (2020). Decomposition of organic matter in caves. Frontiers in Ecology and Evolution, 8, 554651.

Vanderwolf, J. K., et al. (2014) A world review of fungi, yeasts, and slime molds in caves. Int J Speleol, 42(3):77-96

Venarsky, M. P., & Huntsman, B. M. (2018). Food webs in caves. Cave ecology, 309-328.

Wiseschart, A., & Pootanakit, K. (2020). Metagenomic-based approach to a comprehensive understanding of cave microbial diversity. Recent advancements in microbial diversity, 561-586.

CHAPTER 2. Microbial diversity and proxy species for human impact in Italian karst caves

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Abstract

To date, the highly adapted cave microbial communities are challenged by the expanding anthropization of these subterranean habitats. Although recent advances in characterizing show-caves microbiome composition and functionality, the anthropic effect on promoting the establishment, or reducing the presence of specific microbial guilds has never been studied in detail. This work aims to investigate the whole microbiome (Fungi, Algae, Bacteria and Archaea) of four Italian show-caves, displaying different environmental and geo-morphological conditions and one recently discovered natural cave to highlight potential human-induced microbial traits alterations. Results indicate how show-caves share common microbial traits in contrast to the natural one; the first are characterized by microorganisms related to outdoor environment and/or capable of exploiting extra inputs of organic matter eventually supplied by tourist flows (i.e. *Chaetomium* and *Phoma* for fungi and *Pseudomonas* for bacteria). Yet, variation in microalgae assemblage composition was reported in show-caves, probably related to the effect of the artificial lighting. This study provides insights into the potential microbiome cave contamination by human-related bacteria (e.g. *Lactobacillus* and *Staphylococcus*) and commensal/opportunistic human associated fungi (e.g. *Candida*) and dermatophytes. This work is critical to untangle caves microbiome towards management and conservation of these fragile ecosystems.

Keywords: Caves, Microbial diversity, Amplicon sequencing, Subterranean ecosystems, Fungi, Bacteria

2.1 Introduction

Caves are spatially confined, subsurface environments widespread on Earth, where peculiar abiotic factors (i.e. oligotrophy, total darkness and high mineral concentrations) and microclimatic conditions (i.e. scarcely fluctuating low temperature and very high humidity) [1;2] impose special adaptations to microbial life-forms [3]. Microbial assemblages in caves include archaea, bacteria, fungi and other micro-eukaryotes [4]; these highly adapted microbial communities represent the living-backbone of cave ecosystems and play a key role in shaping structures (via direct and indirect metabolic activities) and sustaining trophic networks [5;7]. They live in constant balance between coping with unfavorable environmental conditions and maintaining microbe-microbe interactions, either competitive or cooperative [1;8]. Yet, the growing speleological and touristic interest in these underground settings has led to a major anthropization of caves (thereafter show -caves), which have been turned into real attractions. To date, showcaves, with 1,440 sites in 148 countries, among which 60 are in Italy (www.showcaves.com), represent an expanding worldwide scenario that results in a constant anthropization of a large number of natural hypogean settings. The presence of logistic interventions, such as the establishment of walkways, barriers and artificial lighting systems, coupled with tourist flows may alter the pristine local environmental conditions, by changing the local microclimate and introducing nutrients (i.e. soil, hair, skin, lint from clothing) and allochthonous microbial species [9;13]. Recently, researchers started to characterize show -caves microbiome, analyzing different types of samples (sediments, speleothems, and biofilms) and comparing natural and showcaves to discern a potential human impact on microbial composition and functionality [e.g. 13;17]. Potential bioindicators of altered conditions due to human activities have been identified in showcaves; for instance, the proliferation of lampenflora component, due to artificial light, is more abundant but generally less diverse than in natural caves [18]. Although some predominant microbial taxa reported in pristine settings have been detected in anthropized caves [8;13], the effect of human impact on promoting the establishment or reducing the presence of specific microbial traits has never been investigated in detail. Therefore, the consequence of tourist flows and human activities in general on microbial assemblages composition

remains largely unknown. To address this knowledge gap, our study intends to provide novel comprehensive insights into the microbial diversity and composition of 5 caves that include 4 show-caves and 1 recently discovered natural cave, displaying different environmental and geo-morphological conditions across the Italian peninsula. Through taxonomic comparison, we have characterized the principal microbial traits found in anthropized and pristine habitats, starting to shed light on potential microbial taxa that may be considered as indicative of human impact.

2.2 Results

2.2.1 Microbial community composition in cave sediments

The ITS1 dataset generated 5,793,980 raw sequence reads, resulting in 5,458,895 gene quality-filtered reads, ranging from 1252 up to 540,803 per sample. After singletons and rare taxa (<5 reads) removal (1,108 out of 10,595 ASVs total), a total of 9, 176 high-quality ASVs were obtained (Table S1). A total of 5,453,881 raw reads were generated from 16S rDNA dataset and accounted for a total of 4,806,902, which were grouped into 31,878 ASVs (out of a total of 65,037 ASVs) after quality filtering, with sequencing depths between samples ranging from 2,066 to 265,442 reads. Subsequently, the total 16S dataset was splitted by grouping the bacterial (31,015 ASVs) and archaeal (863 ASVs) ASVs separately for the downstream analyses (Table S2; S3). The 18S dataset was processed through a specific evaluation, extracting and analyzing only the sequences related to the microalgal component. A total of 97 ASVs were mapped for a total of 10,084 quality filtered reads, spread over 4 different groups (Alveolata, Chloroplastida, Chromista and Stramenopiles), using the Class taxonomic rank as the threshold for taxonomic identification as a consequence of the weak accuracy in the assignment towards higher ranks (Table S4).

Considering the total composition of microbial communities in cave sediments as a unique dataset (Fig. 1A), fungi represented the most abundant group (51.4%), followed by bacteria (46.6%), while archaea (1.8%) and micro-algae (0.2%) constituted minor fractions.

When analyzing the assemblage composition of each cave individually (Fig. 1B), Bossea cave was characterized by an almost equal presence of fungi (50.22%) and bacteria (49.16%). Caudano and

Vento caves were dominated by fungi (57.17% and 61.46%, respectively), while the bacterial community accounted for 42.37 and 37.22% of the total assemblage, respectively. Conversely, Costacalda and the Pertosa-Auletta caves showed a higher bacterial presence (66.66% and 52.70%, respectively) compared to fungi (32.05 and 41.48%, respectively). Concerning the archaea domain, Pertosa-Auletta cave was the most representative with 5.74% of the total microbial assemblage, followed by Costacalda (1.27%), Vento (0.85%), Bossea (0.52%) and Caudano (0.41%) caves. The algal component was scarcely represented along all caves, with a relative abundance ranging from 0.47 for Vento cave to 0.01% for Costacalda cave.

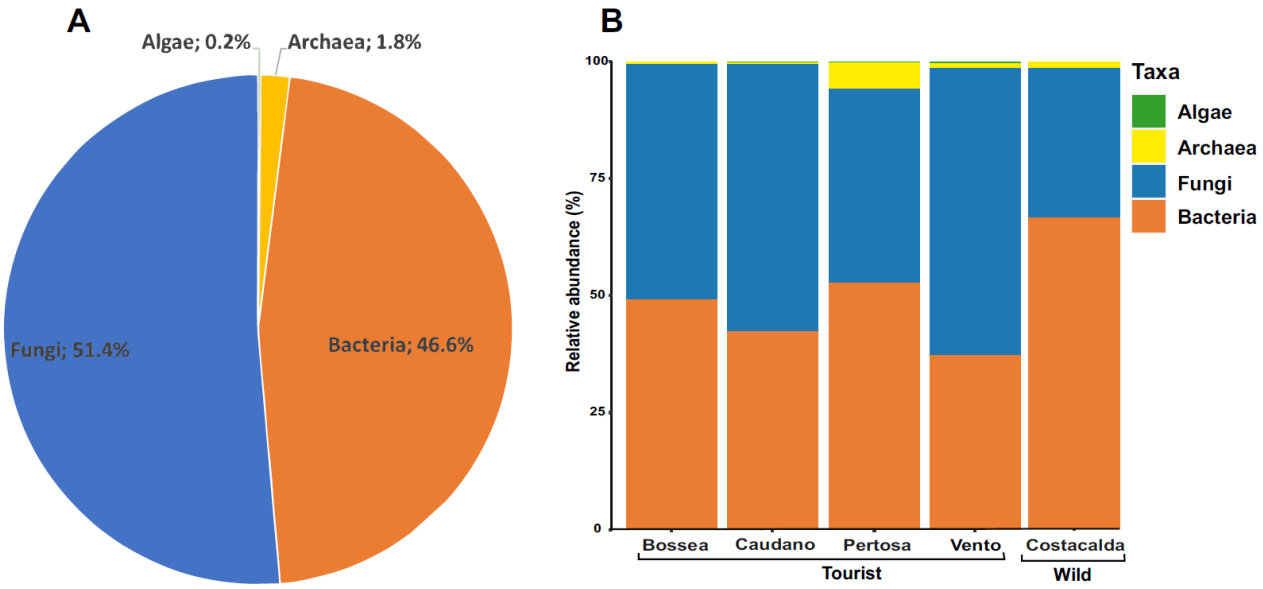

Figure 1. Total microbial cave community composition: as a unique dataset (A) and per single cave perspective (B).

2.2.2 Fungal community composition

In all caves, the dominant phylum was Ascomycota (Fig. 2A) (ranging from 60% in Costacalda to 76.25% in Pertosa Auletta), followed by Basidiomycota and Mortierellomycota. A broad different distribution of fungal taxa pattern emerged between the four showcaves and the wild cave (Fig. 2: A,B,C,D). Notably, the genus *Mortierella* (Fig. 2D) was particularly abundant in the Costacalda wild cave (up to 31.8%), while in the showcaves did not exceed 10% (from 2.31% in Caudano up to 9.30% in Vento). The opposite was observed for the genus *Candida* (class Saccharomycetes), which was more abundant in the tourist caves (ranging from 13.36% in Caudano to 37.32% in Vento). Also, the genus *Dipodascus* was mainly present in the anthropized settings, even if at much lower

extent compared to *Candida*, ranging from 0.14% in Pertosa-Auletta to 4% in Bossea. The genus *Archaeorhizomyces* (class Archaeorhizomycetes) was reported in showcaves only, albeit with scarce relative abundance (e.g. 0.01% Bossea up to 2.70% Vento). A number of cave-specific genera in the class Sordariomycetes have been recorded: for instance, *Chaetomium* (7.50%) in Caudano, *Sarocladium* (6.4%) in Pertosa-Auletta, *Cephalotrichum* (6.30%) and *Rodentomyces* (5.60%) in Costacalda; differently, the genus *Humicola* (average abundance 1.39%) was found in all caves (Fig. 2). The class Eurotiomycetes was mainly represented by the genera *Aspergillus* (ranging from 0.01% in Costacalda to 6.77% in Pertosa-Auletta) and *Penicillium* (ranging from 0.17% in Vento to 6.45% in Pertosa-Auletta), while the genus *Gymnoascus* was most abundant in Costacalda and Bossea caves, accounting for 3.72% and 1.50% respectively. Likewise, the genera *Tetracladium* and *Pseudogymnoascus* (not shown in the Top 15) were the dominant members of Leotiomycetes throughout the dataset, up to 1.96% and 1.12% in Costacalda, respectively (Fig. 2D). Agaricomycetes class was particularly abundant in show-caves (Caudano 12.6%, Pertosa-Auletta 8.25%, Bossea 4.44%, Vento 4.30%, Costacalda 1.2%), with the genus *Bjerkandera* being the only one reported among the top 15 (Fig. 2D). Yet, despite a considerable proportion of classes Tremellomycetes (ranging from 1.41% at Pertosa-Auletta to 12.7% at Bossea) and Dothideomycetes (ranging from 1.64% at Pertosa-Auletta to 7.50% at Caudano) among caves, they were mainly represented by two genera, i.e. *Apiotrichum* (Trichosporonaceae) and *Phoma* (Didymellaceae). *Apiotrichum* was spotted across all sites investigated, while *Phoma* appeared more prevalent among tourist caves (Vento: 3.45%, Caudano: 1.51%, Bossea: 1.10%, Pertosa-Auletta: 0.07%, Costacalda: 0.004%). Some other fungal classes showed different abundance patterns between tourist and wild cave, such as: Malasseziomycetes, (over 1% in show-caves vs. 0.15% of Costacalda), Microbotryomycetes (Bossea: 0.93%, Vento: 0.3%, Caudano: 0.16%, Pertosa-Auletta: 0.13%, vs. Costacalda: < 0.001%) and Geminibasidiomycetes (Costacalda: 1.52%, vs. all tourist caves: < 0.2%). Finally, lichenized fungi class of Lecanoromycetes was especially reported in Bossea (0.85%) and Vento (0.52%) caves.

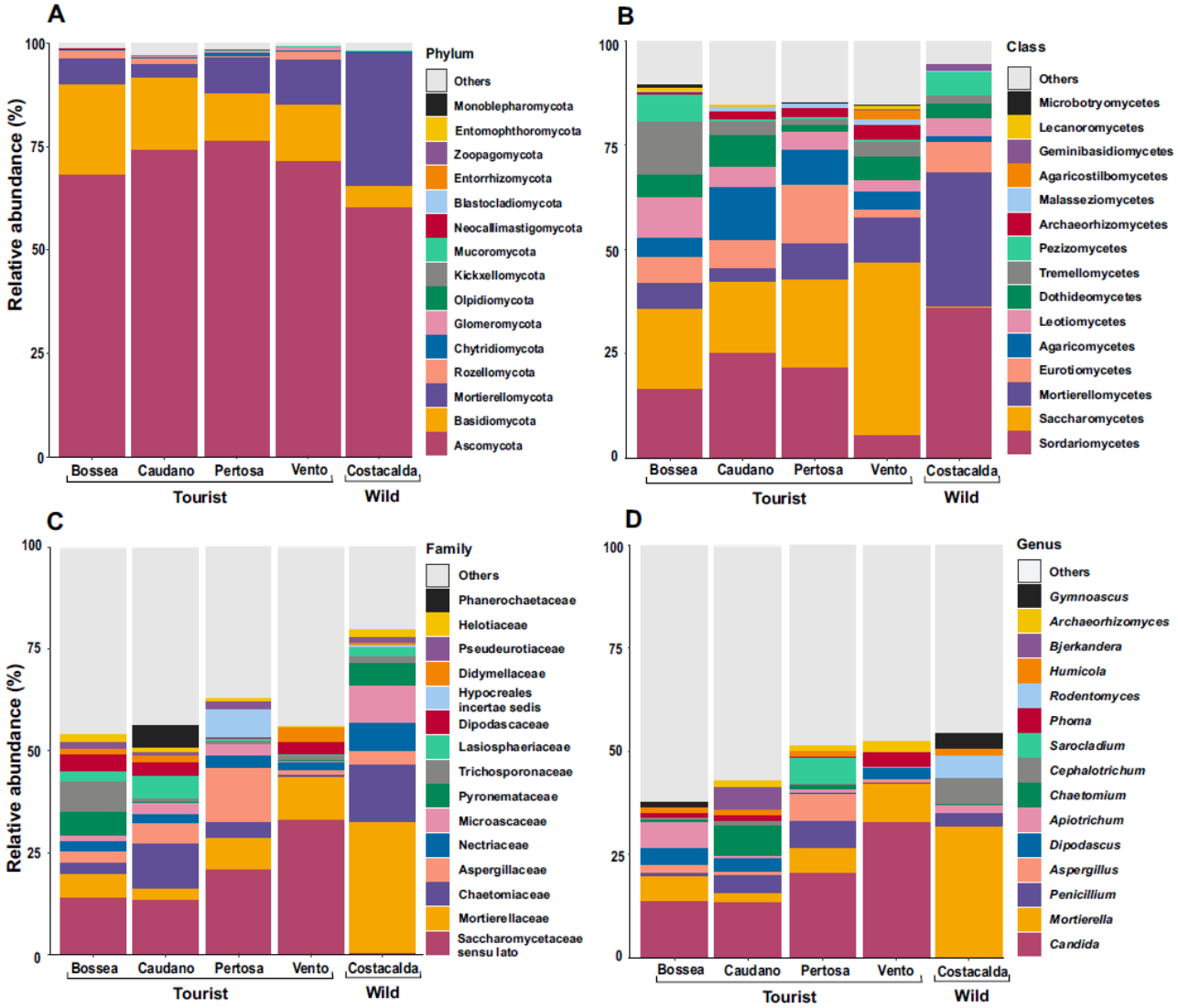

Figure 2. Fungal community composition of 5 examined caves: Bossea, Caudano Pertosa-Auletta, Vento (showcaves) and Costacalda (wild cave). The top 15 most abundant taxa are shown for taxonomic ranks: phylum (A), class (B), family (C) and genus (D).

2.2.3 Bacterial community composition

A different bacterial distribution was found in each cave (Fig. 3 A-D). For instance, the phylum Pseudomonadota dominated throughout the dataset, with remarkable presence of its main classes (Gamma, Alpha, Beta and Delta-Proteobacteria) and by different genera reported in the top 15 (Fig. 3). The class Gamma-Proteobacteria was mainly represented by the genus *Pseudomonas,* particularly abundant in tourist caves (ranging from 11.1% in Bossea to 17.78% in Pertosa-Auletta, vs. Costacalda 2.95%). Conversely, the genera *Sphingomonas* (class Alpha-Proteobacteria, 2.46%), *Lysobacter* (class Gamma-Proteobacteria, 2.34%) and *Polaromonas* (class Beta-Proteobacteria, 1.8%) were prevalent in the pristine site, while *Hyphomicrobium* genus (class Alpha-Proteobacteria)

was largely spread across all habitats (average of 0.6%). Three Acidobacteria sub-groups, i.e. Gp6 (mean 6.0%; highest in Bossea: 8.2%), Gp17 (mean 2. 0%), Gp16 (mean 1.74%), Gp4 (mean 1.35%) and the two main Actinobacteriota genera, i.e. *Gaiella* (with 0.78% in Pertosa-Auletta up to 4.51% in Vento caves) and *Arthrobacter* (ranging from 0.24% in Vento up to 2% in Pertosa-Auletta caves) showed a broad distribution across all caves. Yet, among Actinobacteriota, the genus *Bifidobacterium* was recorded only in show-caves (between 0.3% in Caudano and 2.5% in Pertosa-Bossea caves). However, similar abundance trends across all surveyed sites were also reported for the family Planctomycetaceae (ranging from 2.7% in Vento up to 4.27% in Bossea caves) and the genus *Nitrospira* (between 0.7% for Caudano and 1.75% of Costacalda caves). Other remarkable compositional spectra were represented by Bacillota with classes Clostridia (ranging from 0.48% in Caudano up to 1.62% in Bossea, vs. 0.07% in Costacalda) and Bacilli (from 3.15% in Caudano up to 7.66% in Pertosa-Auletta and 1.81% for Costacalda caves) with genus *Lactobacillus* (1.64% mean in show-caves, vs. 0.007% in Costacalda) all typically found in anthropized settings. Conversely, the *Flavobacterium* genus (Phylum Bacteroidota, 2.47% in Costacalda) was more than 8 times higher in the natural one than in showcaves.

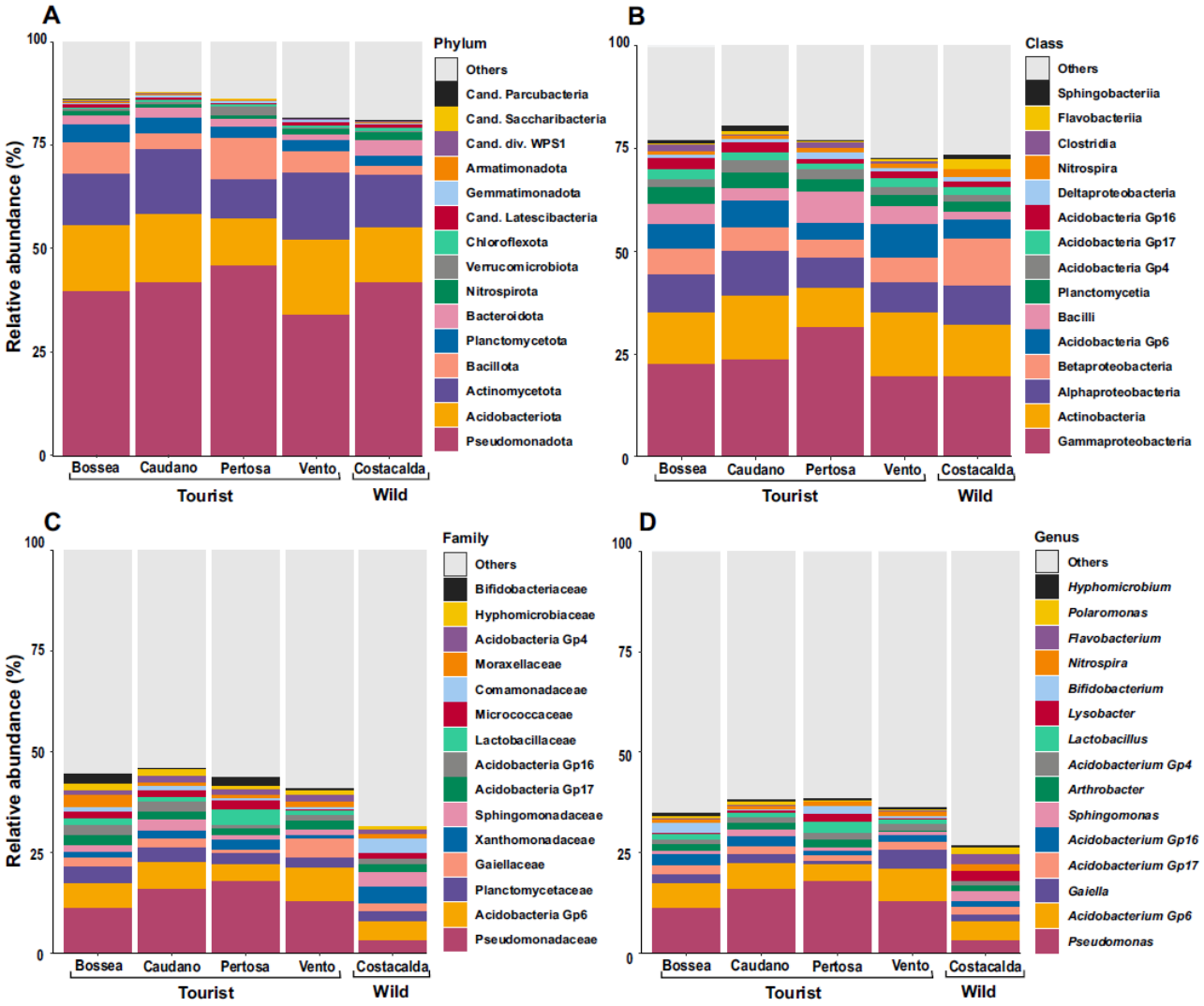

Figure 3. Bacterial community composition of 5 caves studied: Bossea, Caudano Pertosa-Auletta, Vento (showcaves) and Costacalda (wild cave). The top 15 most abundant taxa are shown for taxonomic ranks: phylum (A), class (B), family (C) and genus (D).

2.2.4 *Archaeal community composition*

Archaea showed 2 main compositional trends describing the archaeome throughout the investigated caves (Fig. 4: A,B,C,D). The first group is recurrent in all investigated caves and includes Thaumarchaeota and Euryarchaeota as the most abundant phyla, with the genera *Nitrososphaera* (ranging from 21.38% in Bossea to 41.35% in Pertosa-Auletta caves), *Nitrosopumilus* (between 14.23% and 41.76% in Bossea e Pertosa-Auletta) and *Methanomassiliicoccus* (ranging from 3% in Pertosa-Auletta to 21.35% in Costacalda). The same distribution across all caves was observed for taxa in the phylum Woesearchaeota, even if far less represented. The second group included taxa more frequent in showcaves as for the genera *Acidianus* (Phylum Chrenarcheota, class Thermoprotei, ranging from 2.62% Bossea to 11.34% in Pertosa-Auletta), *Ignicoccus* (average in

show-caves 2.04%) and *Thermocladium* (4.14% highest value in Pertosa-Auletta); the genera *Methanothermobacter* and *Methanobacterium* (Phylum Euryarcheota, class Methanobacteria) were also found mainly in showcaves, the first with an average of 2.33% and the second with a frequency ranging between 0.55% and 3.9%. Other less abundant genera such as *Methanosphaera* and *Thermococcus* could be included in this second group.

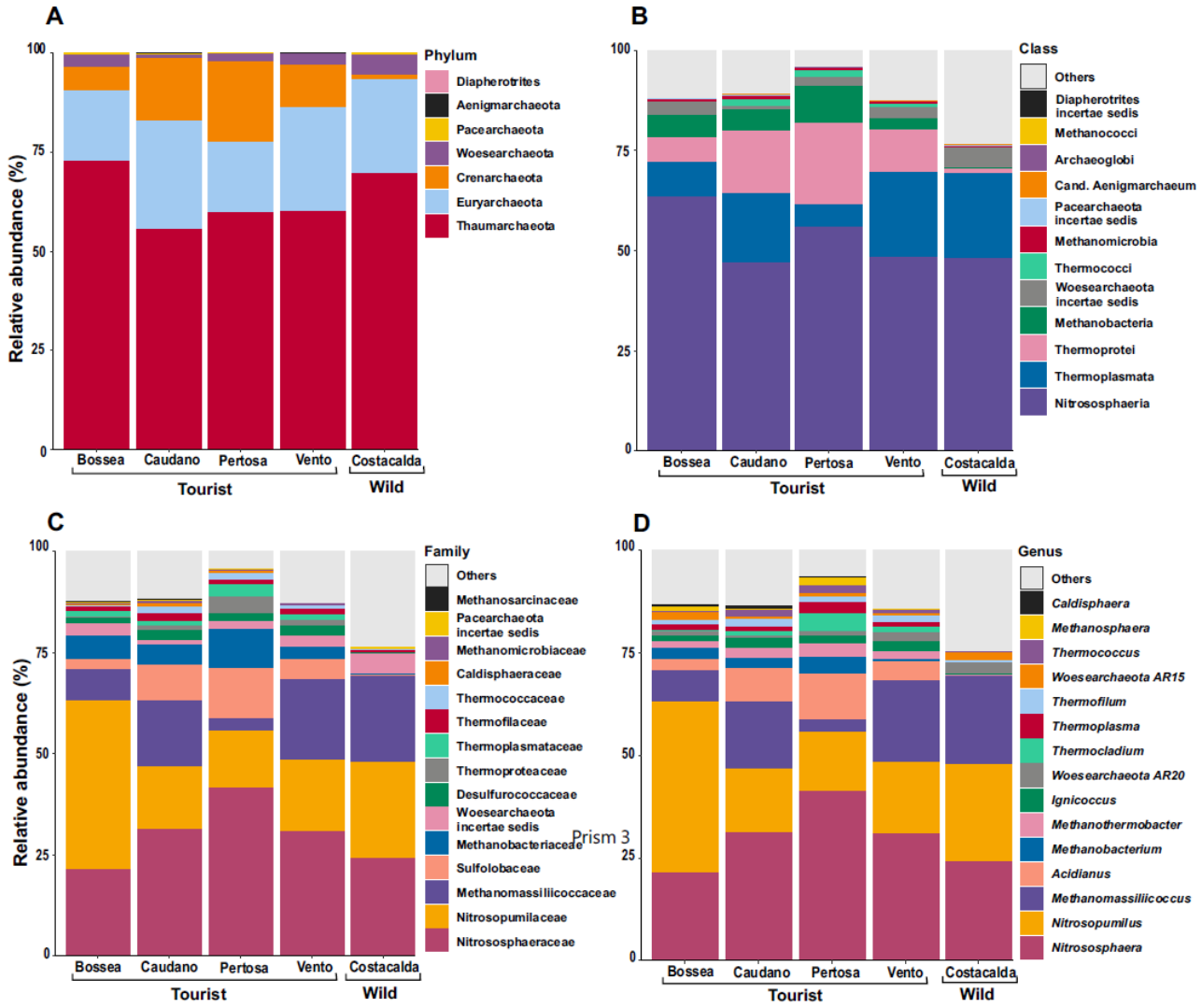

Figure 4. Archaeal community composition of 5 caves studied: Bossea, Caudano Pertosa-Auletta, Vento (showcaves) and Costacalda (wild cave). The top 15 most abundant taxa are shown for taxonomic ranks: phylum (A), class (B), family (C) and genus (D).

2.2.5 Micro-algal community composition

Algae is by definition a polyphyletic group; therefore, the micro-algal assemblage (Fig. 5: A,B) was derived by clustering members belonging to different kingdoms in order to investigate the micro-photosynthetic component. The compositional scenario was characterized by the Class Chrysophyceae (phylum Ochrophyta) dominating in most of the caves, in particular in Costacalda (81.5%) and Pertosa-Auletta (39.76%) caves. Vento cave was dominated by Cryptophyceae (35.3%) members of the phylum Cryptophyta. Thereafter, community composition became patchy as no other widespread classes were found across the dataset. For instance, Dinophyceae showed a high abundance in the Caudano cave (25.86%), but it dropped in Vento (12.1%), Pertosa-Auletta and Costacalda (11.57%) caves; on the other hand, Trebouxiophyceae showed a decreasing trend along Caudano (20.10%) del Vento (17.37%), Bossea (11.47%) and Pertosa-Auletta (7.25%) caves. The class Diatomea was particularly present in Bossea cave instead (24.48%).

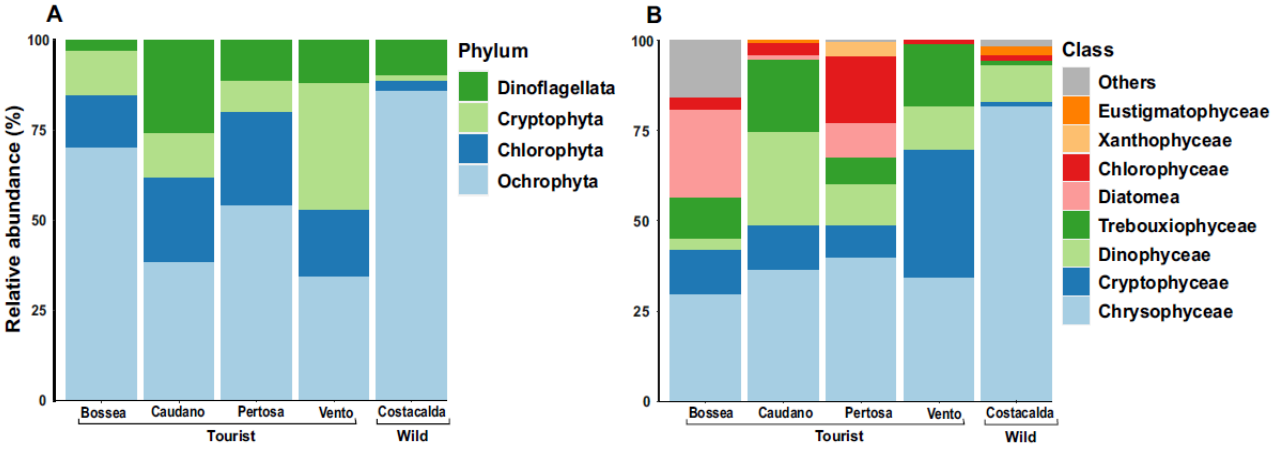

Figure 5. Micro-algal assemblage composition of 5 caves studied: Bossea, Caudano Pertosa-Auletta, del Vento (showcaves) and Costacalda (wild cave). All taxa observed are shown for taxonomic ranks: phylum (A) and class (B).

2.2.6 Shared and unique taxa

Venn diagrams (Fig. 6) displayed the ASVs that typically characterized each cave (Unique ASVs) and those that are representative of the community core, shared throughout the dataset. Regarding the fungal community, the core was scarcely populated (200 unique ASVs), accounting for only 2.2% of the total composition. The ASVs shared between all caves were represented by 54 genera; in particular *Mortierella* (13%) and *Candida* (10%). Followed by *Penicillium* (5.4%), *Humicola*

(3.8%), *Cephalotrichum* (3.1%), *Geomyces* (2.3%), *Pseudogymnoascus* (1.5%) and *Aspergillus* (1.5%) genera commonly found in cave environments. When considering the unique ASVs, Bossea cave had the highest number of unique ASVs (1,281) representing 14% of the total composition, spread over 178 fungal genera. The other caves, showed a conspicuous number of unique ASVs, in particular the Caudano cave (1,144 ASVs, 12.5% of the total), displayed the highest number of genera (192) identified among the typical ASVs, followed by Vento cave showed 1,124 unique ASVs (12.2%) and Pertosa-Auletta cave characterized by 999 unique ASVs (10.9%) with 171 identified genera. The wild Costacalda cave counted 102 unique ASVs (1.1%), with only 30 genera among the site-specific ASVs reported.

The bacterial community (Fig. 6) gravitated around a robust core of 4,674 ASVs (15.1%) and 283 genera, among which *Acidobacteria* Gp6 (15.8%), *Gaiella* (6%) and *Nitrospira* (1.9%) were the most abundant. However, the entire cluster of subgroups-*Acidobacteria* accounted for 37% of the shared ASVs. On the other hand, there were few unique ASVs for each cave, with Pertosa-Auletta cave showing 2,292 ASVs (7.4%), followed by Bossea cave (1,870 ASVs, 6%), Vento cave (1,260 ASVs, 4.1%), Caudano cave (742 ASVs, 2.4%) and Costacalda cave (262 ASVs, 0.8%). Moreover, some peculiar genera were uniquely reported for the different caves, such as *Legionella* (3.3%) in Bossea, *Gemmatimonas* (4.5%) in Caudano, *Lactobacillus* (9.5%) *Desulfomicrobium* (3.8%) and *Clostridium sensu stricto* (3.6%) in Pertosa-Auletta.

The distribution of ASVs belonging to the archaeal component among caves (Fig. 6) was sharply defined. Indeed, while the core group (79 ASVs, 9.1%) and unique ASVs of Bossea (20 ASVs, 2.3%), Vento (15 ASVs, 1.7%), Costacalda (9 ASVs, 1%) and Caudano (3 ASVs, 0.3%) caves were represented by a low number of ASVs, the Pertosa-Auletta cave was characterized by 355 unique ASVs (41%), taxonomically labeled by *Acidianus* (33%) and *Thermoplasma* (14%) genera. However, the genera of archaeal methanogens constituted a solid cluster either across the common core (24.3%) and unique ASVs for each cave.

The distribution of taxa detected for the phototrophic assemblage (Fig. 6) was extremely polarized between caves. One pole was represented by Costacalda Cave for which no unique ASVs were

recorded. The other one was the cave of Pertosa-Auletta which accounted for 28 unique ASVs (28.9%), mostly belonging to the Chlorophyceae (39%), Chrysophyceae (32%) and Diatomea (18%) classes.

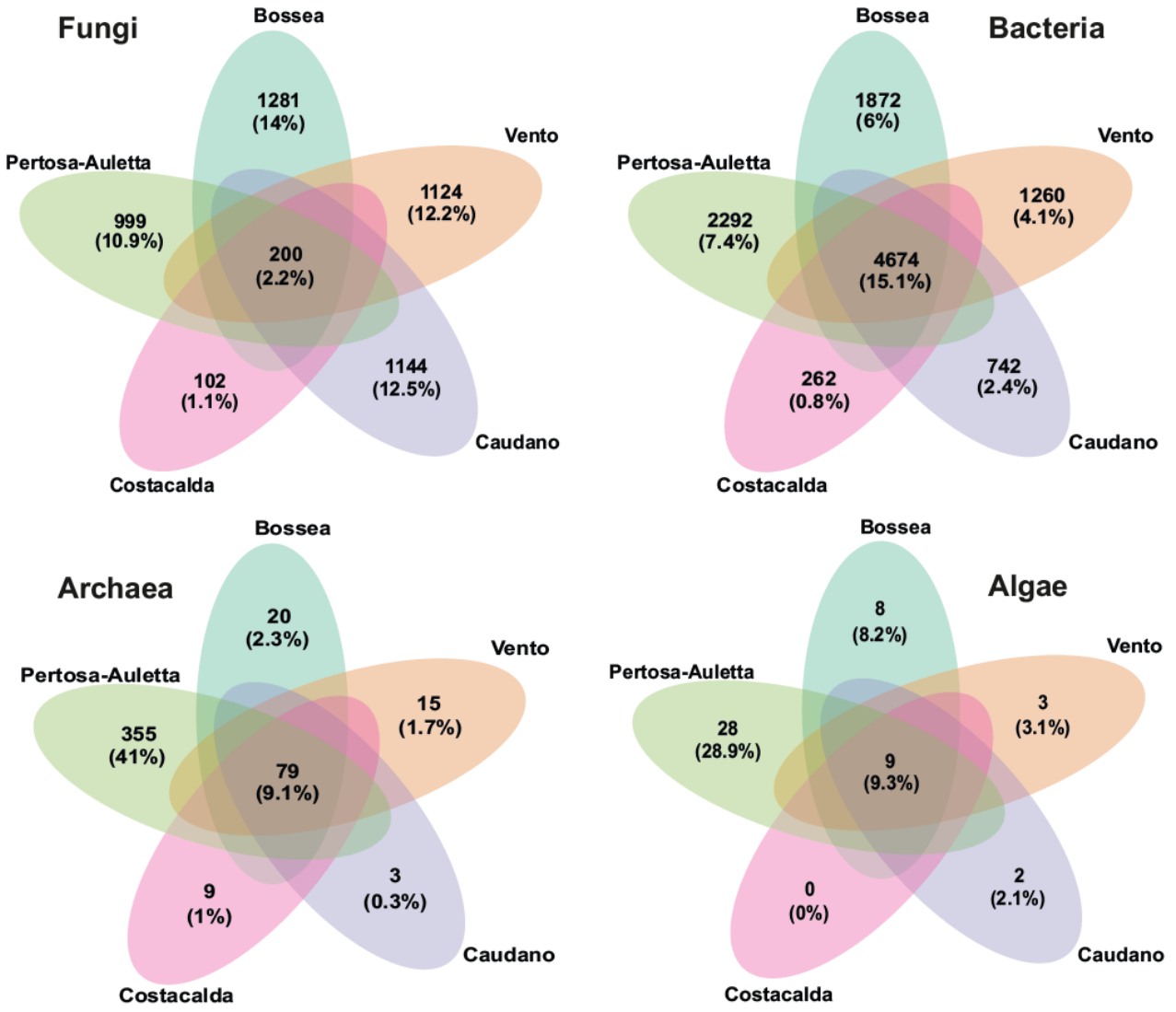

Fig 6. Venn diagrams show shared and unique taxa distribution for each microbial component studied: Fungi, Bacteria, Archaea and Micro-Algae, among the 5 caves investigated: Bossea, Caudano Pertosa-Auletta, Vento (showcaves) and Costacalda (wild cave).

2.2.7 Human related microbial taxa

Due to the high abundance of some common human-related microbial taxa found in the showcaves investigated (i.e. Malasseziomycetes class and *Candida* genus for fungi, *Lactobacillus* and *Bifidobacterium* genera for bacteria), we decided to explore the occurrence of less abundant guilds too as potential proxies for direct human impact.

Throughout the fungal dataset (Fig. 7A) tourist caves were especially characterized by an higher significant presence of dermatophytes with the genus *Trichosporon* (Bossea 0.26%, Vento 0.084%, Caudano 0.0054% and Pertosa-Auletta 0.0001%, vs. 0% in Costacalda; $p<0.05$) and *Cutaneotrichosporon* on average 0.05% for the show -caves, vs. 0% in Costacalda ($p<0.05$). The genus *Arthroderma*, here recorded in all caves analyzed, is instead a geophilic dermatophyte mostly occurring in soil, but also frequent in cave environments, and it is rarely associated with human and animal cutaneous infections [19]. Moreover, *Malassezia* spp., here mainly found in human impacted caves (Bossea 0.07%, Caudano 1.02%, Pertosa-Auletta 1.10% and Vento 1.30%, vs. Costacalda 0.15%; $p>0.05$), currently exist as a commensal fungi of the mammalian skin, and associated with atopic dermatitis in inflammatory skin disorders only.

Similarly, for bacterial assemblage (Fig. 7B), many widespread human tissues-inhabiting members of Bacilli class characterized the tourist caves, in contrast to Costacalda wild cave, where their relative abundance never exceeded the 0.007%; in particular *Staphylococcus* (ranging from 0.082% in Pertosa-Auletta to 0.48% in Bossea; $p<0.05$), *Streptococcus* (from 0.061% in Pertosa-Auletta to 0.47% in Bossea; $p<0.01$), *Lactococcus* (as a mean of 0.21% in the showcaves, vs. 0.003% in Costacalda; $p<0.05$) and *Lactobacillus* (with 1.7% on average in showcaves, vs. 0.007% in Costacalda; $p<0.05$) showed a higher significant occurrence within show-caves. Furthermore, even the *Clostridium* genus (from 0.5% in Caudano to 1.6% in Bossea, vs. 0.07% in Costacalda; $p>0.05$) seemed to exhibit a higher occurrence across the anthropized settings.

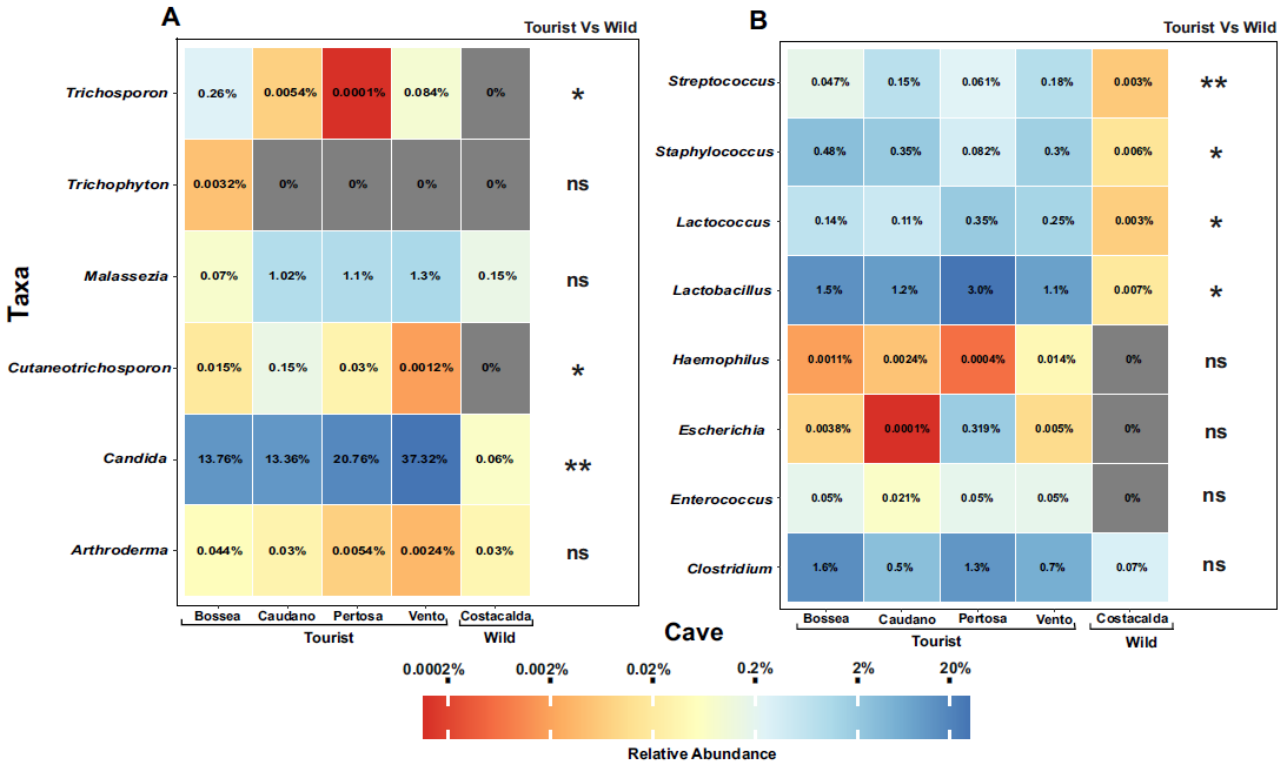

Fig 7. Heatmaps showing the relative abundances of fungal (A) and bacterial (B) taxa commonly associated to the human body, among the 5 caves investigated: Bossea, Caudano Pertosa-Auletta, Vento (showcaves) and Costacalda (wild cave). Kruskal-Wallis test results comparing tourist caves vs. wild cave are shown as follows: *** ($p<0.001$); ** ($p<0.01$); * ($p<0.05$); ns ($p>0.05$).

2.3 Discussion

The effects of alterations occurring on structure, biodiversity, microclimate, energetic condition [11;12] during the conversion of a natural cave into a showcave are still almost unexplored. However, the increasing anthropization of new hypogean sites associated with human exploitation constantly impacts cave microbiomes, making their composition and functionalities unpredictable for future conservation and management [9;10;13;20]. Herein, we deeply investigate, for the first time, microbiomes of four Italian showcaves using next generation sequencing approaches. We provided a first attempt to identify potential human-induced alterations by comparing showcaves with a recently discovered natural cave. The extensive sampling performed, together with the amplicon-sequencing analysis, led to a wide identification of microbial diversity, encompassing 17 phyla, 52 classes and 554 genera for the ITS dataset. On the other hand, the 16S barcode recorded 37 and 7 phyla, 73 and 8 classes, 1,339 and 49 genera for Bacteria and Archaea domains, respectively. For algae, a total of 4 phyla and 9 classes were identified; this low diversity of the

phototropic counterpart is likely due to the condition of darkness characterizing the ground sampling area, although abundant algal growths were reported at the level of the rock formations in the examined showcaves [21].

The mycobiome composition and the distribution of unique fungal ASVs across the 4 surveyed showcaves appeared more complex and diversified than those observed in the natural one. The predominance of *Candida* genus, which includes many species known as common colonizers of humans, in showcaves may reflect a relation with touristic flow. *Candida* has also been reported as an oligotrophic multi-stress tolerant fungus, skilled to colonize a wide range of natural, urbanized and human environmental contexts [22;23]. Cave visitors, as previously reported, may act as primary vector in the spreading of this genus, by bio-aerosols formation (breathing, sneezing or coughing), surface contact and shedding of skin scales, hair and lint from clothing [24]. Potential direct contamination by human-related species [25] of cave mycobiome are further supported by the higher abundance of dermatophyte fungal genera within tourist caves (e.g. *Cutaneotrichosporon* and *Trichosporon*). Conversely, the mycobiome of the natural cave was dominated by taxa belonging to the genera *Mortierella* and *Cephalotricum*, which include psychrotolerant cellulose-degrading fungi [26;27], and by the genera *Rodentomyces* and *Gymnoascus*, which include psychrotolerant, coprophilic and keratinolytic species [28;29], that may be related to the main caves macro-fauna components, i.e. rodents and bats. Furthermore, fast-growing saprophytic fungal genera (*Penicillium*, *Chaetomium* and *Humicola*) were also reported, even if in lower relative abundance.

Such specific fungal traits have been reported as early wild cave colonizers [30], able to cope with the constraining low-temperature and oligotrophic conditions in these habitats. On the other hand, within the 4 investigated showcaves, the notable abundance of Agaricomycetes (and generally Basidiomycota) may be the result of increasing ventilation during cave opening. In fact, airborne fungal spore contamination is facilitated by the atmospheric continuum between the outdoor and underground environment [31;32]. Moreover, the high occurrence of fast-growing and heavily-sporulating fungi (*Aspergillus* and *Penicillium*), or common competent degraders and plant pathogens (*Chaetomium*, *Phoma*, *Sarocladium* and *Dipodascus*) could be the result of combined

effects of tourist flows, spreading fungal alien species and extra complex organic matter input, and the implement of nutrients due to the consumption of food and beverages during events hosted in caves [33;34]. Finally, artificial lighting may have promoted the growth of lichenized fungi (Lecanoromycetes) inside the caves, as reported in previous studies [18;35]. In particular, their colonization of carbonatic rock substrate and speleothems may result in severe aesthetic and structural damage, due to the large extent of thalli and the high lichen acids production. The genus *Tetracladium* was always present in all caves, even if at low frequency. It belongs to the group of "Ingoldian fungi" or aquatic hyphomycetes, phylogenetically diverse fungi growing on decaying leaves and plant litter in streams [36]; their presence in our environmental samples may be related to the presence of water flows present in all caves.

The compositional similarity found throughout the dataset for the Bacteria and Archaea domains, reflects their paramount role in biogeochemical cycles of caves biosphere: C, N, CH_4-genesis and metabolism and S oxide-reduction, sustaining trophic networks and peculiar caves' geochemical processes, even in these deeply impacted habitats. In fact, a wide range of polymers degraders particularly adapted to cope with low temperature and oligotrophic conditions were found. For instance, Acidobacteria-subgroups (37% of the shared ASVs among the caves), *Sphingomonas*, *Lysobacter*, *Polaromonas* and a few widespread Mn-Fe oxidizing bacteria, such as family Planctomycetaceae members, *Hyphomicrobium* (Bacteroidota), *Flavobacterium* and *Pseudomonas* (Pseudomonadota) genera. Yet, the *Pseudomonas* high abundance reported in show -caves might be due to the indirect effects of human activities in these habitats. Extra nutrient inputs by tourist flows might stimulate fungal metabolic activity [37] and lead to high secretion of acids (i.e. oxalate and pyrophosphate), which in turn could trigger the metabolic activity and proliferation of *Pseudomonas* by chelating Mn [38]. Also related to the biogenic carbon cycle, the group of Methane-oxidizing bacteria (MOB) found along α,β,γ-Proetobacteria classes were widely present throughout the caves, underlining their key role in continuous CH_4 consumption in these subterranean ecosystems [39]. In contrast, *Methanobacterium* and *Methanothermobacter* were more present in tourist caves among the most abundant genera of the Archaea-methanogens counterpart.

This compositional alteration could be the result of the indirect effects of tourist flows, which may increase indoor CO_2 levels up to 200% [40], promoting the proliferation of these microorganisms, which use H_2 to reduce carbon dioxide molecules into methane. Compositional variations for the archaeal community have already been reported in touristic caves, due to anthropic pressure and after simulated organic matter treatment on cave microcosms [13;41]. The similar compositional pattern throughout the dataset of the main bacterial and archaeal players involved in the N cycle, i.e. *Nitrospira* (nitrite-oxidant), *Gaiella* (nitrate-reducing), *Nitrososphaera* and *Nitrosopumilus* (ammonia-oxidant), underlines how human cave activities did not impact these microbial traits. Besides, some bacterial taxa could be also considered as bioindicators of human presence being a result of contamination from the entrance [30;42]. For instance, many human-related genera as , *Lactobacillus* (also as unique ASVs), *Lactococcus*, *Legionella* (only as unique ASVs), *Staphylococcus* and *Streptococcus* were mainly, or in some cases uniquely, found in show -caves. Similarly, the recurrence of opportunistic human pathogenic fungi such as *Candida* and dermatophytes (i.e *Trichosporon* and *Cutaneotrichosporon*) throughout tourist caves, emphasize a probable direct mycobiome cave contamination as the result of the extensive human exploitation of these subterranean settings.

Concerning microalgae, except for a widespread presence among caves of Ochrophyta phylum and gold-brown algae Chrysophyceae related class, the eukaryotic photosynthetic abundance background appeared patchy. Some caves were largely characterized by presence of particular classes, e.g. Bossea cave by Diatomea, Caudano by Dinophyceae and Trebouxiophyceae, Pertosa-Auletta by Chlorophyceae and Vento cave with Cryptophyceae and Trebouxiophyceae. However, the only notable differences were related to the relative abundance values and lack of unique ASVs of microalgal components harbored by the wild Costacalda cave, dominated by the class Chrysophyceae. This class is composed of taxa that prefer oligotrophic conditions and most species in this group show a mixotrophic metabolism, being able to shift between photosynthesis and ingesting smaller organisms or particles for food. We may hypothesize that the micro-algal component in pristine caves was based on a poorly biodiverse common core [18], populated by

species well adapted to cope with the typical challenges of the cave environment, such as strict oligotrophy and darkness [43]. The subsequent anthropization (artificial lighting and tourist flows) of these settings would have had a key role, both in importing allochthonous species and spreading the local and alien microalgal component even in cave's zones previously uncolonized [18;44]. Yet, the ASVs cores recorded in bacteria, archaea and algae indicate a high degree of adaptation and specialization for these microbial compartments in caves. Conversely, the narrow core observed for the fungal component may indicate a less strict adaptation to the cave environment and broad capacity to colonize different habitats, according to the high physiological, metabolic and stress-tolerance plasticity of these organisms.

In conclusion, this study provides for the first time a multi-spatial and extensive microbiota characterization of 5 Italian caves, among which 4 tourist and 1 pristine cave, shedding light on microbial diversity of different rapidly evolving subterranean environments. We highlighted how the 4 investigated showcaves share common microbial traits, by filtering microorganisms derived from the outside or human-related, multi-stress tolerant or capable of exploiting the extra supplies of organic matter or degradable compounds provided by tourist flows. Although human activities have been affecting these caves for a long time, the principal common microbial traits, related to the biogeochemical key processes, are still clearly detectable. Finally, we have provided some insights into the potential direct microbiome cave contamination by human-related microbial species.

It is worth expanding this dataset by sampling additional underground environments, including both showcaves and wild caves, at local (Italian) geographic scales, to confirm the trends of microbial diversity pattern here found in anthropized caves with respect to the wild one. This study represents the groundwork for further microbiome cave analyses to shed light also on the functional guilds of each microbial component and their distribution and variation in both pristine and impacted settings.

2.4 Materials and Methods

2.4.1 Study area

Five Italian karst caves were selected from north to south Italy (Fig. 8A): the Caudano (44°17'34.3"N; 7°47'25.7"E), Bossea (44°14'31.0"N; 7°50'24.0"E) and Costacalda (44°14'24.8"N; 7°50'54.9"E) caves, in the Maritime Alps complex (Piedmont region); Vento cave (44°02'02.0"N; 10°21'28.2"E) in the Apuan Alps (Tuscany) and the Pertosa-Auletta cave (40°32'14.7"N; 15°27'17.9"E) in the mountain region of the Campanian Apennines.

Besides sharing the same karstic origin, the caves investigated, with the exception of Costacalda, are characterized by a long human exploitation background. Bossea was the first Italian show cave established in 1875 (https://www.grottadibossea.com), while the Caudano cave was permanently opened to the public in 2002 (https://grottedelcaudano.com). Besides regular tourist flows, these caves have experienced exceptional human activity episodes over the last century, like the "700 hours underground" experiment (Caudano cave in 1961), where 12 speleologists and some domestic animals lived inside the cave for one month, aiming to understand the effect of underground life on human and animals. In addition, Bossea cave has been the setting of many music and food-wine festivals. The Vento cave has been known since the 17th century and its cold air currents were used by local people as a natural cooler to preserve food b. The cave was later explored in the 19th century and opened to the public for the first time in 1967 (http://grottadelvento.com). The Pertosa-Auletta cave has been exploited by humans since the Neolithic, successively used during the Iron Age (the ruins of a stilt village were found), then converted into a Christian church during the 12th century and into an air-raid shelter during WWII It was permanently turned into a tourist cave in the second half of the 1900s (https://fondazionemida.com/grotte-pertosa-auletta). Costacalda is a natural (wild; i.e. not impacted) cave discovered in the spring of 2018, when it appeared as a hole about 10 cm wide on the ground. To date, the cave is only partially explored and entirely preserves its pristine structural condition (http://www.speleologiassi.it/82-sommari).

2.4.2 Sampling design

We applied an extensive sampling of sediments along the whole extension of each cave (Fig. 8B) according to Piano et al. (2022) [20], starting from the areas near the entrance, towards the deepest zones and at different distances from the tourist/speleological paths. A total of 12 *in situ* sampling sites were selected for the 4 show -caves, while 3 sites were selected in the Costacalda wild cave. For each sample, 9 technical sediment replicates subdivided into 3 triplets, up to 5 cm depth, were collected using sterile Falcon® tubes (50 ml), for a total of 459 samples. Samples were then stored in a cooler-bag until arrival at the laboratory, where the 3 replicates for each sample were pooled and homogenized. Twelve final samples were assembled for each show cave (Bossea, Caudano, del Vento and Pertosa-Auletta) and 3 for Costacalda, for a total of 51 samples. Sampling collection was carried out between June and September 2020.

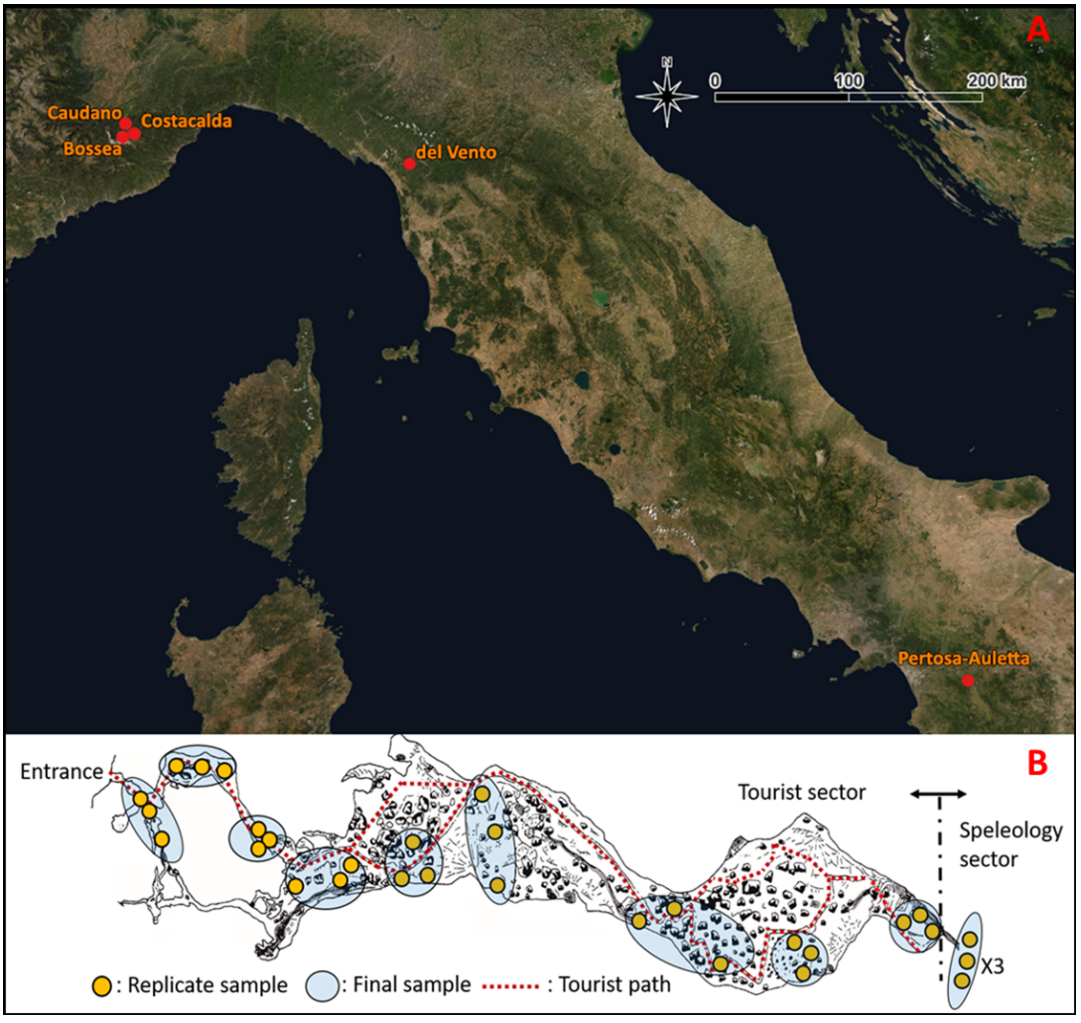

Figure 8. Geographic map of Italy with locations of caves surveyed (A) and schematic overview of sampling method applied (B). Images were produced using qGIS 3.22 [45].

2.4.3 Metagenomic DNA extraction and amplicon sequencing

Sediment samples were sieved, under sterile conditions, by removing coarse rock debris and metagenomic DNA was extracted from 0.5 g of sample using Qiagen DNeasy PowerSoil Pro Kit (Carlsbad, CA, USA). The Internal Transcribed Sequence 1 ribosomal region (ITS1), Eukaryotic SSU rRNA (18S) and hypervariable region V4 of 16S ribosomal gene were targeted to assess the fungal, general eukaryotic and prokaryotic community composition, respectively. The ITS1 region was amplified using barcoded primers ITS1F/ITS2, suitable for shorter read length [46], the 18S region with the Euk_1391f/EukBr primers (http://www.earthmicrobiome.org), while for the V4 region of 16S, barcoded F515/R806 primer set was used according to Caporaso et al. (2012) [47]. PCRs was performed with 1 μL of each primer, 12.5 μL of Taq DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA), 9.5 μL of nuclease-free water (Sigma–Aldrich, St. Louis, MO, USA) and 5 ng of DNA template by an automated thermal cycler (BioRad, Hercules, CA, USA), for a total volume of 25 μL. The ITS1 locus, the eukaryotic SSU rRNA and 16S V4 region were amplified following the protocols and technical specifications according to Coleine et al. (2021) [48]. Amplicons were quantified by a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) and then pooled. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq platform.

2.4.4 Bioinformatic processing data and downstream analysis

Demultiplexed ITS, 18S and 16S sequence datasets were processed using AMPtk [49] v.1.5.4 software. Briefly, reads were merged using VSEARCH 2.21.1 [50] and barcodes/indexes and primer sequences were removed from raw data; reads were then subjected to quality trimming to a maximum of 250 bp and discarding reads less than 100 bp in length, and sequencing artifacts were dropped by using USEARCH v.9.1.13 with default parameters [51]. Sequence quality filtering was performed with the expected error parameter of 0.9 [52] and the cleaned reads were clustered at 99% similarity using DADA2 denoising algorithm [53] that uses a statistical error model to correct sequencing errors to infer the Amplicon Sequence Variants (ASVs). Global singletons and rare taxa (<5 reads) were skipped as likely false positives due to sequencing errors, following Lindahl et al.

(2013) [54]. Finally, taxonomic identification was performed with a sequence identity of 97% as threshold, using hybrid database Global Alignment and SINTAX [51] on reference databases UNITE 8.2. [55] and RDP 11 [56]. All samples from each cave were analyzed as replicates to describe the global microbial diversity of each underground environment. The relative abundance data shown in this paper represent the average of the abundance values among all samples for each cave. Kruskal-Wallis test was used to assess significant abundance differences among tourist and natural settings, for the potential human-associated microbial taxa. All analyses were performed using the following R packages: "phyloseq" [57], "microeco" [58].

Data availability

Raw sequences were deposited in Figshare's repository and are openly available at https:// doi. org/ 10. 6084/ m9. figsh are. 21354 537.

2.5 References

[1] Gabriel, C. R., & Northup, D. E. Microbial ecology: caves as an extreme habitat. In *Cave microbiomes: a novel resource for drug discovery,* (ed. Springer, New York, NY), 85-108; (2013).

[2] Zhang, Z. F., Zhao, P., Cai, L. Origin of cave fungi. *Frontiers in microbiology*, **9**, 1407; (2018).

[3] Culver, D. C., & Pipan, T. *The biology of caves and other subterranean habitats*. (*Oxford University Press)*; (2019).

[4] Romero, A. Cave biology: life in darkness. (*Cambridge University Press); (2019)*.

[5] Engel A. S. Chemoautotrophy. In: White WB, Culver DC, eds. *Encyclopedia of Caves,* (ed. Oxord, UK, Academic Press), 125–34; (Elsevier, 2012).

[6] Miller, A. Z., Dionísio, A., Jurado, V., Cuezva, S., Sanchez-Moral, S., Cañaveras, J. C., Saiz-Jimenez, C. Biomineralization by cave dwelling microorganisms. *Advances in Geochemistry Research*, 77-105; (2013).

[7] Venarsky, M. P., & Huntsman, B. M. Food webs in caves. (In *Cave ecology* Springer), 309-328; (Cham, 2018).

[8] Wiseschart, A., & Pootanakit, K. Metagenomic-based approach to a comprehensive understanding of cave microbial diversity. *Recent advancements in microbial diversity*, 561-586; (2020).

[9] Falasco, E., Ector, L., Isaia, M., Wetzel, C. E., Hoffmann, L., Bona, F. Diatom flora in subterranean ecosystems: a review. *International Journal of Speleology*, **43**(3); (2014).

[10] Mulec, J. Human impact on underground cultural and natural heritage sites, biological parameters of monitoring and remediation actions for insensitive surfaces: Case of Slovenian show caves. *Journal for Nature Conservation*, **22**(2), 132-141; (2014).

[11] Cigna, A. A. Tourism and show caves. *Zeitschrift für Geomorphologie*, **60**(2), 217-233; (2016).

[12] Cigna, A. A. Show caves. (In *Encyclopedia of caves*, 909-921, Academic Press); (2019).

[13] Alonso, L., Pommier, T., Kaufmann, B., Dubost, A., Chapulliot, D., Doré, J., '*et al.*'. Anthropization level of Lascaux Cave microbiome shown by regional‐scale comparisons of pristine and anthropized caves. *Molecular ecology*, **28**(14), 3383-3394; (2019).

[14] Leuko, S., Koskinen, K., Sanna, L., D'Angeli, I. M., De Waele, J., Marcia, P., '*et al.*'. The influence of human exploration on the microbial community structure and ammonia oxidizing potential of the Su Bentu limestone cave in Sardinia, Italy. *PloS one*, **12**(7); (2017).

[15] Pfendler, S., Karimi, B., Maron, P. A., Ciadamidaro, L., Valot, B., Bousta, '*et al.*'. Biofilm biodiversity in French and Swiss show caves using the metabarcoding approach: First data. *Science of the total environment*, **615**, 1207–1217; (2018).

[16] Thompson, B., Richardson, D., Vangundy, R. D., Cahoon, A. B. Metabarcoding comparison of prokaryotic microbiomes from Appalachian karst caves to surface soils in southwest Virginia, USA. *Journal of Cave & Karst Studies*, **81**(4); (2019).

[17] Bontemps, Z., Alonso, L., Pommier, T., Hugoni, M., Moënne-Loccoz, Y. Microbial ecology of tourist Paleolithic caves. *Science of the Total Environment*, **816**, 151492; 10.1016/j.scitotenv.2021.151492 (2021).

[18] Burgoyne, J., Crepeau, R., Jensen, J., Smith, H., Baker, G., & Leavitt, S. D. Lampenflora in a show cave in the Great Basin is distinct from communities on naturally lit rock surfaces in nearby wild caves. *Microorganisms*, **9**(6), 1188; 10.3390/microorganisms9061188 (2021).

[19] Hainsworth, S., Kučerová, I., Sharma, R., Cañete-Gibas, C. F., Hubka, V. Three-gene phylogeny of the genus *Arthroderma*: basis for future taxonomic studies. *Medical Mycology*, **59**(4), 355-365; (2021).

[20] Piano, E., Biagioli, F., Nicolosi, G., Coleine, C., Poli, A., Prigione, V., Isaia, M., '*et al.*' Human disturbance drives differential diversity patterns of microbial communities in hypogean habitats. Preprint at https://www.authorea.com/doi/full/10.22541/au.165538108.89649607 (2022).

[21] Piano, E., Bona, F., Falasco, E., La Morgia, V., Badino, G., Isaia, M. Environmental drivers of phototrophic biofilms in an Alpine show cave (SW-Italian Alps). *Science of the Total Environment*, **536**, 1007-1018; (2015).

[22] Kulesza, K., Biedunkiewicz, A., Nowacka, K., Dynowska, M., Urbaniak, M., & Stępień, Ł. Dishwashers as an extreme environment of potentially pathogenic yeast species. *Pathogens*, **10**(4), 446; (2021).

[23] Papon, N., Courdavault, V., Clastre, M., & Bennett, R. J. Emerging and emerged pathogenic Candida species: beyond the Candida albicans paradigm. *PLoS pathogens*, **9**(9); (2013).

[24] Mulec, J., Oarga-Mulec, A., Šturm, S., Tomazin, R., Matos, T. Spacio-temporal distribution and tourist impact on airborne bacteria in a cave (Škocjan Caves, Slovenia). *Diversity*, **9**(3), 28; (2017).

[25] Theelen, B., Cafarchia, C., Gaitanis, G., Bassukas, I. D., Boekhout, T., Dawson Jr, T. L. Malassezia ecology, pathophysiology, and treatment. *Medical mycology*, **56**(1), 10-25; (2018).

[26] Werner, S., Peršoh, D., Rambold, G. New aspects of the biology of Mortierella alliacea. *Mycological progress*, **15**(12), 1293-1301; (2016).

[27] Jiang, J. R., Cai, L., & Liu, F. Oligotrophic fungi from a carbonate cave, with three new species of Cephalotrichum. *Mycology*, **8**(3), 164-177; (2017).

[28] Voyron, S., Lazzari, A., Riccucci, M., Calvini, M., Varese, G. First mycological investigation on Italian bats; (2011).

[29] Gupta, P., Vakhlu, J., Sharma, Y. P., Imchen, M., & Kumavath, R. Metagenomic insights into the fungal assemblages of the northwest Himalayan cold desert. *Extremophiles*, **24**(5), 749-758; (2020).

[30] Jones, D., Lavoie, K., Barton, H. A., Ortiz-Ortiz, M., Neilson, J. W., Legatzki, A., Sanchez-Moral, S., '*et al.*'. *Microbial life of cave systems* (ed. Walter de Gruyter GmbH & Co KG), **3**; (2015).

[31] Dominguez-Moñino, I., Jurado, V., Rogerio-Candelera, M. A., Hermosin, B., Saiz-Jimenez, C. Airborne fungi in show caves from Southern Spain. *Applied Sciences*, **11**(11), 5027; 10.3390/app11115027 (2021).

[32] Sanchez-Moral, S., Jurado, V., Fernandez-Cortes, A., Cuezva, S., Martin-Pozas, T., Gonzalez-Pimentel, J. L., Saiz-Jimenez, C., '*et al.*' Environment-driven control of fungi in subterranean ecosystems: the case of La Garma Cave (northern Spain). *International Microbiology*, **24**(4), 573-591; (2021).

[33] Griffin, D. W., Gray, M. A., Lyles, M. B., & Northup, D. E. The transport of nonindigenous microorganisms into caves by human visitation: a case study at Carlsbad Caverns National Park. *Geomicrobiology Journal*, **31**(3), 175-185; (2014).

[34] Marques, E. L. S., Dias, J. C. T., Silva, G. S., Pirovani, C. P., Rezende, R. P. Effect of organic matter enrichment on the fungal community in limestone cave sediments. *Genetics and Molecular Research*, **15**(3); 10.4238/gmr.15038611 (2016).

[35] D'agostino, D., Beccarisi, L., Camassa, M., Febbroriello, P. Microclimate and microbial characterization in the zinzulusa show cave (south italy) after switching to led lighting. *Journal of Cave & Karst Studies*, **77**(3); (2015).

[36] Kravetz, S., Gonzalez, B., Marano, A., Giorgi, A. The genus Tetracladium in Pampean streams (Buenos Aires, Argentina). *Phytotaxa*, **338**(3), 276-284; (2018).

[37] Carmichael, M. J., Bräuer, S. L., Engel, A. S. Microbial diversity and manganese cycling: a review of manganese oxidizing microbial cave communities. *Microbial life of cave systems, life in extreme environments*, **3**, 137-60; (2015).

[38] Carmichael, S. K., Zorn, B. T., Santelli, C. M., Roble, L. A., Carmichael, M. J., Bräuer, S. L. Nutrient input influences fungal community composition and size and can stimulate manganese (II) oxidation in caves. *Environmental microbiology reports*, **7**(4), 592-605; (2015).

[39] Martin-Pozas, T., Cuezva, S., Fernandez-Cortes, A., Cañaveras, J. C., Benavente, D., Jurado, V., Sanchez-Moral, S. '*et al.*'. Role of subterranean microbiota in the carbon cycle and greenhouse gas dynamics. *Science of the Total Environment*, **831**, 154921; 10.1016/j.scitotenv.2022.154921 (2022).

[40] Baker, A., & Genty, D. Environmental pressures on conserving cave speleothems: effects of changing surface land use and increased cave tourism. *Journal of Environmental Management*, **53**(2), 165-175; (1998).

[41] Marques, E. L. S., Dias, J. C. T., Silva, G. S., Pirovani, C. P., Rezende, R. P. Organic matter enrichment affects archaea community in limestone cave sediments. *Journal of Cave & Karst Studies*, **79**(2); (2017).

[42] Bercea, S., Năstase-Bucur, R., Moldovan, O. T., Kenesz, M., Constantin, S. Yearly microbial cycle of human exposed surfaces in show caves. *Subterranean Biology*, **31**(1); (2019).

[43] Czerwik-Marcinkowska, J., Wojciechowska, A., Massalski, A. Biodiversity of limestone caves: aggregations of aerophytic algae and cyanobacteria in relation to site factors. *Polish Journal of Ecology*, **63**(4), 481-499; (2015).

[44] Borderie, F., Tête, N., Cailhol, D., Alaoui-Sehmer, L., Bousta, F., Rieffel, D., Alaoui-Sossé, B., '*et al.*'. Factors driving epilithic algal colonization in show caves and new insights into combating biofilm development with UV-C treatments. *Science of the Total Environment*, **484**, 43-52; (2014).

[45] QGIS.org, %Y. QGIS 3.22 Geographic Information System. QGIS Association.

http://www.qgis.org

[46] Smith, D. P., & Peay, K. G. Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. *PloS one*, **9**(2); (2014).

[47] Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Knight, R., '*et al.*'. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*, **6**(8), 1621-1624; (2012).

[48] Coleine, C., Biagioli, F., de Vera, J. P., Onofri, S., Selbmann, L. Endolithic microbial composition in Helliwell Hills, a newly investigated Mars‐like area in Antarctica. *Environmental Microbiology*, **23**(7), 4002-4016; (2021).

[49] Palmer, J. M., Jusino, M. A., Banik, M. T., Lindner, D. L. Non-biological synthetic spike-in controls and the AMPtk software pipeline improve mycobiome data. *PeerJ*, **6**; (2018).

[50] Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, **4**; (2016).

[51] Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, **26**(19), 2460-2461; (2010).

[52] Edgar, R. C., & Flyvbjerg, H. Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics*, **31**(21), 3476-3482; (2015).

[53] Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., Holmes, S. P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods*, **13**(7), 581-583; (2016).

[54] Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kauserud, H., '*et al.*'. Fungal community analysis by high‐throughput sequencing of amplified markers–a user's guide. *New phytologist*, **199**(1), 288-299; (2013).

[55] Abarenkov, K., Zirk, A., Piirmann, T., Pöhönen, R., Ivanov, F., Nilsson, R.H., Kõljalg, U. UNITE top50 release. Version 04.02.2020. UNITE Community. https://doi.org/10.15156/BIO/786374; (2020).

[56] Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Tiedje, J. M., '*et al.*'. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic acids research*, 42, 633-642; (2014).

[57] McMurdie, P. J., & Holmes, S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*, **8**(4); (2013).

[58] Liu, C., Cui, Y., Li, X., Yao, M. Microeco: an R package for data mining in microbial community ecology. *FEMS microbiology ecology*, **97**(2); (2021).

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Competing interests

The authors declare no competing interests.

Additional information

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CHAPTER 3. Tourism affects microbial assemblages in show caves

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Abstract

Anthropogenic disturbance on natural ecosystems is growing in frequency and magnitude affecting all ecosystems components. Understanding the response of different types of biocoenosis to human disturbance is urgently needed and it can be achieved by adopting a metacommunity framework. With the aid of advanced molecular techniques, we investigated sediment communities of Fungi, Bacteria and Archaea in four Italian show caves, aiming to disentangle the effects induced by tourism on their diversity and to highlight changes in the driving forces that shape their community composition. We modelled diversity measures against proxies of tourism pressure. With this approach we demonstrate that the cave tourism has a direct effect on the community of Bacteria and an indirect influence on Fungi and Archaea. By analysing the main driving forces influencing the community composition of the three microbial groups, we highlighted that stochastic factors override dispersal-related processes and environmental selection in show caves compared to undisturbed areas. Thanks to this approach, we provide new perspectives on the dynamics of microbial communities under human disturbance suggesting that a proper understanding of the underlying selective mechanisms requires a comprehensive and multi-taxonomic approach.

Keywords: show caves, Fungi, Bacteria, Archaea, beta diversity, microbiome

3.1 Introduction

Anthropogenic disturbance on natural ecosystems is growing in frequency and magnitude worldwide, with consequent negative repercussions on all levels of biodiversity (Ceballos et al., 2015; Pimm et al., 2014). In light of the pivotal role of microbiomes in driving ecosystem processes and delivering ecosystem services (Correa-Garcia et al., 2022), research on the impact of human pressures on the diversity and structure of microbial communities is particularly important. Insights into the main processes that determine changes in local microbial communities following external perturbations may be obtained by examining variation in species richness and composition in a metacommunity ecology perspective (Chase & Leibold, 2017).

In this context, the operational framework proposed by Stegen et al. (2013, 2015), based on the conceptual synthesis proposed by Vellend (2010), allows understanding the relative contribution of the main selective processes shaping the composition of microbial communities. These processes can be related to Selection (i), when niche assembly rules represent the major force determining the community species composition (e.g. Graham et al., 2017) that may evolve either in a scenario of *Variable Selection* when the species composition is determined by multiple variables or in a scenario of *Homogeneous Selection* when species are filtered by a dominant environmental force; to Dispersal (ii), when the dispersal level of the species represents the most important driving force in a community, leading either to a scenario of *Dispersal Limitation* in case of low dispersal levels (e.g. Evans et al., 2020) or to a scenario of *Homogenizing Dispersal* (e.g. Bottos et al., 2018); or to Drift (iii), when stochastic forces, namely random birth/death rates and priority effects, determine the composition of community (e.g. Brislawn et al., 2019).

Notably, a proper understanding of the underlying mechanisms shaping microbial communities is limited by their rapid evolution (Niehus et al., 2015), high taxonomic richness (Shoemaker et al., 2017), functional redundancy (Curtis & Sloan, 2004), and dormancy (Locey et al., 2020). Being characterised by highly predictable gradients in their environmental conditions, simplified trophic webs, climatic stability, and spatial confinement (Culver & Pipan, 2019; Poulson & White, 1969), subterranean ecosystems represent ideal ecological laboratories in this regard (Mammola, 2019).

Among the main threats affecting subterranean ecosystems (Mammola et al., 2022), the ever-increasing conversion of natural caves into tourist attractions, i.e. the so-called 'show caves', imposes significant anthropogenic pressure on the subterranean ecosystem (Cigna, 2016). Well-documented effects on abiotic components include changes in microclimate (e.g. Addesso et al., 2022a; Šebela et al., 2019), carbon dioxide concentration (e.g. Addesso et al., 2022a; Lang et al., 2015), and geochemical properties (e.g. Addesso et al., 2019), with cascade effects on the subterranean fauna (e.g. Nicolosi et al., 2021; Pacheco et al., 2021) and energy fluxes (e.g. Addesso et al., 2022b; Fernandez-Cortes et al., 2011). In addition, the proliferation of a photosynthetic community is often recorded due to the installation of artificial lights (e.g. Borderie et al., 2014; Havlena et al., 2021; Piano et al., 2015, 2021).

Changes in microbial communities have been documented in caves due to tourists vehiculating microbial propagules on their clothes, shoes and skin, with consequent biological pollution of the cave atmosphere (Martin-Sanchez et al., 2014; Porca et al., 2011), water (Ando & Murakami, 2020, Moldovan et al., 2020), soil (Kukla et al., 2018; Mammola et al., 2017) and speleothems (Bercea et al., 2019), and to human-induced changes in substrate composition (Kukla et al., 2018). Yet, studies show inconsistent responses of the microbial communities to human disturbance, pointing out either a positive (Mammola et al., 2017; Marques et al., 2016), negative (Alonso et al., 2019; Shapiro & Pringle, 2010) or contrasting effects (Bercea et al., 2019; Mulec & Oarga-Mulec, 2012). Whether tourism significantly influences the composition of microbial communities in subterranean ecosystems have never been tested within a metacommunity framework to date.

To gain insights into the underlying mechanisms that determine the composition of subterranean microbial communities in relation to possible tourism-induced perturbations we here set up a case study encompassing four show caves in Italy. Using advanced molecular techniques, we examined changes in diversity and composition of three microbial components that naturally inhabit cave sediments, i.e. Fungi, Bacteria and Archaea. By adopting a replicated factorial design, we tested: i) to what extent microbial diversity and composition is determined by tourism pressure; and ii) which mechanisms determine the composition of Fungi, Bacteria and Archaea. We hypothesised that: i)

diversity patterns of the three examined communities are influenced by tourism pressure; and ii) the contribution of the different mechanisms in explaining the community composition varies across the three groups.

3.2 Materials And Methods

3.2.1 Sampling design

We performed our study in four Italian show caves (Fig. 1). In each cave, we adopted a replicated factorial sampling design wherein sediment samples were collected in three areas placed at progressive lateral distance from the tourist path, which was intended as proxy of tourism pressure. We focused on soil as it proved to be the compartment with the highest microbial diversity (see Alonso et al., 2019). Specifically, we identified i) the High Pressure (HP) area at 0-3 m from the tourist path; ii) the Medium Pressure (MP) area at 3 to 5 m from the tourist path; and iii) the Low Pressure (LP) area at > 5 m from the tourist path. In each of them, we identified three sampling sectors at progressive linear distance from the cave entrance, whose extension was obtained by dividing the tourist path into three parts of equal length.

	1. Grotta di Bossea	2. Grotte del Caudano	3. Grotta del Vento	4. Grotte di Pertosa-Auletta
Municipality	Frabosa Soprana	Frabosa Sottana	Fornovolasco	Pertosa
Province	Cuneo	Cuneo	Lucca	Salerno
Long.	7.836°E	7.786°E	10.355°E	15.452°E
Lat.	44.240°N	44.294°N	44.033°N	40.537°N
Elevation	844 m asl	720 m asl	640 m asl	240 m asl
Planimetric development	2,800 m	3,000 m	4,500 m	3.000 m
Length of the touristic path	1,000m	700 m	2,200 m	1,900 m
Cave temperature	9°C	9°C	11°C	16°C
Opened to public since	1874	1902	1967	1932
Visitors/year	16,000	2,900	43,000	50,000
Lamp type	LED	Halogen	LED	LED

Fig. 1 - Map of the investigated show caves with information about geographic (municipality, province, coordinates and elevation), morphological, physical and touristic features. The number of visitors refers to 2019. Photo credits: AGTI (Grotta del Vento and Grotta di Pertosa-Auletta) and Simone Marzocchi (Grotta di Bossea and Grotta del Caudano).

Within each sampling sector, we identified 3 plots in three homogeneous deposits of sediment of 1 m^2, and in each of them we collected 3 random replicates (Fig. 2). This procedure allowed us to obtain a representative sample of the entire sampling area for each sampling sector. Each replicate was collected on the ground within an area of 10 cm^2 up to 3 cm depth with sterile Falcon® tubes (50 ml) and the 9 replicates were then pooled together to obtain a composite sediment sample. In each cave, we also identified three random plots in an area closed to the public (control area, C), where we collected sediment samples following the procedure described above to obtain a representative view of the natural conditions of the cave, for a total of 48 samples (4 show caves × 4 areas × 3 sampling sectors) (see Fig. S1-S4 for a detailed view of the sampling sectors within each cave). As seasonal differences have been observed in the subterranean microbiota (Mammola et al., 2017), samples were collected during a single occasion in each cave in summer 2020, to avoid seasonal bias.

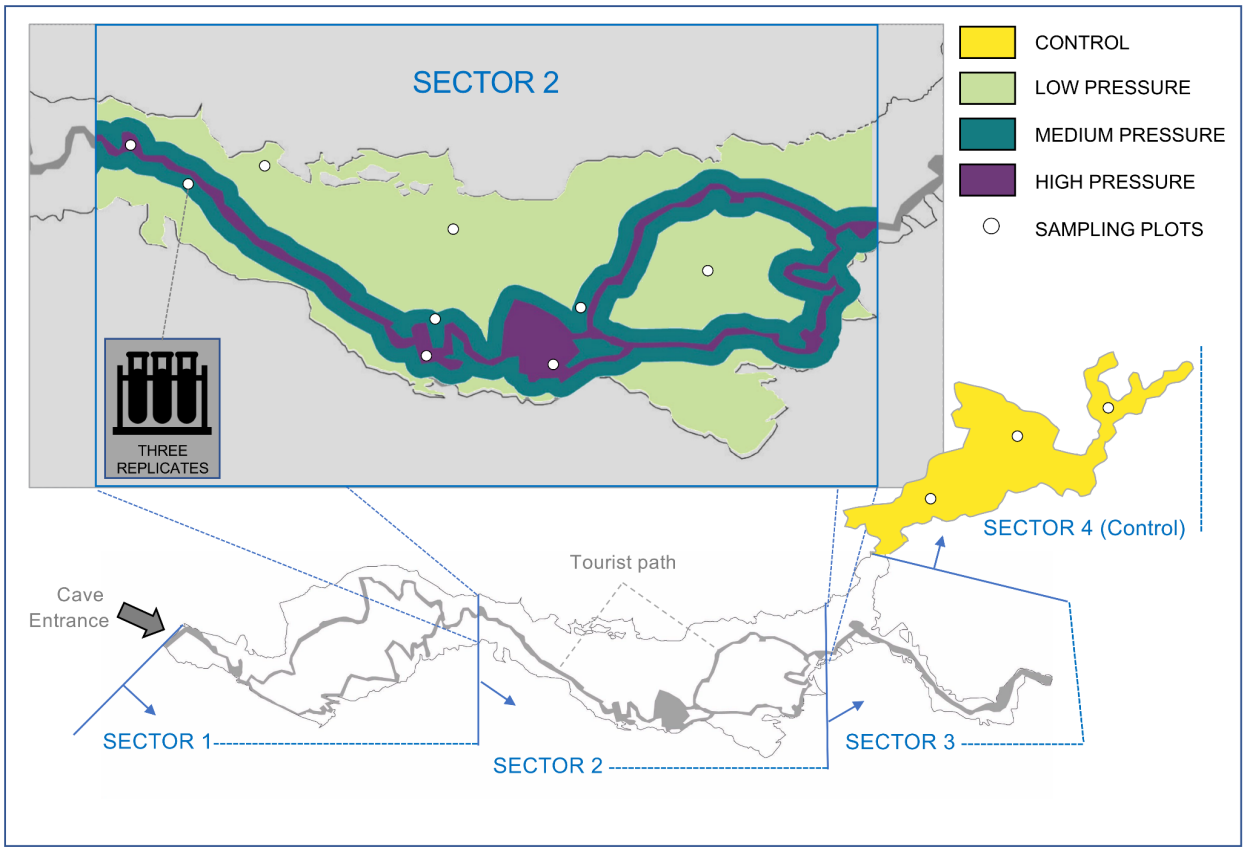

Fig. 2 – Schematic representation of the sampling design. The cave was subdivided in four sectors: Sectors 1 to 3 included the area open to tourism and Sector 4 encompassed the Control area, closed to the public. Within each touristic sector, three areas of tourism pressure were identified: the High Pressure area corresponded to the tourist path, the Medium Pressure area was a buffer of 5 m from the tourist path and the Low Pressure area encompassed the remaining area within the Sector. Sampling plots, exemplified by white dots, were composed by three replicates, successively pooled by pressure level. The map depicts an imaginative cave (graphic purpose only).

3.2.2 Sediment analysis

Samples were preserved in a thermal bag until the arrival at the laboratory, where the 9 replicates were pooled together and homogenised. Sediment samples were then sieved under sterile conditions to remove coarse rock debris. The physical and chemical properties of each sediment sample were evaluated by measuring pH, concentration of organic Carbon, total Nitrogen and the percentage of sand (%Sand), silt (%Silt) and clay (%Clay) with standard protocols by Regione Piemonte Laboratorio Agrochimico Settore Fitosanitario e Servizi Tecnico-Scientifici. To estimate the deviation of sediment collected in tourist areas, we calculated the dissimilarity (Bray-Curtis distance) among the sediment composition of each sample collected in the tourist areas from the mean sediment composition of the three samples in the control area for each cave. The obtained variable (Substrate) summarises the differences in the substrate conditions between samples collected in the tourist areas and the control samples.

3.2.3 Metagenomic DNA extraction, amplicon sequencing and bioinformatics

Metagenomic DNA was extracted from 0.5 g of sample using Qiagen DNeasy PowerSoil Pro Kit (Carlsbad, CA, USA). The Internal Transcribed Sequence 1 ribosomal region (ITS1) and hypervariable region V4 of 16S ribosomal gene were targeted to assess the fungal and prokaryotic community composition, respectively. The ITS1 region was amplified using barcoded primers ITS1F/ITS2, suitable for shorter read length (Smith & Peay, 2014), while for the V4 region of 16S, barcoded F515/R806 primer set, amplifying both Bacteria and Archaea, was used according to Caporaso et al. (2012). PCR reactions consisted of 1 μL of each primer, 12.5 μL of Taq DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, MA, USA), 9.5 μL of nuclease-free water (Sigma–Aldrich, St. Louis, MO, USA) and 5 ng of DNA for a total volume of 25 μL and occurred in an automated thermal cycler (BioRad, Hercules, CA, USA). The ITS1 locus and V4 region were amplified according to Coleine et al. (2021). Amplicons were quantified by a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA) and then pooled. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq platform at the Edmund Mach Foundation (San Michele all'Adige TN, Italy). Demultiplexed ITS and 16S sequence datasets were processed using

AMPtk (Palmer et al., 2018) v.1.5.1 software. Briefly, barcodes/indexes and primer sequences were removed from raw data. Reads were subjected to quality trimming to a maximum of 250 bp, discarding those shorter than 100 bp; sequencing artefacts were dropped by using USEARCH v.9.1.13 with default parameters (Edgar, 2010). Sequence quality filtering was performed with the expected error parameter of 0.9 (Edgar & Flyvbjerg, 2015); the cleaned reads were merged and clustered at 99% similarity using VSEARCH (Rognes et al., 2016) v.2.15.1, with DADA2 (Callahan et al., 2016). Global singletons and rare taxa (<5 reads) were skipped as likely false positives due to sequencing errors (Lindahl et al., 2013). Finally, taxonomic identification was performed with a sequence identity of 97% as threshold, using hybrid database Global Alignment and SINTAX (Edgar, 2010) on reference databases UNITE 8.2. (Abarenkov et al., 2020) and RDP 11 (Cole et al., 2014).

3.2.4 Diversity measures

Amplicon Sequence Variants (ASVs) obtained from the ITS were considered as proxies of fungal taxonomic units, while ASVs obtained from the 16S were considered as a proxy of taxonomic units of the two prokaryotic groups, after differentiating between bacterial and archaeal ASVs. Being interested in deviations from natural conditions, we aimed at quantifying, for each cave and for each examined group, the distance of tourist samples from control samples: i) first, we obtained the ratio between the number of unique ASVs in tourist samples divided by the number of ASVs in the control samples (hereafter fraction of unique ASVs); ii) then, using the function 'trans_beta' in the *microeco* package (Liu et al., 2021), we obtained the Bray Curtis pairwise dissimilarity (hereafter ASV turnover) of tourist samples from control samples, ranging from 0 (samples sharing all ASVs) to 1 (samples sharing no ASVs).

3.2.5 Data analysis

We performed all statistical analyses in the R environment (R Core Team, 2022). Statistical models were performed with the function 'glmmTMB' from the *glmmTMB* package (version 1.1.2.3; Brooks et al., 2017). Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson, 2001) was performed with the function 'adonis' from the *vegan* package (Oksanen et al., 2019). The identification of selective mechanisms was performed with the functions reported in the *microeco* package (Liu et al., 2021). Graphical representations were made with the *ggplot2* (Wickham, 2016), *ggpubr* (version 0.5.0; Kassambara, 2022) and *factoextra* (version 1.0.7; Kassambara & Mundt, 2020) packages.

Sediment analysis. In a first step, we analysed the contribution of chemical and physical parameters of collected sediments by means of a Principal Component Analysis (PCA). To detect possible shifts in physical and chemical parameters of collected sediments among levels of tourism pressure, we applied a PERMANOVA based on Euclidean distances, specifying tourism pressure levels as factor and cave identity as strata to keep into account the spatial autocorrelation. Based on the assumption that tourism may influence sediment composition in caves (e.g. Kukla et al., 2018), we tested possible shifts in physical and chemical parameters along the gradient of tourism pressure using Generalized Linear Mixed Models (GLMMs) with a Gaussian error distribution. Before computing statistical models, we converted the levels of tourism pressure into ordinal variables, where High Pressure = 1, Medium Pressure = 2 and Low Pressure = 3.

Community analysis. We first visually inspected whether community composition of the three examined groups differed according to the pressure levels by means of a PCoA using the Bray Curtis dissimilarity index with Hellinger transformation. We then tested the observed differences in order composition against a random distribution across pressure levels with a permutational multivariate analysis of variance (PERMANOVA) using the cave identity as strata to keep into account possible spatial autocorrelation among samples (Anderson, 2001).

Diversity analysis. By adopting Generalized Linear Mixed Models (GLMMs), we tested the effect of tourism pressure on the diversity patterns of three examined groups by considering the following

variables: (i) fraction of unique ASVs; and ii) ASV turnover. We included as covariates in the models the following parameters: i) distance from the tourist path, here intended as a proxy of direct pressure exerted by tourism; and ii) variation in sediment composition with respect to control samples as a proxy of indirect tourism pressure. We assumed a Gamma error distribution for the set of models performed on the fraction of unique ASVs and a Beta error distribution for the set of models performed on ASV turnover. To account for the spatial dependency of samples within the same cave, a cave identifier (CaveID) was incorporated as a random factor. Model validation was performed with the function 'check_model' from the *performance* package (Lüdecke et al., 2021) and by visually inspecting the distribution of the residuals (Zuur et al., 2010). Results were retained significant with a p-value < 0.05.

Identification of selective processes. The identification of the main selective processes acting on the examined microbial communities was performed by applying the analytical framework presented in Stegen et al. (2013). More in detail, we implemented the null modelling approach to quantitatively estimate the degree to which spatial turnover in community composition is influenced by Selection, Drift or Dispersal. In a first step, we inferred the strength of Selection by means of the pairwise phylogenetic turnover between communities calculated using the mean-nearest-taxon-distance (β-MNTD) metric (Webb et al., 2008; Fine and Kembel, 2011). The contribution of Selection was evaluated by means of the beta-nearest taxon index (β-NTI) that was obtained by comparing observed β-MNTD values to the mean of a null distribution of β-MNTD values normalized by its standard deviation (Stegen et al., 2012). Values of β-NTI < -2 indicated significantly less turnover than expected, i.e. *Homogeneous Selection*, while values of β-NTI > 2 were representative of significantly more turnover than expected, i.e. *Variable Selection*. The calculation of β-MNTD was performed with the function 'cal_ses_betamntd', while the calculation of β-NTI was obtained by implementing the function 'cal_NTI'. In a second step, the role of Dispersal was tested on pairwise community comparisons that did not deviate from the null model distribution by calculating the Raup-Crick metric extended to account for species relative abundances, i.e. RC_{bray} (Stegen et al., 2015). Observed Bray-Curtis dissimilarities were compared to the null distribution to derive RC_{bray}.

Values of RC_{bray} >0.95 were interpreted as indicating *Dispersal Limitation*, whereas values <-0.95 were interpreted as indicating *Homogenizing Dispersal*. This calculation was performed with the function 'cal_rcbray'. Those pairwise dissimilarities that showed no significant effect either of Selection or Dispersal were interpreted as to be dominated by Drift. The relative contributions of the different components that determine the composition of a microbial community were obtained with the function 'cal_process'.

This approach was applied to: i) all samples to obtain an overall view of the driving forces acting in show caves; and ii) to control samples only to gain insights into the selective processes occurring in subterranean ecosystems without human disturbance.

3.3 Results

3.1. Sediment analysis

The results of the PCA (Fig. 3) showed that the first three axes explain 90% of the total variance (Axis 1 = 52.1%; Axis 2 = 26.4%; Axis 3 = 11.5%; Axis 4 = 0.08%; Axis 5 = 0.02%). The variable loadings to each axis demonstrate that total Nitrogen concentration, %Clay, pH and %Sand equally contribute to the variance of the first axis. While the contribution of total Nitrogen concentration and %Clay is positive, the contribution of pH and %Sand is negative. Regarding the second axis, most of the variance is explained by the %Sand and %Silt, with positive and negative contribution, respectively. The variance of the third axis is mostly explained by the concentration of organic Carbon, which shows a negative contribution (see Tab. S1 for PCA statistics). Results of the PERMANOVA performed on the physical and chemical parameters revealed no significant differences among levels of tourism pressure, but significant differences among caves (Tab. 1). Results of the statistical model performed on the compositional variation of sediment samples in tourist areas compared to control samples showed a significant effect of the gradient of tourism pressure. More in detail, at increasing distance from the tourist path, we observed significantly lower sediment dissimilarity (β-est = -0.955, SE = 0.206; z = -2.89; P = 0.004).

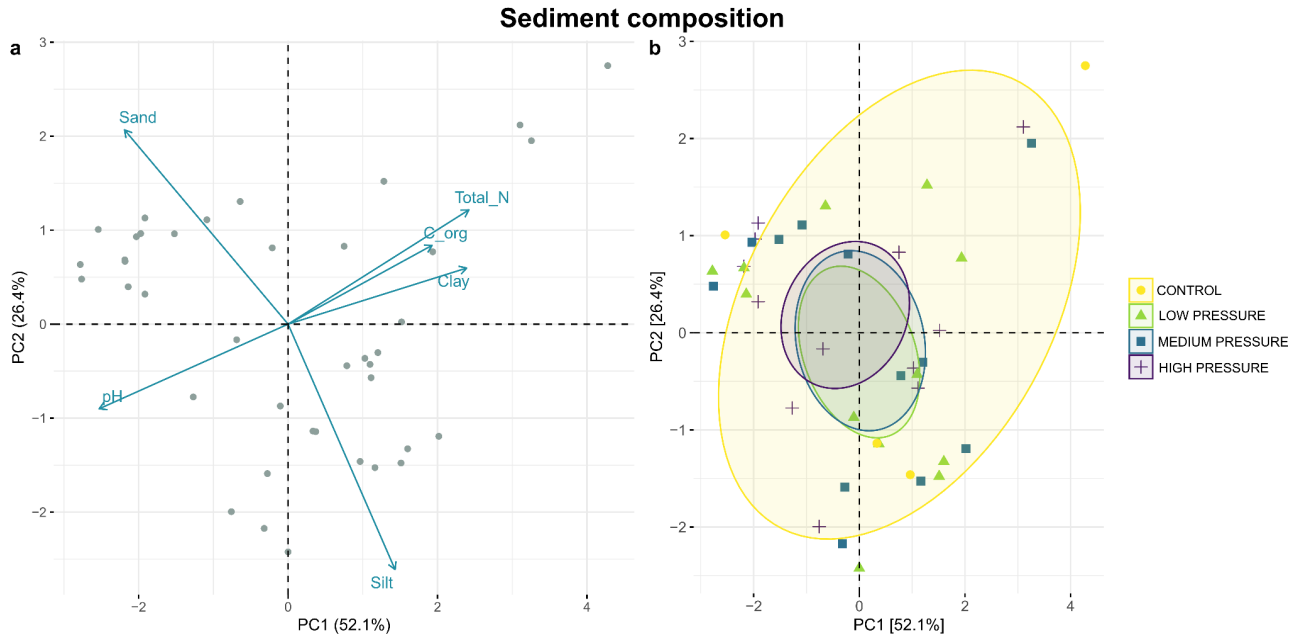

Fig. 3 – Results of PCA performed on sediment parameters (Euclidean space): a) eigenvectors of environmental variables; b) samples, grouped by tourism pressure. Ellipses represent standard deviations around the centroid of each group.

3.3.2 Community analysis

The ITS1 dataset generated 5,793,980 raw sequence reads, resulting in 5,458,895 gene quality-filtered reads, ranging from 1,252 up to 540,803 per sample. After singletons and rare taxa (<5 reads) removal (1,108 out of 10,595 ASVs total), a total of 9,487 high-quality ASVs were obtained. A total of 5,453,881 raw reads were generated from 16S rDNA dataset and accounted for a total of 4,806,902, which were grouped into 47,367 ASVs (out of a total of 65,037 ASVs) after quality filtering, with sequencing depths between samples ranging from 2,066 to 265,442 reads. Subsequently, the total 16S dataset was split by grouping the bacterial (31,015 ASVs) and archaeal (863 ASVs) ASVs separately for the downstream analyses. See Biagioli et al. (2023) for further details on relative composition of the different orders for the three examined microbial groups.

Visual inspection of the ordinations depicts no clear separation of communities in relation to the levels of tourism pressure (Fig. 4).

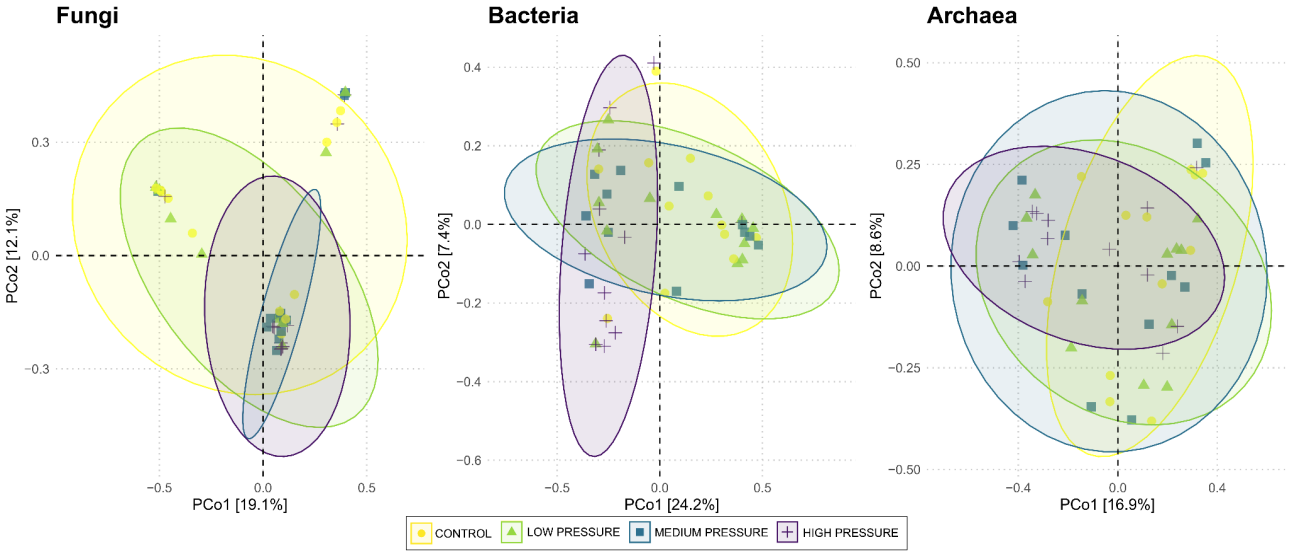

Fig. 4 – Ordination according to the first two PcoA axes of the communities of Fungi, Bacteria and Archaea (from left to right). Samples are grouped by levels of tourism pressure. Ellipses represent standard deviations around the centroids of each group.

This pattern is also highlighted by the PERMANOVA, except for Bacteria that showed a significant difference among levels of tourism pressure. Differences were remarkably significant among caves for all the three groups (Tab. 1).

Group	Variable	$F_{3,41}$	P
Substrate	Tourism pressure	1.44	0.212
	Cave identity	55.3	**0.001**
Fungi	Tourism pressure	1.02	0.407
	Cave identity	2.04	**0.001**
Bacteria	Tourism pressure	1.52	**0.032**
	Cave identity	2.29	**0.001**
Archaea	Tourism pressure	1.01	0.446
	Cave identity	2.49	**0.001**

Tab. 1 – F-values (F) and p-values (P) obtained from PERMANOVA performed on sediment composition and community of Fungi, Bacteria and Archaea to test their response to levels of tourism pressure and cave identity. Significant results are highlighted in bold.

3.3.3 Diversity analysis

In most cases, the mean fraction of unique ASVs was lower than one for the three examined groups, despite some very high values were observed (Tab. S2). The results of the models (Tab. 2a and Fig. 5) showed that the fraction of unique ASVs of Fungi was significantly affected by sediment composition but not by the level of tourism pressure. In other words, the number of unique ASVs in the touristic areas increased when sediment composition differed from the control area. Regarding Bacteria, we observed a significant effect of tourism pressure, with significantly lower values in the lower pressure level but no effect of sediment composition was recovered. No significant responses were recorded for Archaea in relation to tourism pressure or sediment composition.

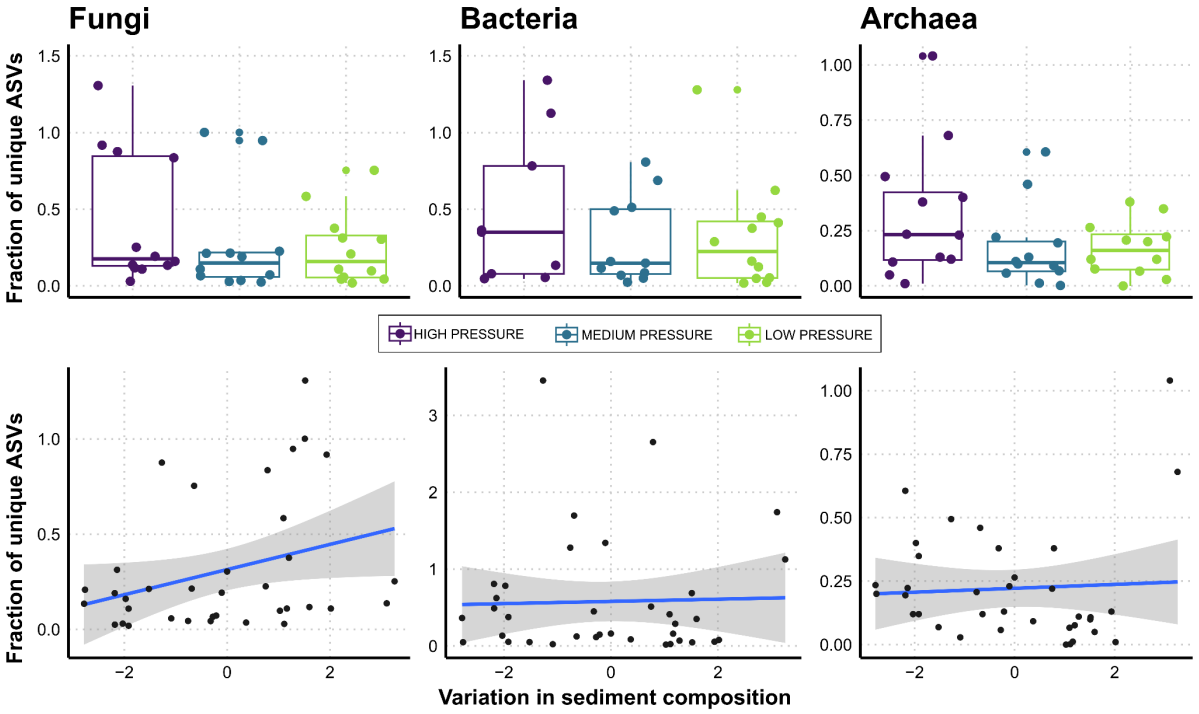

Fig. 5 – Boxplots representing the fraction of unique ASVs in the three levels of tourism pressure (purple = HP; light blue = MP; green = LP) with respect to control areas of the three examined microbial groups (upper panel). Regression lines with confidence intervals representing the relation between the fraction of unique ASVs and the variation of sediment composition with respect to the control areas for the three examined microbial groups (lower panel).

The overall ASV turnover of communities was extremely high (Fungi = 0.89 ± 0.16; Bacteria = 0.83 ± 0.09; Archaea = 0.83 ± 0.15). The results of the models (Tab. 2b and Fig. 6) showed a significant effect of the tourism pressure on Bacteria, with significant lower values of ASV turnover in sites at medium or low pressure compared to high. Sediment composition affected both Bacteria and Archaea, with significant decreasing values of ASV turnover with increasing dissimilarity of

sediment composition from control samples. Conversely, the ASV turnover of Fungi was not affected by any of the covariates.

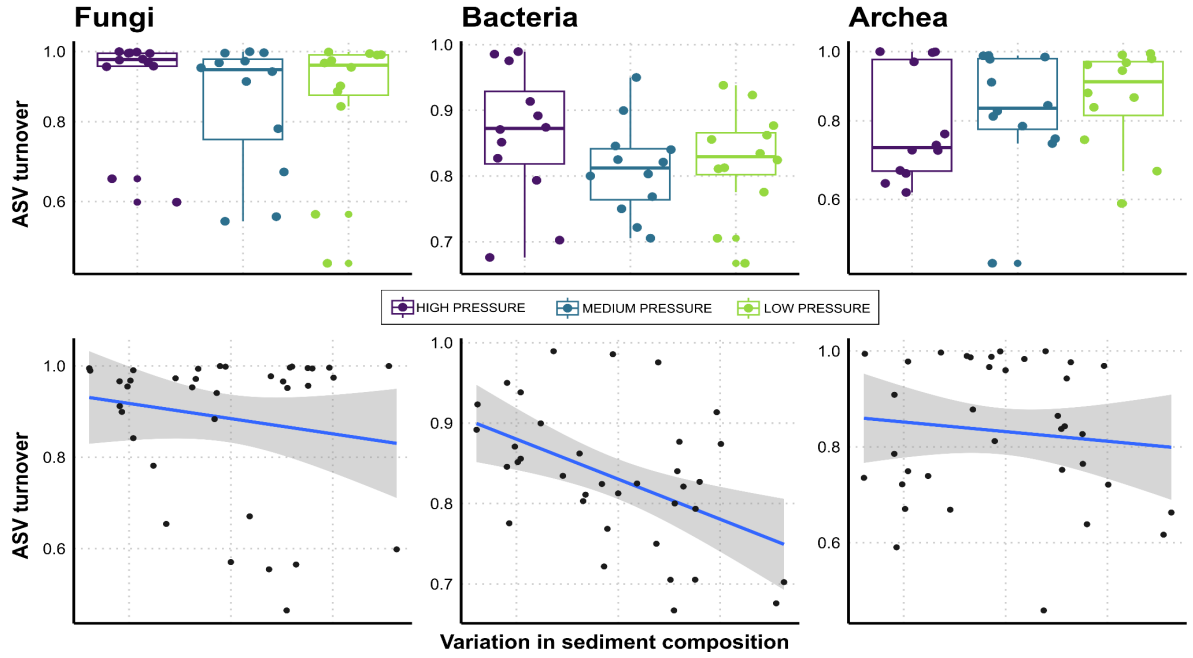

Figure 6 – Upper panel reporting boxplots representing the ASV turnover in the three levels of tourism pressure (purple = HP; light blue = MP; green = LP) with respect to control areas of the three examined microbial groups (upper panel). Lower panel reporting regression lines with confidence intervals representing the relation between ASV turnover and the variation of sediment composition with respect to the control areas for the three examined microbial groups (lower panel).

Group	Variable	*β-est*	*SE*	*z*	P
a) Fraction of unique ASVs					
Fungi	Distance_path	−0.158	0.196	−0.804	0.421
	Substrate	0.227	0.109	2.08	**0.037**
Bacteria	Distance_path	−0.487	0.247	−1.97	**0.048**
	Substrate	0.071	0.192	0.372	0.710
Archaea	Distance_path	−0.232	0.200	−1.16	0.246
	Substrate	0.069	0.137	0.505	0.614
b) ASV turnover					
Fungi	Distance_path	−0.169	0.213	−0.795	0.427
	Substrate	0.016	0.106	0.147	0.883
Bacteria	Distance_path	−0.396	0.119	−3.32	**<0.001**
	Substrate	−0.261	0.071	−3.67	**<0.001**
Archaea	Distance_path	−0.034	0.169	0.201	0.841
	Substrate	−0.274	0.127	−2.16	**0.031**

Tab. 2 – Results of the GLMMs analysis for the three examined microbial groups on a) the fraction of unique ASVs, and b) ASV turnover. Estimated parameters (β-est), standard errors (SE), z-values (z) and p-values (P) for each covariate are reported. Distance_path = Distance from the tourist path; Substrate = Sediment variation from Control areas. Significant results are reported in bold.

3.3.4 Identification of selective processes

When considering all sites, the analysis of the different contribution of selective processes (Tab. 3 and Fig. 7) displayed a dominant role of Drift, which contributed around 50%. The role of Selection was extremely reduced in Fungi, but more important in Bacteria and Archaea with a dominant role of Variable Selection in Bacteria and of Homogeneous Selection in Archaea. Dispersal showed a high contribution for Fungi, with a dominant role of Homogenizing Dispersal compared to Dispersal Limitation, while its contribution was lower for the prokaryotic component, with a more intense effect of Dispersal Limitation than Homogenizing Dispersal.

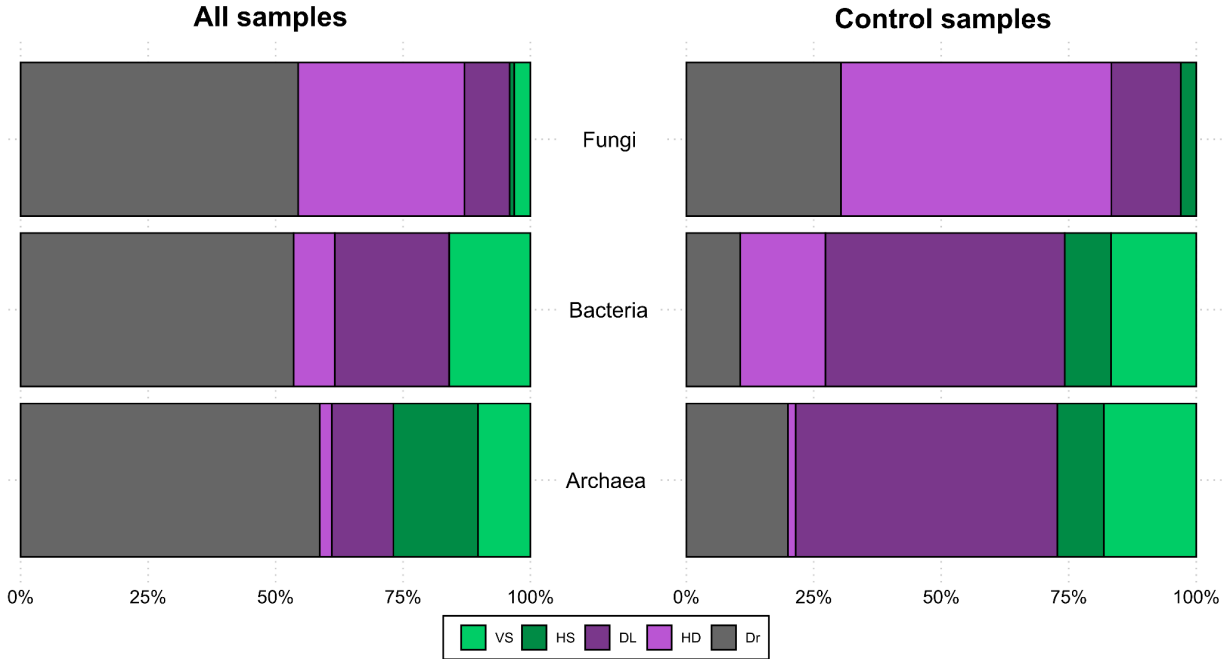

Fig.7 – Barplots representing the different contribution of selective processes to the community composition of the three examined microbial groups calculated by considering: a) all samples; and b) control samples (VS = Variable Selection; HS = Homogeneous Selection; DL = Dispersal Limitation; HD = Homogenizing Dispersal; Dr = Drift).

When analysing control samples separately, the contribution of the Drift component was drastically reduced, ranging from 10% in Bacteria to 30% in Fungi. Conversely, Dispersal resulted in the dominant process, contributing around 50% in shaping the three examined microbial communities. More in detail, Homogenizing Dispersal represented the most important driving force for Fungi, while the prokaryotic component was dominated by Dispersal Limitation. The Selection process resulted almost absent in Fungi, while it contributed for around 25% to the prokaryotic component

Group	Variable	All samples (%)	Control samples (%)
Fungi	Variable Selection	3.01	0.00
	Homogeneous Selection	0.798	3.03
	Dispersal Limitation	8.87	13.6
	Homogenizing Dispersal	32.9	53.0
	Drift	54.3	30.3
Bacteria	Variable Selection	15.9	16.7
	Homogeneous Selection	0.090	9.10
	Dispersal Limitation	22.4	47.0
	Homogenizing Dispersal	8.07	16.7
	Drift	53.5	10.6
Archaea	Variable Selection	10.3	18.2
	Homogeneous Selection	16.6	9.10
	Dispersal Limitation	12.1	51.5
	Homogenizing Dispersal	2.39	1.52
	Drift	58.7	20.0

Table 3 – Contribution of the different selective processes shaping community composition of Fungi, Bacteria and Archaea calculated by considering: i) all samples; and ii) control samples.

3.4 Discussion

The conversion of caves into touristic attractions has major impacts on subterranean ecosystems (Fernandez-Cortes et al., 2011; Mulec, 2014). However, extrapolating common patterns of human-induced changes in subterranean microbial communities is often hampered by great differences among caves, which are strictly influenced by local factors such as geography, cave size, geology, morphology, and water dynamics (Saiz-Jimenez, 2012). As demonstrated by our results, environmental differences among caves have great repercussions on sediment composition and microbial communities. We overcame this limitation by relating the microbial diversity of the tourist areas to the one observed in control areas. In addition, by adopting a metacommunity framework, we disentangled the underlying mechanisms that determine the composition of microbial communities in caves opened to tourism for the first time.

Results of regression models pointed out an effect of tourism on the diversity of sediment microbial communities in show cave, with different outcomes depending on the examined microbial group. More in detail, Bacteria emerged as the most sensitive group to tourism pressure as they responded to both direct and indirect pressure, while Fungi and Archaea only responded to indirect pressure.

Also, Fungi only responded in terms of fraction of unique ASVs, while the prokaryotic component responded in terms of ASV turnover. These results suggests that

Both the fraction of unique ASVs and ASV turnover of Bacteria significantly decreased with increasing distance from the tourist path. This suggests a possible replacement of resident species by propagules of microorganisms vehiculated by visitors' shoes and clothes, or by touching cave surfaces during visits (Mulec, 2014; Saiz-Jimenez, 2012; Zhelyazkova et al., 2020), as observed by other authors (Alonso et al., 2019; Dong et al., 2020). Sediment changes induced by tourism significantly increased the fraction of unique ASVs in Fungi and the ASV turnover in the prokaryotic component. We can thus hypothesise that sediment changes induced by tourism favour species (or ecotypes) coping better with new environmental conditions related to the presence of tourists (Cuadros, 2017; Zhu et al., 2019), with a substitution of the original ones for Archea, but not for Fungi. This is in agreement with literature data, which point out a key role of substrate as a driver of species richness and composition of microbial communities in caves (Cailhol et al., 2020; Kukla et al., 2018; Marques et al., 2016, 2017). By examining the contribution of each physical and chemical parameter to the overall variation of sediment composition, we could identify Nitrogen concentration as one of the most important drivers determining changes in sediment composition, as already observed in literature (Tetu et al., 2013). We can therefore hypothesise that tourism indirectly affects microbial communities by introducing high amounts of organic matter —including *lampenflora* biomass proliferating due to artificial lighting system— that favour more competitive species (Addesso et al., 2020; Marquez et al., 2016).

The partitioning of the role of different processes highlighted Drift as the dominant driving force in determining the overall community composition of the three microbial groups here considered. However, when restricting the analysis to control samples, its contribution was drastically reduced. These outcomes advocate that tourism and its related activities increase stochastic processes occurring in microbial communities, overriding the effects of dispersal-related mechanisms and environmental filter. Show caves face profound environmental changes during their set up and during their exploitation as tourist attractions (Cigna, 2016) that may disrupt the key driving forces

shaping the structure of microbial communities, e.g. increased energy input, illumination system, introduction of alien microorganisms, ultimately compromising their interactions with abiotic and biotic compartments in the subterranean ecosystem (Bontemps et al., 2022; Ma et al., 2021). These changes are likely responsible of a random reorganization of microbial communities turning out into the dominant role of Drift.

Although changes in substrate composition demonstrated to influence the ASV composition of Fungi, the contribution of Selection as a process in determining their community composition is extremely limited. This is possibly due to the strong antagonistic interactions among species that are governed by priority effects, thus further emphasising the role of Drift (Powell et al., 2015). This is in accordance with the results obtained by analysing the driving forces of microbial communities in control samples, where the role of stochastic processes is reduced, but still high for Fungi. Conversely, the role of Selection is more important for Archaea and, to a lesser extent, Bacteria, suggesting that these two microbial groups are more influenced by environmental changes than Fungi. In particular, evidence in literature suggests that both groups are influenced by changes in nitrogen cycle (Bercea et al., 2019; Jones et al., 2015), corroborating the results obtained with the analysis of the diversity patterns and likely explaining the role of *Variable Selection*. Also, the composition of Archaea seems to be influenced by the higher concentration of CO_2 induced by visitors (Biagioli et al., 2023) that may explain the higher contribution of *Homogeneous Selection* when analysing all samples compared to the results obtained for control samples only.

Dispersal represented the second most important driving force for Fungi and Bacteria when analysing all samples, and it became the dominant process for all the three microbial groups when considering only control samples. This is in agreement with previous studies that propagules of microorganisms can be disseminated in caves by air circulation (Docampo et al., 2011; Jurado et al., 2021; Ogórek et al., 2016). When differentially analysing the contribution of the two components related to Dispersal, we observed a greater role of *Homogenizing Dispersal* for Fungi, while *Dispersal Limitation* was more important in prokaryotes. This different pattern between Fungi and the prokaryotic component is likely related to their different propagule sizes that vary substantially

within each group —ranging between 2 and 50 µm of diameter for Fungi (Ingold, 1971) and between 0.2 and 20 µm of diameter for Bacteria (Young, 2006)— allowing Fungi to disperse better than prokaryotes, which are in turn limited by their lower dispersal level.

Overall, by adopting a metacommunity framework, we could provide new perspectives on the dynamics and patterns of microbial communities exposed to tourism pressure in show caves. Specifically, our results highlighted that anthropogenic pressure affects all microbial communities but with different effects. Similarly, the three examined groups show differential responses to environmental filtering and dispersal pointing out that a proper understanding of the underlying selective mechanisms require a comprehensive and multi-taxonomic approach.

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3.5 References

Abarenkov, K., Zirk, A., Piirmann, T., Pöhönen, R., Ivanov, F., Nilsson, R.H., Kõljalg, U. (2020). UNITE top50 release. Version 04.02.2020. UNITE Community. https://doi.org/10.15156/BIO/786374.

Addesso, R., Bellino, A., D'Angeli, I. M., De Waele, J., Miller, A. Z., Carbone, C., & Baldantoni, D. (2019). Vermiculations from karst caves: The case of Pertosa-Auletta system (Italy). *Catena*, **182**, 104178. https://doi.org/10.1016/j.catena.2019.104178.

Addesso, R., De Waele, J., Cafaro, S., & Baldantoni, D. (2022). Geochemical characterization of clastic sediments sheds light on energy sources and on alleged anthropogenic impacts in cave ecosystems. *International Journal of Earth Sciences*, **111**, 919-927. https://doi.org/10.1007/s00531-021-02158-x.

Allen, R., Hoffmann, L. J., Larcombe, M. J., Louisson, Z., & Summerfield, T. C. (2020). Homogeneous environmental selection dominates microbial community assembly in the oligotrophic South Pacific Gyre. *Molecular Ecology*, **29**, 4680-4691. https://doi.org/10.1111/mec.15651.

Alonso, L., Pommier, T., Kaufmann, B., Dubost, A., Chapulliot, D., Doré, J., et al. (2019). Anthropization level of Lascaux Cave microbiome shown by regional‐scale comparisons of pristine and anthropized caves. *Molecular Ecology*, **28**, 3383-3394. https://doi.org/10.1111/mec.15144.

Alonso, L., Pommier, T., Simon, L., Maucourt, F., Doré, J., Dubost, A., et al. (2022). Microbiome analysis in Lascaux Cave in relation to black stain alterations of rock surfaces and collembola. *Environmental Microbiology Reports*. 10.1111/1758-2229.13133.

Anderson, M. J. (2001). Permutation tests for univariate or multivariate analysis of variance and regression. *Canadian Journal of Fishery and Aquatic Science*, **58**, 626–39.

Ando, K., & Murakami, T. (2020). Detection of human‐associated bacteria in water from Akiyoshi‐do Cave, Japan. *Water Environment Research*, **92**, 1866-1873. https://doi.org/ 10.1002/wer.1355.

Bercea, S., Năstase-Bucur, R., Moldovan, O. T., Kenesz, M., & Constantin, S. (2019). Yearly microbial cycle of human exposed surfaces in show caves. *Subterranean Biology*, **31**, 1-14. https:/doi.org/10.3897/subtbiol.31.34490.

Biagioli, F., Coleine, C., Piano, E., Nicolosi, G., Poli, A., Prigione, V., et al. (2023). Microbial diversity and proxy species for human impact in Italian karst caves. *Scientific Reports*, **13**, 1-13. https://doi.org/10.1038/s41598-022-26511-5.

Borderie, F., Tête, N., Cailhol, D., Alaoui-Sehmer, L., Bousta, F., Rieffel, D., et al. (2014). Factors driving epilithic algal colonization in show caves and new insights into combating biofilm development with UV-C treatments. *Science of the Total Environment*, **484**, 43-52. http://dx.doi.org/10.1016/j.scitotenv.2014.03.043.

Bottos, E. M., Kennedy, D. W., Romero, E. B., Fansler, S. J., Brown, J. M., Bramer, L. M., et al. (2018). Dispersal limitation and thermodynamic constraints govern spatial structure of permafrost microbial communities. *FEMS Microbiology Ecology*, **94**, fiy110. https://doi.org/ 10.1093/femsec/fiy110.

Brislawn, C. J., Graham, E. B., Dana, K., Ihardt, P., Fansler, S. J., Chrisler, W. B., et al. (2019). Forfeiting the priority effect: turnover defines biofilm community succession. *The ISME Journal*, **13**, 1865-1877. https://doi.org/10.1038/s41396-019-0396-x.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., & Nielsen, A., (2017). *glmmTMB* Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, **9**, 378-400. https://doi.org/10.3929/ethz-b-000240890.

Cailhol, D., Ciadamidaro, L., Dupuy, D., Allegra, S., Girardot, F., & Pfendler, S. (2020). Fungal and bacterial outbreak in the wine vinification area in the Saint-Marcel show cave. *Science of The Total Environment*, **733**, 138756. https://doi.org/10.1016/j.scitotenv.2020.138756.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods*, **13**, 581-583. https://doi.org/10.1038/nmeth.3869.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, **6**, 1621-1624. https://doi.org/10.1038/ismej.2012.8.

Ceballos, G., Ehrlich, P. R., Barnosky, A. D., García, A., Pringle, R. M., & Palmer, T.M. (2015). Accelerated modern human – induced species losses: Entering the sixth mass extinction. *Science Advances*, **1,** e1400253. https://doi.org/10.1126/sciadv.1400253.

Cigna, A. A. (2016). Tourism and show caves. *Zeitschrift für Geomorphologie*, **60**, 217-33. https://doi.org/10.1127/zfg_suppl/2016/00305.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Tiedje, J. M., et al. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, **42**, 633-642. https://doi.org/10.1093/nar/gkt1244.

Coleine, C., Biagioli, F., de Vera, J. P., Onofri, S., & Selbmann, L. (2021). Endolithic microbial composition in Helliwell Hills, a newly investigated Mars-like area in Antarctica. *Environmental Microbiology*, **23**, 4002-4016. https://doi.org/10.1111/1462-2920.15419.

Correa-Garcia, S., Constant, P., & Yergeau, E. (2022). The forecasting power of the microbiome. *Trends in Microbiology*, in press. https://doi.org/10.1016/j.tim.2022.11.013

Cuadros, J. (2017). Clay minerals interaction with microorganisms: a review. *Clay Minerals*, **52**, 235-261. https://doi.org/10.1180/claymin.2017.052.2.05.

Culver, D. C., & Pipan, T. (2019). *The biology of caves and other subterranean habitats.* Oxford University Press. pp. 675. https:/doi.org/10.1093/oso/9780198820765.001.0001.

Curtis, T. P., & Sloan, W. T. (2004). Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. *Current Opinion in Microbiology*, **7**, 221–226. https://doi.org/10.1016/j.mib.2004.04.010.

Docampo, S., Trigo, M. M., Recio, M., Melgar, M., García-Sánchez, J., & Cabezudo, B. (2011). Fungal spore content of the atmosphere of the Cave of Nerja (southern Spain): diversity and origin. *Science of the Total Environment*, **409**, 835-843. https://doi.org/10.1016/j.scitotenv.2010.10.048.

Dong, Y., Gao, J., Wu, Q., Ai, Y., Huang, Y., Wei, W., et al. (2020). Co-occurrence pattern and function prediction of bacterial community in Karst cave. *BMC Microbiology*, **2**, 1-13. https://doi.org/10.1186/s12866-020-01806-7.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Journal of Bioinformatics*, **26**, 2460-2461. https://doi.org/10.1093/bioinformatics/btq461.

Edgar, R. C., & Flyvbjerg, H. (2015). Error filtering, pair assembly and error correction for next generation sequencing reads. *Journal of Bioinformatics*, **31**, 3476-3482. https://doi.org/10.1093/bioinformatics/btv401.

Evans, S. E., Bell‐Dereske, L. P., Dougherty, K. M., & Kittredge, H. A. (2020). Dispersal alters soil microbial community response to drought. *Environmental Microbiology*, **22**, 905-916. https://doi.org/ 10.1111/1462-2920.14707.

Fernandez-Cortes, A., Cuezva, S., Sanchez-Moral, S., Cañaveras, J. C., Porca, E., Jurado, V., et al. (2011). Detection of human-induced environmental disturbances in a show cave. *Environmental Science and Pollution Research*, **18**, 1037-1045. https://doi.org/10.1007/s11356-011-0513-5.

Graham, E. B., Crump, A. R., Resch, C. T., Fansler, S., Arntzen, E., Kennedy, D. W., et al. (2017). Deterministic influences exceed dispersal effects on hydrologically‐connected microbiomes. *Environmental Microbiology*, **19**, 1552-1567. https://doi.org/10.1111/1462-2920.13720.

Havlena, Z., Kieft, T. L., Veni, G., Horrocks, R. D., & Jones, D. S. (2021). Lighting effects on the development and diversity of photosynthetic biofilm communities in Carlsbad Cavern, New Mexico. *Applied and Environmental Microbiology*, **87**, e02695-20. https://doi.org/10.1128/AEM.02695-20.

Jurado, V., Del Rosal, Y., Liñan, C., Martin-Pozas, T., Gonzalez-Pimentel, J. L., & Saiz-Jimenez, C. (2021). Diversity and seasonal dynamics of airborne fungi in Nerja Cave, Spain. *Applied Sciences*, **11**, 6236. https://doi.org/10.3390/app11136236.

Kassambara, A. (2022). *ggpubr*: 'ggplot2' based publication ready plots. https://CRAN.R-project.org/package=ggpubr.

Kassambara, A., & Mundt, F. (2020). *factoextra*: extract and visualize the results of multivariate data analyses. https://CRAN.R-project.org/package=factoextra.

Kukla, J., Holec, M., Trögl, J., Holcová, D., Hofmanová, D., Kuráň, P., et al. (2018). Tourist traffic significantly affects microbial communities of sandstone cave sediments in the protected landscape area “Labské Pískovce” (Czech Republic): implications for regulatory measures. *Sustainability*, **10**, 396. https://doi.org/10.3390/su10020396.

Lang, M., Faimon, J., & Ek, C. (2015). The relationship between carbon dioxide concentration and visitor numbers in the homothermic zone of the Balcarka Cave (Moravian Karst) during a period of limited ventilation. *International Journal of Speleology*, **44**, 167-176. http://dx.doi.org/10.5038/1827-806X.44.2.6.

Leibold, M. A., & Chase, J. M. (2017). *Metacommunity Ecology, Volume 59 (Monographs in Population Biology, 59)*. Princeton University Press, Princeton, USA, pp. 491.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., et al. (2013). Fungal community analysis by high‐throughput sequencing of amplified markers – a user's guide. *New Phytologist*, **199**, 288-299. https://doi.org/ 10.1111/nph.12243.

Liu, C., Cui, Y., Li, X., & Yao, M. (2021). *microeco*: an R package for data mining in microbial community ecology. *FEMS Microbiology Ecology*, **97**, fiaa255. https://doi.org/10.1093/femsec/fiaa255.

Locey, K. J., Muscarella, M. E., Larsen, M. L., Bray, S. R., Jones, S. E., & Lennon, J. T. (2020). Dormancy dampens the microbial distance-decay relationship. *Philosophical Transactions of the Royal Society*, **375**, 20190243. http://dx.doi.org/10.1098/rstb.2019.0243.

Ma, L., Huang, X., Wang, H., Yun, Y., Cheng, X., Liu, D., et al. (2021). Microbial interactions drive distinct taxonomic and potential metabolic responses to habitats in karst cave ecosystem. *Microbiology Spectrum*, **9**, e01152-21. https://doi.org/10 .1128/Spectrum.01152-21.

Madsen, A. M., Larsen, S. T., Koponen, I. K., Kling, K. I., Barooni, A., Karottki, D. G., et al (2016). Generation and characterization of indoor fungal aerosols for inhalation studies. *Applied and Environmental Microbiology*, **82**, 2479-2493. https://doi.org/10.1128/AEM.04063-15.

Mammola, S., Di Piazza, S., Ziotti, M., Badino, G., & Isaia, M. (2017). Human-induced alterations of the mycobiota in an alpine show cave (Italy, SW-Alps). *Acta Carsologica*, **46**, 111-123.

Mammola, S. (2019). Finding answers in the dark: caves as models in ecology fifty years after Poulson and White. *Ecography*, **42**, 1331-1351. https://doi.org/10.1111/ecog.03905.

Mammola, S., Meierhofer, M. B., Borges, P. A., Colado, R., Culver, D. C., Deharveng, L., et al. (2022). Towards evidence‐based conservation of subterranean ecosystems. *Biological Reviews*. https://doi.org/ 10.1111/brv.12851.

Martin-Pozas, T., Nováková, A., Jurado, V., Fernandez-Cortes, A., Cuezva, S., Saiz-Jimenez, C., & Sanchez-Moral, S. (2022). Diversity of microfungi in a high radon cave ecosystem. *Frontiers in Microbiology*, **13**, 869661. https://doi.org/10.3389/fmicb.2022.869661.

Martin-Sanchez, P. M., Jurado, V., Porca, E., Bastian, F., Lacanette, D., Alabouvette, C., et al. (2014). Airborne microorganisms in Lascaux cave (France). *International Journal of Speleology,* **43**, 295-303. http://dx.doi.org/10.5038/1827-806X.43.3.6.

Marques, E. L. S., Dias, J. C. T., Silva, G. S., Pirovani, C. P., & Rezende, R. P. (2016). Effect of organic matter enrichment on the fungal community in limestone cave sediments. *Genetic and Molecular Research*, **15**, 15038611. http://dx.doi.org/10.4238/gmr.15038611.

Marques, E. L. S., Dias, J. C. T., Silva, G. S., Pirovani, C. P., & Rezende, R. P. (2017). Organic matter enrichment affects Archaea community in limestone cave sediments. *Journal of Cave and Karst Studies*, **79**, 95-99. https://doi.org/10.4311/2016MB0138.

Moldovan, O. T., Bercea, S., Năstase-Bucur, R., Constantin, S., Kenesz, M., Mirea, I. C., et al. (2020). Management of water bodies in show caves–a microbial approach. *Tourism Management*, **78**, 104037. https://doi.org/10.1016/j.tourman.2019.104037.

Mulec, J. (2014). Human impact on underground cultural and natural heritage sites, biological parameters of monitoring and remediation actions for insensitive surfaces: Case of Slovenian show caves. *Journal for Nature Conservation*, **22**, 132-141. https://doi.org/10.1016/j.jnc.2013.10.001.

Mulec, J., & Oarga-Mulec, A. (2016). ATP luminescence assay as a bioburden estimator of biomass accumulation in caves. *International Journal of Speleology*, **45**, 207-218. http://dx.doi.org/10.5038/1827-806X.45.3.1975.

Nicolosi, G., Mammola, S., Costanzo, S., Sabella, G., Cirrincione, R., Signorello, G., et al. (2021). Microhabitat selection of a Sicilian subterranean woodlouse and its implications for cave management. *International Journal of Speleology*, **50**, 53-63. https://doi.org/10.5038/1827-806X.50.1.2370.

Niehus, R., Mitri, S., Fletcher, A. G., & Foster, K. R. (2015). Migration and horizontal gene transfer divide microbial genomes into multiple niches. *Nature Communication*, **6**, 8924. https://doi.org/10.1038/ncomms9924.

Ogórek, R., Dyląg, M., Višňovska, Z., Tančinová, D., & Zalewski, D. (2016). Speleomycology of air and rock surfaces in Driny Cave (Lesser Carpathians, Slovakia). *Journal of Cave and Karst Studies*, **78**, 119-127. https://doi.org/10.4311/2015MB0128.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2020). vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan.

Pacheco, G. S. M., de Oliveira, M. P. A., Cano, E., Souza Silva, M., & Ferreira, R. L. (2021). Tourism effects on the subterranean fauna in a Central American cave. *Insect Conservation and Diversity*, **14**, 294-306. https://doi.org/10.1111/icad.12451.

Palmer, J. M., Jusino, M. A., Banik, M. T., & Lindner, D. L. (2018). Non-biological synthetic spike in controls and the AMPtk software pipeline improve mycobiome data. *PeerJ*, **6**, e4925. https://doi.org/10.7717/peerj.4925.

Pfendler, S., Karimi, B., Maron, P. A., Ciadamidaro, L., Valot, B., Bousta, F., et al. (2018). Biofilm biodiversity in French and Swiss show caves using the metabarcoding approach: First data. *Science of the Total Environment*, **615**, 1207-1217. https://doi.org/10.1016/j.scitotenv.2017.10.054.

Piano, E., Bona, F., Falasco, E., La Morgia, V., Badino, G., & Isaia, M. (2015). Environmental drivers of phototrophic biofilms in an Alpine show cave (SW-Italian Alps). *Science of the Total Environment*, **536**, 1007-1018. https://doi.org/10.1016/j.scitotenv.2015.05.089.

Piano, E., Nicolosi, G., & Isaia, M. (2021). Modulating lighting regime favours a sustainable use of show caves: A case study in NW-Italy. *Journal for Nature Conservation*, **64**, 126075. https://doi.org/10.1016/j.jnc.2021.126075.

Pimm, S.L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., et al. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science,* **344**, 1246752. https://doi.org/10.1126/science.1246752

Porca, E., Jurado, V., Martin-Sanchez, P. M., Hermosin, B., Bastian, F., Alabouvette, C., et al. (2011). Aerobiology: An ecological indicator for early detection and control of fungal outbreaks in caves. *Ecological Indicators*, **11**, 1594-1598. https://doi.org/10.1016/j.ecolind.2011.04.003.

Poulson, T. L., & White, W. B. (1969). The cave environment. *Science*, **165**, 971–981.

Powell, J. R., Karunaratne, S., Campbell, C. D., Yao, H., Robinson, L., & Singh, B. K. (2015). Deterministic processes vary during community assembly for ecologically dissimilar taxa. *Nature Communications*, **6**, 1-10.

R Core Team (2022). *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rachid, N. A., & Güngör, N. D. (2021). Human activities' impacts on cave microbial diversity: perspectives for cave microbial diversity conservation. *International Journal of Life Sciences and Biotechnology*, **4**, 311-323. https://doi.org/10.38001/ijlsb.829925.

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, **4**, e2584. https://doi.org/10.7717/peerj.2584.

Saiz-Jimenez, C. (2012). Microbiological and environmental issues in show caves. *World Journal of Microbiology and Biotechnology*, **28**, 2453-2464. https://doi.org/10.1007/s11274-012-1070-x.

Šebela, S., Baker, G., & Luke, B. (2019). Cave Temperature and Management Implications in Lehman Caves, Great Basin National Park, USA. *Geoheritage*, **11**, 1163-1175. https://doi.org/10.1007/s12371-019-00367-0.

Shapiro, J., & Pringle, A. (2010). Anthropogenic influences on the diversity of fungi isolated from caves in Kentucky and Tennessee. *The American Midland Naturalist*, **163**, 76-86. https://doi.org/10.1674/0003-0031-163.1.76.

Shoemaker, W. R., Locey, K. J., & Lennon, J. T. (2017). A macroecological theory of microbial biodiversity. *Nature Ecology and Evolution*, **1**, 0107. https://doi.org/10.1038/s41559-017-0107.

Smith, D. P., & Peay, K. G. (2014). Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. *PLoSONE,* **9**, 90234. https://doi.org/10.1371/journal.pone.0090234.

Stegen, J. C., Lin, X., Fredrickson, J. K., Chen, X., Kennedy, D. W., Murray, C. J., Rockhold, M. L., & Konopka, A. (2013). Quantifying community assembly processes and identifying features that impose them. *The ISME Journal*, **7**, 2069-2079. https://doi.org/ 10.1038/ismej.2013.93.

Stegen, J. C., Lin, X., Fredrickson, J. K., & Konopka, A. E. (2015). Estimating and mapping ecological processes influencing microbial community assembly. *Frontiers in Microbiology*, **6**, 370. https://doi.org/10.3389/fmicb.2015.00370.

Summers Engel, A. (2015). *Microbial Life of Cave Systems*. De Gruyter, Berlin, pp. 355. https://doi.org/10.1515/9783110339888.

Tetu, S. G., Breakwell, K., Elbourne, L. D., Holmes, A. J., Gillings, M. R., & Paulsen, I. T. (2013). Life in the dark: metagenomic evidence that a microbial slime community is driven by inorganic nitrogen metabolism. *The ISME journal*, **7**, 1227-1236. https://doi.org/10.1038/ismej.2013.14.

Vellend, M. (2010). Conceptual synthesis in community ecology. *The Quarterly Review of Biology*, **85**, 183-206.

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis*. Springer-Verlag, New York, pp. 213. https://doi.org/10.1007/978-0-387-98141-3.

Young, K. D. (2006). The selective value of bacterial shape. *Microbiology and Molecular Biology Reviews*, **70**, 660-703. https://doi.org/ 10.1128/MMBR.00001-06.

Zhelyazkova, V., Hubancheva, A., Radoslavov, G., Toshkova, N., & Puechmaille, S. J. (2020). Did you wash your caving suit? Cavers' role in the potential spread of *Pseudogymnoascus destructans,* the causative agent of White-Nose Disease. *International Journal of Speleology*, **49**, 149-159. https://doi.org/10.5038/1827-806X.49.2.2326.

Zhou, Z., Wang, C. & Luo, Y. (2020). Meta-analysis of the impacts of global change factors on soil microbial diversity and functionality. *Nature Communications,* **11**, 3072. https://doi.org/10.1038/s41467-020-16881-7.

Zhu, H. Z., Zhang, Z. F., Zhou, N., Jiang, C. Y., Wang, B. J., Cai, L., & Liu, S. J. (2019). Diversity, distribution and co-occurrence patterns of bacterial communities in a karst cave system. *Frontiers in Microbiology*, **10**, 1726. https://doi.org/10.3389/fmicb.2019.01726.

Zuur, A. F., Ieno, E. N., & Elphick, C. S. (2010). A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution*, **1**, 3-14. https://doi.org/10.1111/j.2041-210X.2009.00001.x

CHAPTER 4. Climate drive diversity patterns of dominant microbial taxa in caves worldwide

Abstract

To date, the expanding anthropization of subterranean habitats increases their exposure to external perturbations, challenging conservation of their cultural and natural heritage. Although recent advances on degradation of microbial diversity and growing exposure of cave ecosystems to climatic perturbations, a holistic ecological perspective on environmental drivers of global cave microbiomes is still lacking. This work represents a first draft of a meta-analysis covering over 1050 microbiomes of caves worldwide, to assess the contribution of geographical, climatic and lithological variables in driving the diversity of fungal and bacterial communities. Our preliminary results seem to indicate a different ecological preference of fungal and bacterial diversity for cave environments placed in temperate-tropical and temperate-continental climatic regions respectively, while arid climate leads to a global reduction in cave microbial diversity. Finally, climatic variables appear as stronger predictors of compositional and diversity patterns of microbial dominant phylotypes, selecting specific dominant taxa across gradients of growing aridity conditions. This work would provide new insights useful for planning protection strategies aimed at conservation of underground environments.

Keywords

Meta-analysis; Cave ecosystems, Cave microbiomes; Microbial ecology; Climate change

4.1 Introduction

Caves are spatially confined, subsurface environments widespread on Earth, where peculiar abiotic factors (i.e. oligotrophy, total darkness and high mineral concentrations) and microclimatic conditions (i.e. scarcely fluctuating low temperature and very high humidity) (Gabriel & Northup, 2013) impose special adaptations to microbial life-forms (Culver & Pipan, 2019). Over the last few decades, the global escalation of tourist and economic interest in cave environments led to a constant increase of visitors and transformation of natural hypogeal sites into authentic tourist attractions, dramatically challenging the preservation of these fascinating underground ecosystems and their cultural and natural heritage (Chiarini et. al, 2022; Cigna, 2019). As a physiological response to expanding phenomena of caves' anthropisation, many multi-disciplinary efforts were paid to evaluate and mitigate the disruptive effects on these fragile geo-ecosystems, focusing in particular on microbiome alterations. Caves microbial communities play a key role in fundamental biogeochemical processes, trophic networks and maintaining the functionality of these underground environments (Engel et al, 2012; Venarsky et al, 2018). Already stressed by challenging environmental constraints like oligotrophy, total darkness and high mineral concentrations (Miller et al 2013, Martin-Pozas et al., 2022), they are further impaired by logistical interventions for tourist fruition and visitor flows (Piano et al., 2023). The consequent caves spatial confinement breach, the bypassing of energy deficit by artificial light and exogenous organic matter inputs (supplied by visitors) have favored the proliferation of photosynthetic components (e.g. cyanobacteria, algae and lichens) and spreading of more competitive microbial species (e.g. fast-growing heterotrophic bacteria and saprophytic fungi) from the outside and/or human-related (Saiz-Jimenez, 2012; Piano et al, 2015; Alonso et al., 2019, Dominguez-Moñino et al, 2021; Biagioli et al, 2023), with consequent reduction of autochthonous microbial diversity and functionality. Furthermore, the extensive structural and microbiological alterations experienced by caves today could make these semi-closed subterranean ecosystems less responsive in buffering the effects of biotic and abiotic environmental external drivers, especially in a future perspective of dramatic environmental alterations driven by climate change (Mammola et al., 2019; Sánchez-Fernández et al., 2021).

However, most of the studies on microbial diversity and ecological responses toward biotic and abiotic factors have been performed by analyzing a small number of caves on a local/country scale with a strictly indoor perspective (Piano et al., 2022). Consequently, a holistic ecological perspective to predict microbiome changes and the relative influence of geographical and climatic factors on cave microbial diversity worldwide is still lacking. Yet, assessing the ecological responses of cave microbial communities towards external environmental drivers could be largely biased either by sporadic microbial species presence or by the continuous reshaping of cave microbiomes due to human activities. Hence, defining caves dominant microbial taxa globally and then assessing their responses to external abiotic factors represents a vital task to gain new insights on forces driving structure, distribution and ecology of caves microbiome, as it was highlighted for global soil microbial communities (Zhou et al., 2018; Delgado-Baquerizo et al., 2020, Egidi et al., 2022). Furthermore, exploring these issues in subsurface environments would provide an important tool to predict shifts in microbiome driven by changing environmental conditions, aiming to develop protection-specific actions of cave habitats. Finally, addressing these topics might turn on the lights on exposure of cave ecosystems to the fluctuating climatic variations amplified by climate-change, whose absence from global biodiversity and climate change agendas marginalizes their ecological, scientific and economic relevance (Mammola et al., 2019; Sánchez-Fernández et al., 2021). To address these knowledge gaps, we assembled a global database of amplicon-sequencing data, including over 1,050 bacterial and fungal microbiomes (as relevant assemblages in biogeochemical processes and trophic webs) from caves worldwide (Fig.1). This analysis includes multiple sample types (sediment, rock, speleothems, biofilms, etc.) collected in habitats experiencing different human presence (tourist and wilde caves), both in karst and volcanic landscapes. From a global ecological perspective, we aim for the first time to explore i) the contribution of sample types and different geographical, climatic lithological conditions in driving responses in diversity of whole fungal and bacterial cave communities ; (ii) elucidate fungal and bacterial taxa dominant caves worldwide and (iii) unravel the relative contribution of climatic variables in predicting structural and diversity variability patterns. Furthermore, this work could

provide useful information for implementing future conservation strategies for cave ecosystems from external environmental alteration phenomena driven by climate change in both natural and anthropized subterranean habitats.

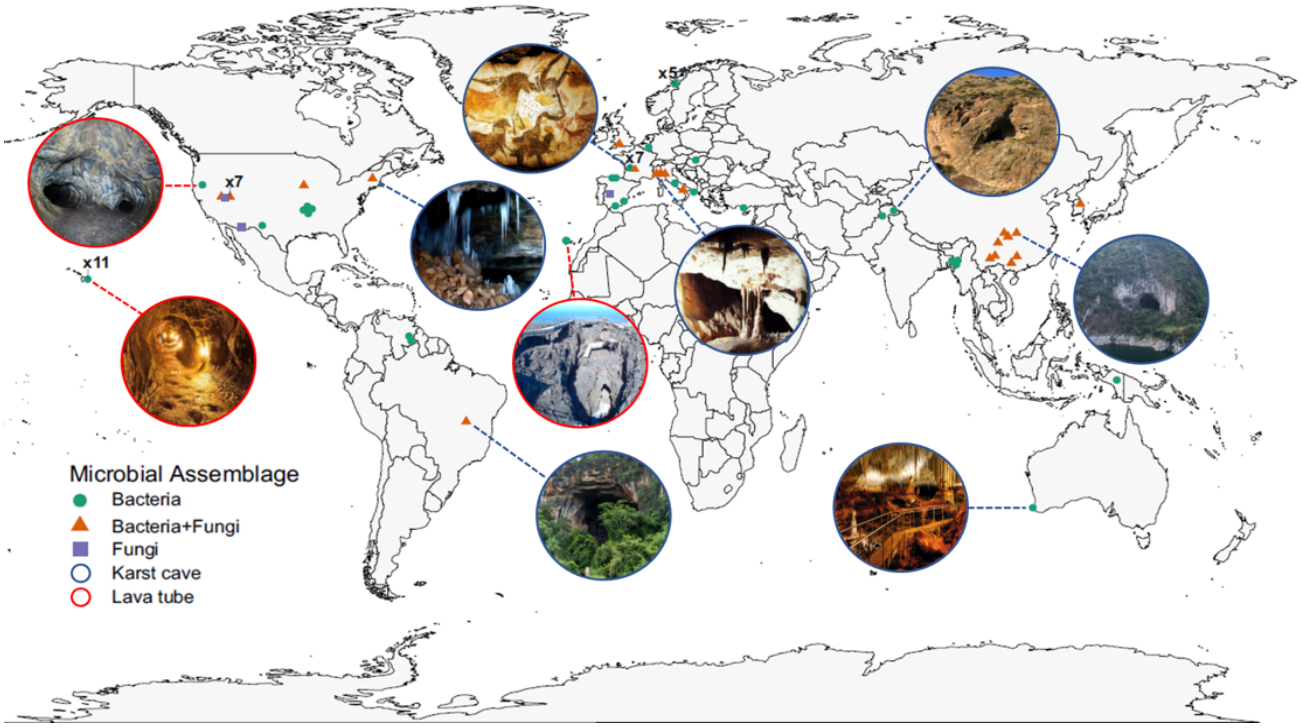

Fig.1: Maps of caves investigated in this work: indicating their location in respective countries, type of cave according to box color and addressed microbial assemblage for each site by shapes. A complete index of the caves is provided in Supplementary Table 1. The pictures in the boxes show some examples of the sites.

4.2 Materials and Methods

4.2.1 Literature and Data selection

The amplicon-sequencing datasets for cave-ecosystems were retrieved from 46 studies for bacteria and 15 studies for fungi, published between 2010 and 2022, searching keywords by environment ("cave," "karst-cave," "ice-cave", "lava-tube"), microbial target ("bacteria," "fungi," "microbes" and "microbial-community"), genomic-target and strategies ("16S," "ITS," “amplicon-sequencing”, "metagenome"). Simultaneously, queries were performed on the NCBI web portal as well as on Google-Scholar for retrieving the corresponding publications and details about : materials-methods applied and metadata (i.e. number and name of sampled environments, sample type, primer sets, platform and sequencing layout, geographic coordinates, etc.). The open-access microbial sequencing raw data were mainly downloaded from the NCBI SRA database using Sratoolkit.

4.2.2 Environmental variable selection

Based on the geographical location of each cave sample, we retrieved bioclimatic information on temperature (e.g. MAT, mean annual temperature; TSEA, temperature seasonality; MDR, mean diurnal temperature range) and precipitation variables (e.g. AI, aridity index; MAP, mean annual precipitation; PSEA, precipitation seasonality) from the Worldclim database (www.worldclim.org), which has a resolution of 1 km (Hijmans et al., 2015). Furthermore we obtained geographic and geological data relative for the Human Influence Index (HII) from Global Human Influence Index Geographic v2 dataset (WCS, C., 2005) and lithological landscape from Global Lithological Map database v1.1 (GLiM, Hartmann & Moosdorf, 2012). The detailed information on the samples with their bioclimatic and geographic models can be found in Supplementary Table 1.

4.2.3 Bioinformatics

Raw 16S and ITS sequence datasets were grouped according to primer-sets, then processed using MICCA (MICrobial Community Analysis) software (Albanese et al., 2015) v.1.7.2. Briefly, reads in Paired-end layout were merged (using the "mergepairs" command), and only sequences with a minimum overlap length of 100 bp and maximum number of 5 allowed mismatches were retained. After this step Paired-end and Single-end reads followed the same processing. Barcodes/indexes and primer sequences were removed from the raw data by quality trimmed step, setting as maximum allowed error rate of 0.1 and dropping reads lacking of forward primers. Parameters for quality filtering step (minimum length filtering, truncation and expected error rate) were selected from results returned by “filterstats” command, choosing the best compromise between read length, expected error rate and number of reads remaining. The Parameters selected were: truncation up to 300 bp, discarding reads less than 100 bp in length and with an expected error rate from 0.25 to 0.5. Because of diverse platforms and layouts sequencing within our dataset, the high quality reads were clustered with 97% similarity into Operational Taxonomic Units (OTUs) using "de novo greedy clustering" protocol and selecting a "greedy clustering strategy distance based" (DGC), skipping global singletons, chimeric sequences and rare taxa (<5 reads) as likely false positives due

to sequencing errors. Finally, taxonomic identification was performed using the assign taxonomy command in AMPtk v.1.5.4 (Palmer et al., 2018) software, with a sequence identity of 97%, using hybrid database Global Alignment and SINTAX algorithm with an ≥80% probability threshold (Edgar et al., 2010) on reference databases UNITE 9.0 (Abarenkov et al., 2020) and RDP 11 (Cole et al., 2014).

4.2.4 Microbial and Statistical analysis

For comparing and exploring cave bacterial and fungal community composition and diversity, the relative abundance of each OTUs was calculated using single samples as replicates for each sample-type and setting investigated, to restrict the weight of sample size across studies. Subsequently, the resulting datasets were rarefied without replacement to mitigate differences in sequencing depth. For each sample, Shannon and Chao1 alpha-diversity indices were calculated. Differences in community diversity and abundance across sample types, continents, climatic zones and lithology, within each site, was assessed in pairwise-comparison by the Wilcoxon test applying a Benjamini–Hochberg FDRmultiple test correction. To test phylogenetic lineages and structure differences in the microbial communities among sample types, geographic (continent, climatic zone) and geological (general lithology context) categories, we applied a permutational multivariate analysis of variance (PERMANOVA) based on a unweighted UniFrac distance matrix mapped by PCoA ordination. Statistical significance was tested by 9,999 random permutations and applying a Benjamini–Hochberg FDR p-value correction. Further, "Cave" factor (~parameter * Cave) was fitted in combination with each single variable for taking into account the effect of samples' origin. To detect and characterize globally dominant fungal and bacterial taxa of caves, we selected most abundant OTUs (with lowest limit of 10% in relative abundance by proportion of total ITS and 16S rRNA reads), frequent (i.e., present in at least 50% of the investigated sites), and with the widest global distribution range (i.e., dominant in at least half of the continents represented). We applied a Redundancy Analysis (RDA) to assess the amount of dominant microbial taxa variation in structure driven by the environmental factors, while the Mantel test (on 9,999 number of permutations) was

used to check for significant correlations between environmental variables and distance matrix used. Moreover, Spearman's correlation analyses were applied to assess the effects of environmental drivers on dominant microbial taxa composition and alpha diversity indices. All tests applied were statistically validated by Benjamini-Hochberg (FDR) p-value correction method. All microbial ecology analyses were performed using the following R packages: "phyloseq" (McMurdie et al., 2013), "microeco" (Liu et al., 2021).

4.3 Results

4.3.1 General description of cave Microbial community

Based on an extensive dataset of amplicon-sequencing analysis, including over 1050 samples from 85 caves worldwide (Supplementary Table 1), this work represents the largest investigation of fungal and bacterial communities of caves existing to date. Six different sample types were analyzed (Soil-Sediment, Parent-rock, Speleothem, Air, Biofilm and Microbial-Mat) from wild and anthropized karstic caves and lava tubes, located in 4 different climatic regions (Arid, Continental, Temperate and Tropical) and multiple geographical contexts (16 countries across 5 continents), gathering multiple environmental data such as the Aridity Index ($0.15 < AI \leq 2.7$), type of superficial lithological landscape (Sedimentary, Igneous and Metamorphic rocks), annual and seasonal average Temperature (MAT, TSEA and MDR) and Precipitation (MAP and PSEA) values, Human Influence Index ($0 < HII \leq 60$) and Altitude ($0 < MASL \leq 3363$). ITS raw sequencing data analysis returned a total of 16,778,890 reads mapped in 11,150 high-quality fungal OTUs, reduced to 7,411 OTUs after (removing 3,739 OTUs) rarefaction step (set to 10,450 reads count threshold) which a final count of 407,550 reads. Of 1,084 genera of fungi recognised, 68% were members of Ascomycota, 29% of Basidiomycota and the remaining 3% were divided across 11 different Phyla (Supplementary Table 2). A total of 22,419,451 reads spanned into 17,440 OTUs were generated from the 16S rDNA dataset and subsequently reduced by rarefaction (with a sequencing depth threshold of 2,574 reads) in 11,891 OTUs (5,549 OTUs removed) for 277,992 of final reads count. Bacteria accounted for 826 genera, 43% ascribed to Pseudomonadota, 17% to Actinomycetota, 14%

to Bacillota, 10% to Bacteroidota and 4% to Acidobacteriota, while the remaining 12% was spread over 21 different Phyla (Supplementary Table 3).

4.3.2 Cave Microbial diversity

Our findings indicated marked differences in sample types, geographic, climatic and lithological factors, to predict changes in distribution and diversity of whole fungal and bacterial communities for caves worldwide (Fig. 2). Fungal diversity within each cave appeared lightly explained by variables taken into account (Supplementary Table 4). In particular, alpha diversity indices showed significant decreases in fungal species and variability in North American caves (Chao1: 214; Shannon: 2.75) as compared to Asian (Chao1: 804; Shannon: 3.988) and European (Chao1: 554; Shannon: 4.10) ones, yet higher values were reported for South American cave (Chao1: 1908; Shannon: 5.1) they appeared not significantly different. Moreover, a declining trend in number of species and variability was observed from wetter to drier environmental conditions, with significant variations recorded when compared arid (Chao1: 186; Shannon: 2.66) with continental (Chao1: 499; Shannon: 3.88) and temperate (Chao1: 761; Shannon: 4.1) climates. Looking at fungal diversity within different specimen substrates, biofilms (Chao1: 226; Shannon: 2.64) appeared to support a reduced number of species and variability than soil-sediment samples (Chao1: 810; Shannon: 4.23), whit latest resulting similar as compared to parent-rocks and speleothems samples. No differences in alpha-diversity were evidenced for mycobiomes located in sedimentary and igneous surficial lithological contexts. In assessing beta-diversity, geographic continental distances (R^2: 0.28; $p<0.001$) and climatic zones (R^2: 0.23; $p<0.001$) were the most powerful factors in predicting shifts across caves fungal communities (Fig.2 AC), especially clustering communities of North America, Europe and Asia sites and those comprised in arid, continental and temperate climates. The influence of sample substrates (R^2: 0.21; $p<0.001$) (Fig. 2B) well characterized fungal assemblages between soil-sediments, parent-rocks and biofilms, while the surface lithological context (R^2: 0.054; $p<0.05$) (Fig. 2D) was less determinant in predicting variations among fungal communities across sedimentary or igneous landscapes.

Bacterial diversity showed significant variation within caves (Supplementary Table 4) in relation to sample types, continental geographic scale, and climatic and lithological context investigated, but reporting a minor contribution of environmental predictors in explaining community differences across sites (Fig. 2E,F,G,H). Sample types displayed the broad bacterial diversity accommodated within soil-sediments (Chao1: 1274; Shannon: 5.26) resulting in a significant higher population and biodiversity, as opposed to biofilm (Chao1: 499) and microbial-mat (Chao1: 370) samples with the lower number in species. Variability in Shannon did not differ significantly among other substrates. Furthermore, specimen substrates (R^2: 0.12; $p<0.001$) led to a spatial separation of bacterial assemblages between caves (Fig. 2F), showing structural and phylogenetic conservation just in Biofilm, Speleothem and Microbial-Mat samples. Lithological scenario characterized a decreasing trend in species count and variability within caves set in metamorphic (Chao1: 1922; Shannon: 5.96), sedimentary (Chao1: 835; Shannon: 4.69) and igneous (Chao1: 633; Shannon: 3.97) bedrock. Partial separation of bacterial lineages between geological contexts (R^2: 0.056; $p<0.001$) was observed (Fig. 2H). Bacterial richness and diversity declined within caves located in arid (Chao1: 527; Shannon: 4.58) and tropical (Chao1: 467; Shannon: 3.7) climate, in contrast to temperate (Chao1: 901; Shannon: 4.7) and continental (Chao1: 1619; Shannon: 5.41) zones. Climate trends also partly predicted (R^2: 0.12; $p<0.001$) structural and phylogenetic divergence of bacteriomes between habitats (Fig. 2G). Geographic continental distances mostly explained bacterial diversity between caves (R^2: 0.10; $p<0.001$) (Fig. 2E) rather than within caves (Supplementary Table 4), showing multiple differences in composition and phylogenetic distance between Asian, European, North and South American caves.

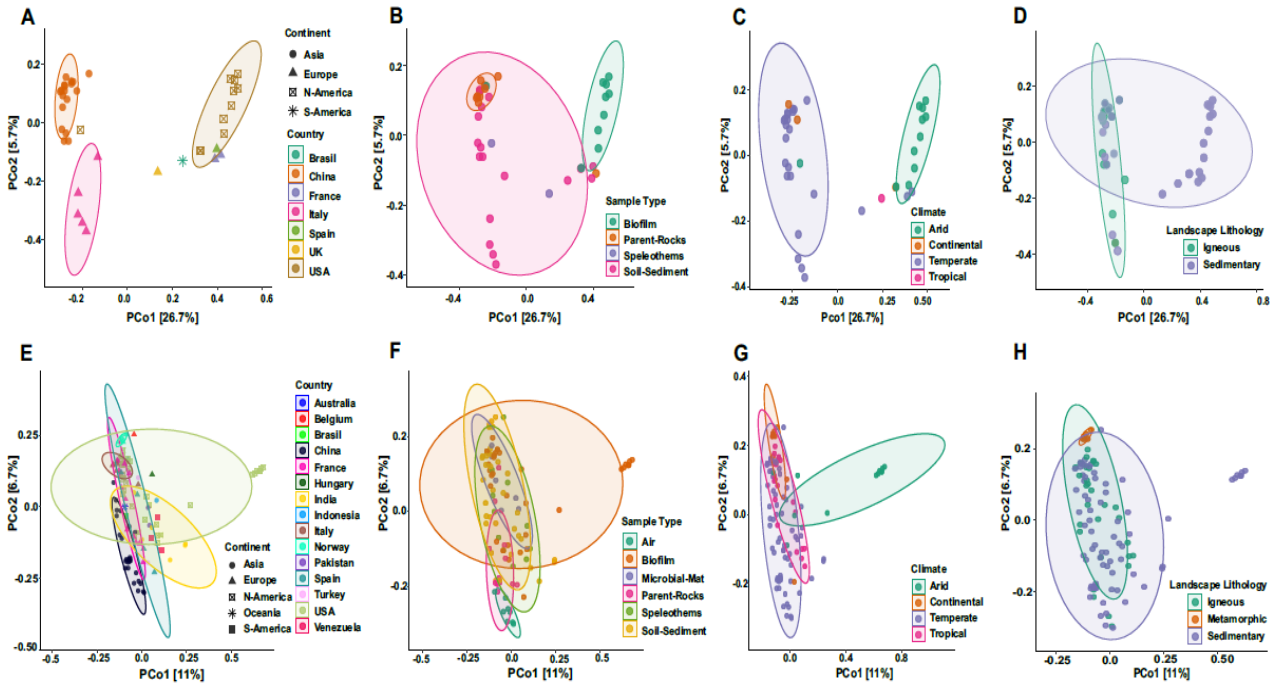

Fig.2: PCoA plots of unweighted UniFrac distances calculated to measure phylogenetic and qualitative distances between cave microbial communities. Fungal (A,B,C,D) and bacterial (E,F,G,H) b-diversity were calculated by clustering sites by geographical locations (A,E), sample types (B,F), climate (C,G) and lithological landscape (D,H). Differences among microbial populations across each variable were tested by PERMANOVA on 9,999 permutations, applying the Benjamini-Hochberg FDR p-value correction test. The significance levels of each variable are *P<0.05; **P<0.01; ***P<0.001.

4.3.3 Cave Top-dominant taxa composition

We found that 50 fungal and 52 bacterial OTUs (Supplementary Table 5,6) were dominant in caves worldwide, representing 0.67% and 0.44% of all fungal and bacterial phylotypes detected and accounting for 36% and 25% of the total reads identified across ITS and 16S datasets respectively. Fungal dominant phylotypes were represented by 3 different phyla, 67.71% by Ascomycota, Mortierellomycota (17.05%) and Basidiomycota (12.68%). 19 different genera were identified (Fig. 3A) accounting for 61% of fungal dominant-taxa, including *Mortierella* (17.05%), *Cladosporium* (11.89%), *Cutaneotrichosporon* (3.89%) *Pseudogymnoascus* (3.48%) and *Cephalotrichum* (3.05%) as the most abundant. Mean abundance values for the dominant fungal phylotypes widely fluctuated across climatic zones and surface lithological contexts (Fig. 3D;E), revealing a larger microbial load among sedimentary bedrocks and temperate and arid climates. Conversely, abundance distribution along continents and sample types (Fig. 3B;C) was less scattered, displaying a significant reduction just across South American subterranean habitats and speleothem samples.

Bacterial dominant-taxa were mainly represented by Pseudomonadota (40.29%), Actinomycetota (33.73%), Nitrospirota (8.62%) and Bacillota (5.27%) phyla for 87.91% and in smaller measure by Deinococcota, Cyanobacteriota, Bacteroidota, Chloroflexota and Acidobacteriota. 31 OTUs were ranked in 28 different genera contributing 43.67% of the total abundance. Among the top 25 abundant genera shown in Fig. 3F (from bottom to top) *Nitrospira* (8.62%), *Pseudomonas* (5.02%), *Herbaspirillum* (2.47%) and *Bacillus* (2.11%) were the most abundant. Between *Acinetobacter* and *Rhodococcus* genera, abundance values ranged between 2% and 1%, while from *Halobacillus* genus values in abundance didn't exceed 1% across the last nine genera (including *Roseiflexus*, *Sinobaca* and *Yersinia* not displayed in the graph). Like reported for fungal core assemblage, population weight of dominant bacterial taxa appeared generally uniform along continental geographic areas and sample types (Fig. 3G,H), with particular differences for South American and Oceanian caves and biofilm samples. Moreover, significant load fluctuations of dominant bacterial phylotypes were reported across the climatic and lithological landscapes (Fig. 3I,J), with temperate and tropical climates and sedimentary and igneous rock bedrock displaying higher levels of abundance.

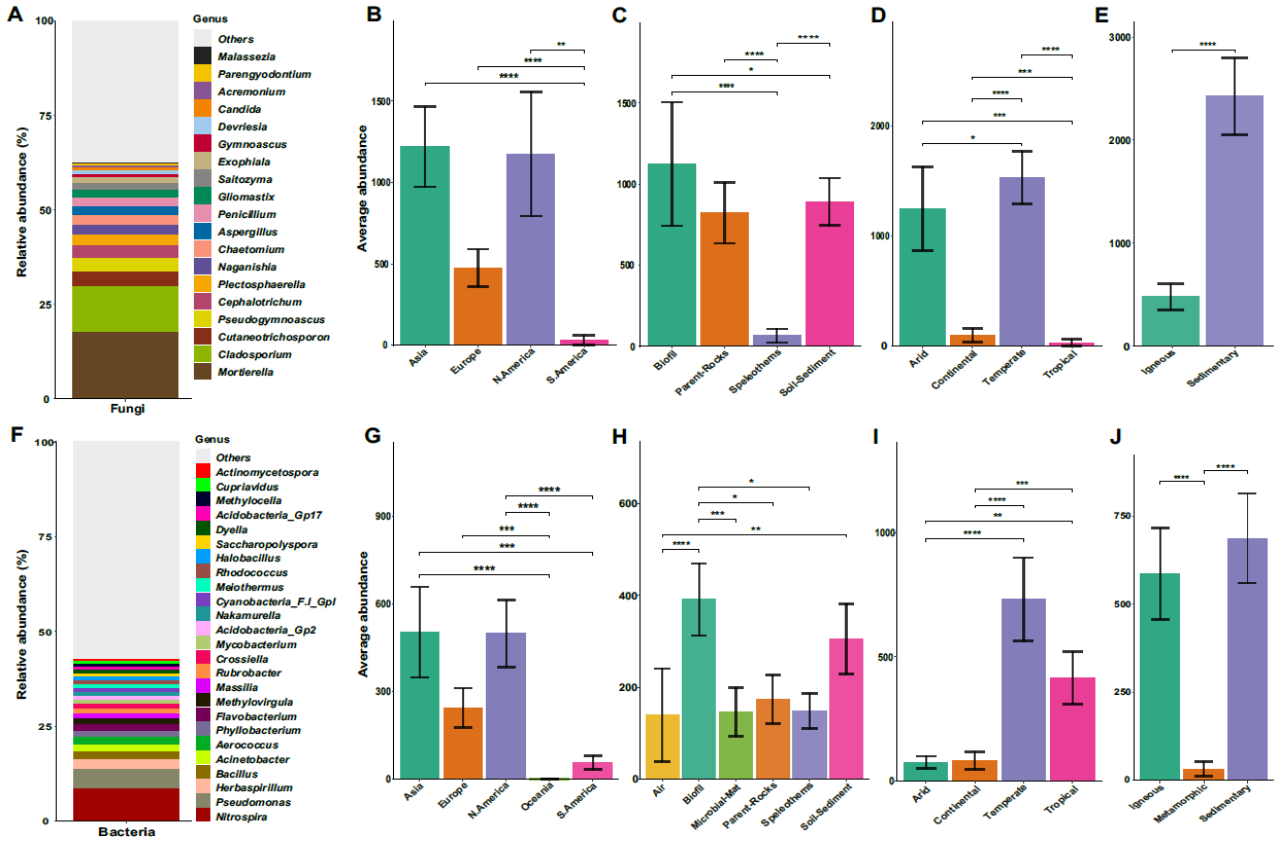

Fig.3: Taxonomic composition of top 20 most abundant genera for fungal (A) and bacterial (F) dominant caves phylotypes. 'Others' includes residual relative abundance of all genera over 20th and phylotypes not identified at genus rank. Mean abundance distribution (mean ± SE) of dominant fungal (B,C,D,E) and bacterial (G,H,I,J) taxa across continents, sample type, climate zones and lithological background. Differences in abundance were addressed by the Wilcoxon test and by applying the Benjamini-Hochberg FDR p-value correction test. The significance levels of each variable are *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Classification of climate regions followed the Köppen-Geiger Systems of Climate Classification (Hijmans et al., 2015), while classification of the lithological landscape followed the Global Lithological Map database v1.1 (GLiM, Hartmann & Moosdorf, 2012).

4.3.4 Driving forces of Top-dominant taxa

Comprehensive correlation analyses on diversity and composition were conducted to elucidate the relationships between microbial cores and environmental drivers.

Results of Mantel test (Supplementary Table 7) applied between unweighted UniFrac distance matrix and environmental parameters showed significant positive correlations, for each variable, to increase differences in structure and phylogenetic distances of fungal core across investigated sites. Considering each single variable, the strongest associations occurred among climatic drivers MDR (r: 0.55, p<0.005), PSEA (r: 0.46, p<0.005), AI (r: 0.44, p<0.005) MAP (r: 0.39, p<0.005) and geographic factor HII (r: 0.46, p<0.005). Spearman's correlation analysis further indicated the broad

contribution of precipitation and temperature range variables to predict variation in alpha-diversity within mycobiome core (Supplementary Table 7). Richness and variability in species were positively and negatively affected by aridity and thermal fluctuation parameters, respectively AI (Chao1: r_s=0.64, p<0.001; Shannon: r_s=0.38, p<0.05) and MDR (Chao1: r_s=-0.65, p<0.001; Shannon: r_s=-0.41, p<0.05). Moreover, precipitation variables MAP (Chao1: r_s=0.54, p<0.01) and PSEA (Chao1: r_s=0.56, p<0.001) had significant positive influences only in expanding the number of species. Redundancy Analysis (Fig. 4A) indicated that the cumulative effect of environmental drivers explained 67.8% of variation in composition of dominant fungal taxa. Strongest significant contribute were by climatic variables MDR (R^2: 0.56, p<0.001), AI (R^2: 0.46, p<0.001) and MAP (R^2: 0.44, p< 0.001), and geographical drivers HII (R^2: 0.50, p<0.001) and Elevation (R^2: 0.31, p<0.005). Moreover, we observed that the 19 dominant fungal genera drifted into 3 clusters over the RDA plot, showing different relationship degrees, positive, negative and null, towards the environmental drivers. This separation was validated by Spearman's correlation analysis results (Fig. 4B), as illustrated by the heatmap and flanking dendrograms. The bottom cluster of 6 fungal genera (from *Mortierella* to *Devresia*) was mainly featured by positive correlations towards AI and precipitation parameters (MAP and PSEA) and partially by mean annual temperature (MAT) and HII, while displaying robust negative relationships with the MDR parameter and sparse ones with TSEA. Conversely, the upper cluster of 6 genera (from *Penicillium* to *Parengyodontium*) showed the reverse pattern in comparison with the first group. A weak influence on global diversity of dominant bacterial taxa across the caves resulted from environmental parameters assessed. Shifts in structure and phylogenetic distances were driven by geographical parameter Elevation (R^2: 0.42, p<0.01) and partially by climatic variable MDR (R^2: 0.23, p<0.01), MAP (R^2: 0.18, p<0.01) and TSEA (R^2: 0.18, p<0.01). Within settings diversity was also mainly influenced by environmental drivers, where MDR (Chao1: r_s=-0.48, p<0.001; Shannon: r_s=-0.23, p<0.05) and PSEA (Chao1: r_s=0.41, p<0.001; Shannon: r_s=0.28, p<0.01) differed in reducing and promoting species richness and variability respectively. However, the weak relations existing between cave bacterial core and outdoor environmental factors was underlined by results of the Redundancy Analysis (Fig. 4C). The

cumulative effects of explanatory variables accounted for 57.7% in driving dominant taxa structure, where TSEA (R^2: 0.29, $p<0.005$), MAP (R^2: 0.28, $p<0.005$) and MAT (R^2: 0.23, $p<0.005$) were the most powerful and significant climatic factors. Furthermore, these explanatory variables, including PSEA (R^2: 0.12, $p<0.005$), established multiple relationships with the most dominant bacterial genera (19 out of 29). Yet, correlation analysis results between bacterial genera and environmental drivers (Fig. 4D) returned a patchy scenario of relevant associations. Interesting were the effect of PSEA, MAP and AI in predicting an increasing prevalence of 8 genera (*Crossiella*, *Aerococcus*, *Massilia*, *Acinetobacter*, *Rhodococcus*, *Halobacillus*, *Mycobacterium* and *Methylovirgula*) when global humidity conditions rise. Conversely, higher abundance rates of *Herbaspirillum*, *Actinomycetospora* and *Rubrobacter* genera were favored under increasing drought conditions, reporting negative associations with AI, PSEA, MAP and positive with MDR and TSEA.

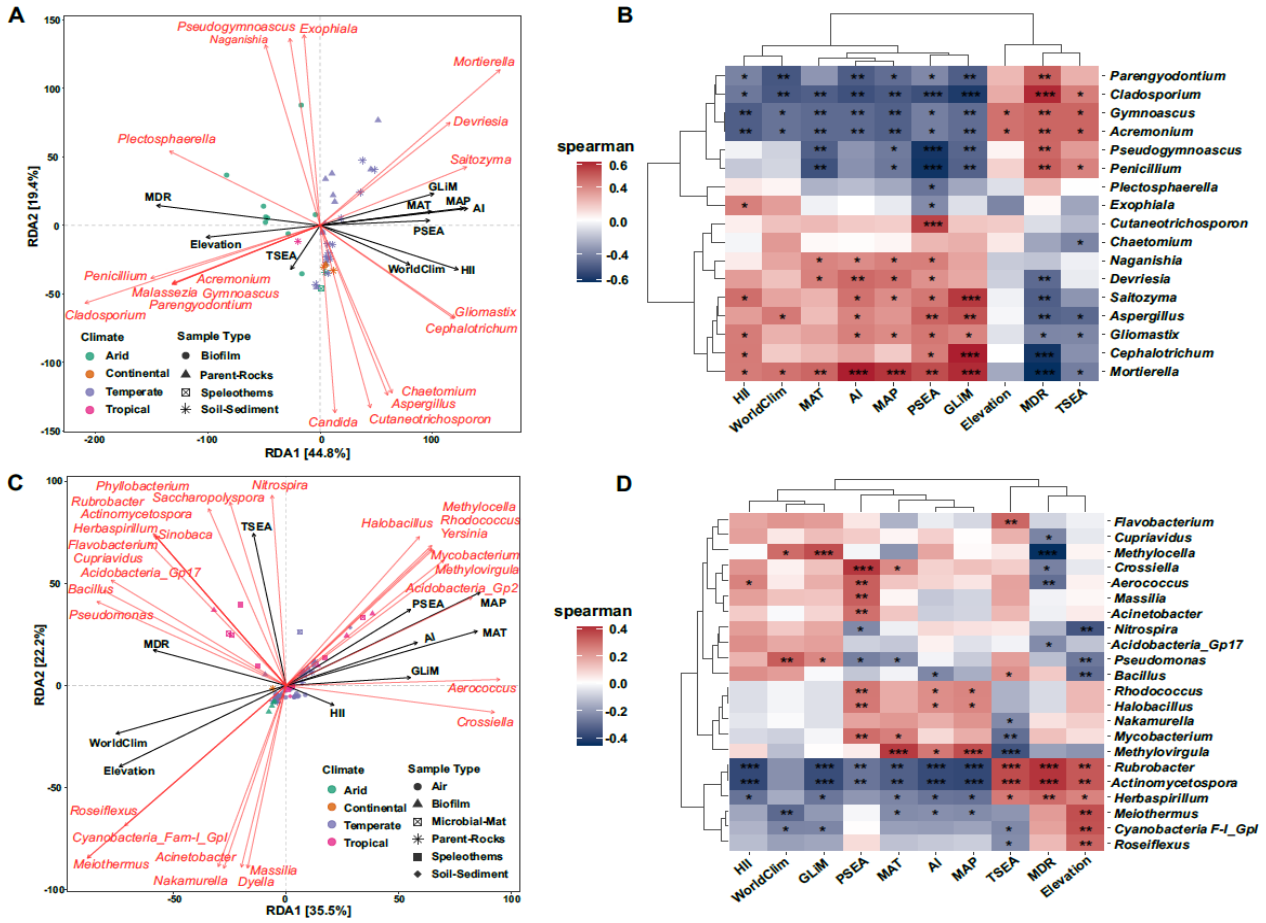

Fig.4: Redundancy analysis (RDA) at the genus level was performed to determine which climatic predictors were most critical in influencing fungal (A) and bacterial (C) dominant taxa distributions. Color of dots represents the climatic region of each site, while shapes define the type of sample collected. Taxa are represented by red arrows in the diagram and climate predictors are represented by black arrows. The statistical significance of each climatic variable's contribution is reported in Supplementary Table 7. Spearman's correlation analysis was performed to investigate the influence of each climatic variable in driving fungal (B) and bacterial (D) dominant phylotypes composition at the genus level. Only genera having at least one significant association with one climatic variable are shown in the heatmaps. The significance levels of each variable are *P<0.05; **P<0.01; ***P<0.001.

4.4 Discussion

A crucial task to assess the integrity and functionality of cave ecosystems and guarantee their future conservation is to understand the ecology and factors driving structural and spatial patterns of their microbiome. Yet, spatial confinement reduction, architectonic modifications and growing tourist flows (Chiarini et. al, 2022; Cigna, 2019) have profoundly disrupted natural environmental, microclimatic and microbiological conditions of caves, making these habitats particularly sensitive to outer disturbances (Mammola et al., 2019). Although knowledge advances about human activities' effects on the cave microbiome and corrective actions developed (Cigna, 2016; Bontemps

et al., 2022; Gillieson et al., 2022; Piano et al., 2023), new uncertainty about long-term conservation of these underground heritages has been spawned by the impending environmental shifts driven by climate change (Sánchez-Fernández et al., 2021). In light of this, an ecological perspective on microbial diversity in global caves and on ecological responses of dominant taxa to environmental drivers could provide new tools aimed to preserve these fragile subterranean ecosystems, including eventual climatic perturbations in future. Surveying cave microbiomes at a global scale represents a hard methodological and technical challenge. The dataset assembly required gathering a large number of microbiomes, including multiple sample types, different typology of caves and selecting a wide array of environmental data, being caves widespread on Earth. Our efforts in investigating fungal and bacterial assemblages produced an extensive global caves microbiome dataset, based on a library of 1058 samples, comprising 6 different substrates, from 85 caves located across 16 countries, 5 continents, 4 climatic zones and 3 different lithological contexts (Fig. 1). Findings revealed a broad microbial diversity represented by 7,411 fungal OTUs and 11,891 bacterial OTUs, identified in 1,084 genera and 13 different phyla for Fungi and 826 genera and 26 phyla for bacteria.

Exploring the global caves microbial communities, our results would suggest how climatic regions were valuable drivers of alpha and beta diversity, outlining a potential divergent climatic pattern for fungal and bacterial assemblages. Caves in wetter environments (from temperate to tropical) or characterized by larger seasonal temperature variation (from temperate to continental) seemed to support a higher fungal and bacterial diversity, respectively. Recent findings revealed how bacteria and fungi could have differing latitudinal diversity gradients on a global scale related to variations in temperature and rainfall, affecting primary productivity and physico-chemical soil properties. Global soils fungal diversity resulted positively associated with high C contents, while bacterial diversity was generally more dependent by soil pH and low C contents (Delgado-Baquerizo & Eldridge, 2019; Liu et al., 2020). Hence, caves placed in continental climates, seemed to support higher bacterial diversity probably for the lower C content and higher pH stability due to the weak seasonal rainfall and productivity of surface soils. Furthermore, we would suggest how higher

oligotrophic conditions might promote specialized bacterial communities in exploiting different nutrient sources (chemolithotrophy) and due to the lower competition for niches along less complex microbial communities (Reboleira et al., 2022). Conversely, fungal diversity would be stimulated by higher organic matter loads sustained by higher primary productivity rates and percolating water across warmer and wetter climates (van Beynen et al., 2002; Marques et al., 2016; Paula et al., 2020). Interestingly, our results seemed to suggest how the differing latitudinal microbial diversity gradients had a point of convergence in ecological responses along settings under arid conditions, reporting a drastic reduction of fungal and bacterial alpha diversity (Supplementary Table 4). Here, where environmental conditions become more challenging (e.g. strong oligotrophy, water depletion and high mineral accumulation), biofilms seemed to represent a successful adaptation strategy, harboring microbial lineages highly-adapted (Fig. 2C,G) to cope with unfavorable conditions and/or to exploit alternative nutrient resources. Their densely packed biological association could provide multi-stress protection (including dryness) and promote interaction between microorganisms (Villa & Cappitelli, 2019). Given the rapid global expansion of drylands due to climate change (Huang et al., 2016), these subterranean ecosystems represent critical hotspots and buffered shelters for biodiversity from external environmental deteriorating conditions.

However, the ecological responses of fungal and bacterial diversity to environmental drivers could be influenced by the presence of sparse and occasional taxa in caves and by the tourism impact on microbiomes. Hence, revealing microbial dominance patterns in global caves could help to unravel relationships between microorganisms and climatic variables, as well as the influence of the surrounding environment in shaping cave microbiomes. The dominant fungal and bacterial taxa in caves worldwide were selected from OTUs with a 10% minimum of presence in at least 50% of the sites and continents surveyed as frequency and distribution parameters. We revealed that 52 bacterial and 50 fungal phylotypes (0.44% and 0.67% of the Bacteria and Fungi recognised) were dominant in caves worldwide. The dominant bacterial phylotype composition resulted more diversified, with 28 genera (out of a total of 826 reported) and 9 different phyla mainly represented by Pseudomonadota (40.29%), Actinomycetota (33.73%) and Nitrospirota (8.62%). Conversely,

fungal dominant taxa structure appeared more uniform, with 19 genera (out of a total of 1084 reported) and represented by Ascomycota (67.71%), Mortierellomycota (17.05%) and Basidiomycota (12.68%) phyla. To the best of our knowledge, this is the first study that attempts to characterize the fungal and bacterial core communities across a broad range of caves and environmental conditions at a global scale. For the dominant taxa identified, biofilm and soil-sediment samples (Fig. 3C,H) seemed to represent the largest diversity reservoirs accommodating higher microbial loads, probably due to the higher protection, nutrients, minerals and moisture levels provided. Moreover, climatic conditions seemed to support a different proportion of dominant phylotypes (Fig. 3D,I), higher in mild climatic conditions and lower in areas with large temperature and precipitation seasonality. The only exception was the high coverage of dominant fungal taxa under arid habitats, probably supported by a broad multi-stress tolerance and skills to degrade different sources of organic matter.

The correlation patterns seemed to suggest a dominant contribution of climate drivers in predicting changes in diversity and structure of cave microbiome cores at the global scale (Fig. 4 A,C; Supplementary Table 7), along which dominant fungal phylotypes showed stronger associations than bacterial assemblage. Usually, lower values of aridity and seasonal precipitation indices (AI, MAP and PSEA) in contrast to higher values of seasonal temperature parameters and diurnal temperature fluctuations (MAT, TSEA and MDR) outline a shift from humid to drier environmental conditions and vice versa. Our results seemed to suggest a contrasting contribution of temperature parameters (MDR, MAT/TSEA) and seasonal precipitation with aridity index (MAP/PSEA and AI), in reducing and increasing the microbial core diversity respectively, speculating how caves microbiome core would qualitatively related to the productivity patterns of surface ecosystems. Probably, the low productivity and C content of soils under growing drought conditions (Maestre at al., 2015; Delgado‐Baquerizo et al., 2016) and lack of precipitation could limit the crucial supplies of organic matter and water to underground ecosystems, distressing their biodiversity and functionality (Venarsky & Huntsman, 2018). Moreover, the contrasting effects of humid and dry conditions could drive the spreading of alien species inside caves. Higher outdoor temperatures and

low humidity were associated with a higher spreading of airborne microbial spores and propagules into the caves from the outside and contrasted by wetter and rainy conditions (Wang et al., 2010a, 2010b). The climate modulating effects on caves energy supply from donor environments seemed to drive the compositional responses of dominant fungal phylotypes (Fig. 4A,B). Our results seemed to highlight how increasing arid conditions may promote the proliferation of specific fungal dominant taxa (*Pseudogymnoascus*, *Acremonium*, *Gymnoascus*, *Cladosporium* and *Parengyodontium*) probably adapted to cope with the challenging environmental conditions in arid caves.) (Biagioli et al., 2023). Interestingly, among the dominant fungal phylotypes were reported strains (e.g. *Parengyodontium*, *Cladosporium*, *Exophiala* and *Devriesa*) commonly associated with tourist exploitation and/or harmful for caves cultural and structural properties conservation (Dominguez-Moñino et al., 2021; Isola et al., 2022). Moreover, dominant bacterial genera displayed a patchy compositional pattern towards climatic drivers, where heterotrophic biomineralization-related (e.g. *Crossiella*, *Massili*a and *Rhodococcus*) and methanotrophic (e.g. *Methylocella*, *Methylovirgula* and *Mycobacterium*) strains seemed favored by wetter climatic conditions. Conversely, strains commonly related to tourism exploitation (*Cyanobacteria* and *Roseiflexus*) and biodeterioration phenomena (*Rubrobacter* and *Actinomycetospora*) in caves were positively associated with higher arid conditions (Saiz-Jimenez, 2012; Farda et al., 2022; Dimkić et al., 2023). Finally, our work aims to provide some preliminary insights into the performance of climate variables in predicting changes in the diversity of cave fungal and bacterial communities and their globally dominant taxa. Because of fundamental trophic and biogeophysical relationships between caves and surrounding ecosystems, we suggest that shifts in microbiome cores should be taken into account to predict variations in functions of subterranean habitats in perspective of eventual climate alterations in future. Taken together, these preliminary results would provide new insights about composition, distribution and ecological responses of microbial taxa dominating global caves. This understanding could be helpful to develop long-term approaches and strategies aimed at preserving the astounding natural and cultural heritage of caves worldwide.

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Competing interest

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

Data Availability

4.5 References

Abarenkov, K., Zirk, A., Piirmann, T., Pöhönen, R., Ivanov, F., Nilsson, R.H., Kõljalg, U. UNITE top50 release. Version 04.02.2020. UNITE Community. https://doi.org/10.15156/BIO/786374; (2020).

Albanese, D., Fontana, P., De Filippo, C., Cavalieri, D., & Donati, C. (2015). MICCA: a complete and accurate software for taxonomic profiling of metagenomic data. *Scientific reports*, *5*(1), 1-7.

Alonso, L., Pommier, T., Kaufmann, B., Dubost, A., Chapulliot, D., Doré, J., ... & Moënne‐Loccoz, Y. (2019). Anthropization level of Lascaux Cave microbiome shown by regional‐scale comparisons of pristine and anthropized caves. Molecular ecology, 28(14), 3383-3394.

Biagioli, F., Coleine, C., Piano, E., Nicolosi, G., Poli, A., Prigione, V., ... & Selbmann, L. (2023). Microbial diversity and proxy species for human impact in Italian karst caves. Scientific Reports, 13(1), 689.

Bontemps, Z., Alonso, L., Pommier, T., Hugoni, M., & Moënne-Loccoz, Y. (2022). Microbial ecology of tourist Paleolithic caves. Science of the Total Environment, 816, 151492.

Chiarini, V., Duckeck, J., & De Waele, J. (2022). A global perspective on sustainable show cave tourism. Geoheritage, 14(3), 82.

Cigna, A. A. (2016). Tourism and show caves. Zeitschrift für Geomorphologie, 60(2), 217-233.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Tiedje, J. M., '*et al.*'. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic acids research*, 42, 633-642; (2014).

Culver, D. C., & Pipan, T. (2019). The biology of caves and other subterranean habitats. Oxford University Press.

Delgado‐Baquerizo, M., Maestre, F. T., Reich, P. B., Trivedi, P., Osanai, Y., Liu, Y. R., ... & Singh, B. K. (2016). Carbon content and climate variability drive global soil bacterial diversity patterns. Ecological Monographs, 86(3), 373-390.

Delgado-Baquerizo, M., & Eldridge, D. J. (2019). Cross-biome drivers of soil bacterial alpha diversity on a worldwide scale. Ecosystems, 22, 1220-1231.

Delgado-Baquerizo, M., Guerra, C. A., Cano-Díaz, C., Egidi, E., Wang, J. T., Eisenhauer, N., ... & Maestre, F. T. (2020). The proportion of soil-borne pathogens increases with warming at the global scale. *Nature Climate Change*, *10*(6), 550-554.

Dimkić, I., Ćopić, M., Petrović, M., Stupar, M., Savković, Ž., Knežević, A., ... & Unković, N. (2023). Bacteriobiota of the Cave Church of Sts. Peter and Paul in Serbia—Culturable and Non-Culturable Communities' Assessment in the Bioconservation Potential of a Peculiar Fresco Painting. International Journal of Molecular Sciences, 24(2), 1016.

Dominguez-Moñino, I., Jurado, V., Rogerio-Candelera, M. A., Hermosin, B., & Saiz-Jimenez, C. (2021). Airborne fungi in show caves from Southern Spain. Applied Sciences, 11(11), 5027.

Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, **26**(19), 2460-2461; (2010).

Egidi, E., Delgado‐Baquerizo, M., Berdugo, M., Guirado, E., Albanese, D., Singh, B. K., & Coleine, C. (2022). UV index and climate seasonality explain fungal community turnover in global drylands. Global Ecology and Biogeography.

Engel, A. S. Chemoautotrophy. In Encyclopedia of Caves (eds White, W. B. & Culver, D. C.) 125–134 (Elsevier, 2012).

Farda, B., Djebaili, R., Vaccarelli, I., Del Gallo, M., & Pellegrini, M. (2022). Actinomycetes from caves: an overview of their diversity, biotechnological properties, and insights for their use in soil environments. Microorganisms, 10(2), 453.

Gabriel, C. R., & Northup, D. E. (2013). Microbial ecology: caves as an extreme habitat. Cave microbiomes: a novel resource for drug discovery, 85-108.

Gillieson, D. S., Gunn, J., Auler, A., & Bolger, T. (Eds.). (2022). Guidelines for cave and Karst protection. International Union of Speleology (UIS) and the International Union for Conservation of Nature (IUCN).

Hartmann, J., & Moosdorf, N. (2012). The new global lithological map database GLiM: A representation of rock properties at the Earth surface. Geochemistry, Geophysics, Geosystems, 13(12).

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G., & Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. International Journal of Climatology: A Journal of the Royal Meteorological Society, 25(15), 1965-1978.

Huang, J., Yu, H., Guan, X., Wang, G., & Guo, R. (2016). Accelerated dryland expansion under climate change. Nature climate change, 6(2), 166-171.

Isola, D., Bartoli, F., Meloni, P., Caneva, G., & Zucconi, L. (2022). Black fungi and stone heritage conservation: Ecological and metabolic assays for evaluating colonization potential and responses to traditional biocides. Applied Sciences, 12(4), 2038.

James, E. W., Banner, J. L., & Hardt, B. (2015). A global model for cave ventilation and seasonal bias in speleothem paleoclimate records. Geochemistry, Geophysics, Geosystems, 16(4), 1044-1051.

Liu, C., Cui, Y., Li, X., Yao, M. Microeco: an R package for data mining in microbial community ecology. *FEMS microbiology ecology*, **97**(2); (2021).

Liu, S., Wang, H., Tian, P., Yao, X., Sun, H., Wang, Q., & Delgado-Baquerizo, M. (2020). Decoupled diversity patterns in bacteria and fungi across continental forest ecosystems. Soil Biology and Biochemistry, 144, 107763.

Maestre, F. T., Delgado-Baquerizo, M., Jeffries, T. C., Eldridge, D. J., Ochoa, V., Gozalo, B., ... & Singh, B. K. (2015). Increasing aridity reduces soil microbial diversity and abundance in global drylands. Proceedings of the National Academy of Sciences, 112(51), 15684-15689.

Mammola, S., Piano, E., Cardoso, P., Vernon, P., Domínguez-Villar, D., Culver, D. C., ... & Isaia, M. (2019). Climate change going deep: The effects of global climatic alterations on cave ecosystems. The Anthropocene Review, 6(1-2), 98-116.

Marques, E. L. S., Dias, J. C. T., Silva, G. S., Pirovani, C. P., & Rezende, R. P. (2016). Effect of organic matter enrichment on the fungal community in limestone cave sediments. Genet Mol Res, 15(10.4238).

Martin-Pozas, T., Cuezva, S., Fernandez-Cortes, A., Cañaveras, J. C., Benavente, D., Jurado, V., ... & Sanchez-Moral, S. (2022). Role of subterranean microbiota in the carbon cycle and greenhouse gas dynamics. Science of the Total Environment, 831, 154921.

McMurdie, P. J., & Holmes, S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*, **8**(4); (2013)

Miller, A. Z., Dionísio, A., Jurado, V., Cuezva, S., Sanchez-Moral, S., Cañaveras, J. C., & Saiz-Jimenez, C. (2013). Biomineralization by cave dwelling microorganisms. Adv. Geochem. Res, 77-105.

Mulec, J., Oarga-Mulec, A., Šturm, S., Tomazin, R., & Matos, T. (2017). Spacio-temporal distribution and tourist impact on airborne bacteria in a cave (Škocjan Caves, Slovenia). Diversity, 9(3), 28.

Palmer, J. M., Jusino, M. A., Banik, M. T., Lindner, D. L. Non-biological synthetic spike-in controls and the AMPtk software pipeline improve mycobiome data. *PeerJ*, **6**; (2018).

Paula, C. C. P. D., Bichuette, M. E., & Seleghim, M. H. R. (2020). Nutrient availability in tropical caves influences the dynamics of microbial biomass. MicrobiologyOpen, 9(7), e1044.

Piano, E., Nicolosi, G., Mammola, S., Balestra, V., Baroni, B., Bellopede, R., ... & Isaia, M. (2022). A literature-based database of the natural heritage, the ecological status and tourism-related impacts in show caves worldwide. Nature Conservation, 50, 159-174.

Piano, E., Biagioli, F., Nicolosi, G., Coleine, C., Poli, A., Prigione, V., ... & Isaia, M. (2023). Tourism affects microbial assemblages in show caves. Science of The Total Environment, 162106.

Reboleira, A. S., Bodawatta, K. H., Ravn, N. M., Lauritzen, S. E., Skoglund, R. Ø., Poulsen, M., ... & Jønsson, K. A. (2022). Nutrient-limited subarctic caves harbour more diverse and complex bacterial communities than their surface soil. Environmental Microbiome, 17(1), 1-17.

Saiz-Jimenez, C. (2012). Microbiological and environmental issues in show caves. World Journal of microbiology and biotechnology, 28, 2453-2464.

Sánchez-Fernández, D., Galassi, D. M., Wynne, J. J., Cardoso, P., & Mammola, S. (2021). Don't forget subterranean ecosystems in climate change agendas. Nature Climate Change, 11(6), 458-459.

Shabarova, T., Widmer, F., & Pernthaler, J. (2013). Mass effects meet species sorting: transformations of microbial assemblages in epiphreatic subsurface karst water pools. Environmental microbiology, 15(9), 2476-2488.

van Beynen, P. E., Schwarcz, H. P., Ford, D. C., & Timmins, G. T. (2002). Organic substances in cave drip waters: studies from Marengo Cave, Indiana. Canadian Journal of Earth Sciences, 39(2), 279-284.

Villa, F., & Cappitelli, F. (2019). The ecology of subaerial biofilms in dry and inhospitable terrestrial environments. Microorganisms, 7(10), 380.

Venarsky, M. P., & Huntsman, B. M. (2018). Food webs in caves. Cave ecology, 309-328.

Wang, W., Ma, X., Ma, Y., Mao, L., Wu, F., Ma, X., ... & Feng, H. (2010a). Seasonal dynamics of airborne fungi in different caves of the Mogao Grottoes, Dunhuang, China. International Biodeterioration & Biodegradation, 64(6), 461-466.

Wang, W., Ma, Y., Ma, X., Wu, F., Ma, X., An, L., & Feng, H. (2010b). Seasonal variations of airborne bacteria in the Mogao Grottoes, Dunhuang, China. International Biodeterioration & Biodegradation, 64(4), 309-315.

WCS, C. (2005). Last of the Wild Project, Version 2, 2005 (LWP-2): Global Human Influence Index (HII) Dataset (Geographic). NASA Socioeconomic Data and Applications Center (SEDAC), Palisades, NY.

Zhou, Z., Wang, C., & Luo, Y. (2018). Effects of forest degradation on microbial communities and soil carbon cycling: a global meta‐analysis. *Global Ecology and Biogeography*, *27*(1), 110-124.

CHAPTER 5. Synthesis and Conclusions

In Chapter 1, we introduced the cave ecosystems, as spatially confined extreme environments characterized by peculiar microclimatic conditions and governed by a delicate balance of geochemical, hydrogeological and atmospheric dynamics. Here, highly adapted microbial communities feed on organic matter fluxes from surface donor ecosystems, and play a key role in nutrient recycling and fundamental biogeochemical processes (Venarsky et al, 2018; Wiseschart & Pootanakit, 2020),supplying important services for caves development (e.g. rock dissolution and mineral precipitation), and contributing to cave structures formation (Martin-Pozas et al., 2020). Furthermore, we provided an overview of key patterns of disturbance on cave microbiomes due to tourist exploitation. The coupled effects of visitors and artificial lighting have a relevant impact in spreading and proliferation of allochthonous microorganisms (such as Bacteria and Fungi) and photosynthetic components (Falasco et al., 2014; Vanderwolf et al. 2014), threatening the functionality and conservation of cave ecosystems (Mulec et al., 2017; Alonso et al., 2019).

In Chapter 2, we used an amplicon-sequencing analysis approach to investigate, for the first time, the whole microbiomes (including Fungi, Algae, Bacteria and Archaea) of four Italian show-caves and recently discovered natural cave, applied an extensive sampling of sediments along the whole extension of each cave. The study indicated how the 4 investigated showcaves share common microbial traits in contrast to the natural one, by filtering microorganisms related to outdoor environment and/or capable of exploiting extra inputs of organic matter eventually supplied by tourist flows (i.e. *Chaetomium* and *Phoma* for fungi and *Pseudomonas* for bacteria). Concerning indirect impacts of cave tourism, these results expand the understanding of how the atmospheric continuum between outdoor and underground environment due to caves opening and extra complex organic matter input by tourist flows represent crucial determinants in reshaping the native microbiome. Furthermore, we have provided some novel insights into the potential direct microbiome cave contamination by human-related bacteria (e.g. *Lactobacillus* and *Staphylococcus*) and commensal/opportunistic human associated fungi (e.g. *Candida*) and dermatophytes. Although cave visitors, as previously reported (Mulec et al., 2017), may act as primary vector in the spreading

of human-related microbial species (by bio-aerosols formation, surface contact and shedding of skin scales and lint from clothing) direct evidence was still lacking. Finally, the large spread of the microalgal component and presence of lichen taxa even in caves' zones previously uncolonised emphasized the coupled effect of artificial lighting and tourist flows in spreading and promoting proliferation of allochthonous species. Overall, our findings provided significant inputs to shedding light on complex alteration patterns of native microbiomes in tourist caves (Borderie et al., 2014; Griffin et al., 2014; Carmichael et al., 2015; Mulec et al., 2017; Burgoyne et al., 202; Dominguez-Moñino et al., 2021).

In Chapter 3, we started from an extensive sequencing analysis of sediment from 4 Italian showcaves. By adopting an advanced ecological framework approach (Vellend, 2010; Stegenet al., 2013, 2015) we aimed to gain insights into mechanisms that determine the composition of subterranean microbial communities in relation to possible tourism-induced perturbations, investigating changes in diversity and composition of fungi, bacteria and archaea components from the 4 show-caves sediments. Sediment samples were collected in three areas placed at progressive lateral distance from the tourist path, which was intended as a proxy of tourism pressure (HP, MP and LP) and for each of them, we identified 3 sampling sectors at progressive linear distance from the cave entrance. In each cave, we collected sediment samples in an area closed to the public to obtain a (control area, C) representative view of the natural conditions of the cave. By physical and chemical properties of each sediment sample we obtained variable (Substrate) summarizing the differences in the substrate conditions between samples collected in the tourist areas and the control samples. As major results, we highlighted how bacteria emerged as the most sensitive group to tourism pressure as they responded to both direct and indirect pressure, while fungi and archaea only responded to indirect pressure. In particular, bacterial diversity significantly decreased with increasing distance from the tourist path. This suggests a possible replacement of resident species by propagules of microorganisms vehiculated by visitors (Mulec, 2014; Saiz-Jimenez, 2012; Zhelyazkova et al., 2020). The replacement of bacterial species, resulting from direct human impact, might dramatically affect the sustainability of whole cave ecosystems, as native bacteria

play a key role in fundamental biogeochemical and energetic processes. Furthermore, sediments variation induced by tourism significantly predicted different shifts in fungal (increment of unique ASVs fraction) and archeal diversity (increase ASVs turnover), probably selecting tourism-favor species (or ecotypes) coping better with new environmental conditions determined by presence of tourists, with a substitution of the original ones for archaea, but not for fungi (Cuadros, 2017; Zhu et al., 2019; Zhou et al., 2020). Besides representing an important advance in knowledge of indirect impacts of human activities on cave microbiomes, these results highlight for the first time the archaeal assemblage sensibility to tourism pressure. Concerning the selective processes in determining the overall microbiome composition, Drift resulted as the dominant driving force, where stochastic processes occurring in microbial communities, overriding the effects of dispersal-related mechanisms and environmental filters. This may depend on the extensive logistical interventions experienced by showcaves, that may disrupt the key driving forces shaping the structure of microbial communities (e.g. increased energy input, illumination system, introduction of alien microorganisms). Furthermore, Homogenizing Dispersal for fungi e Dispersal Limitation for bacteria represented the second most important driving forces among selective processes after Drift. This different pattern is likely related to different propagule sizes that vary substantially within two microbial assemblages (Young, 2006; Madsen et al., 2016), allowing fungal propagules to disperse better than bacteria in caves by air circulation (Docampo et al., 2011; Ogórek et al., 2016; Jurado et al., 2021). These results, for the first time, provided new insights into the selective processes shaping cave microbial components under tourism pressure, underlining the analytical framework's efficiency in unraveling ecological responses of cave microbiomes in anthropised cave ecosystems.

In Chapter 4, we presented a first draft of a meta-analysis of cave fungal and bacterial communities worldwide, aiming at exploring their ecological responses in relation to biological, geographic and climatic factors. For our purposes we assembled an extensive global caves fungal and bacterial sequence dataset, based on a library of 1,058 samples from amplicon sequencing, comprising 6 different substrates, from 85 caves located across 16 countries, 5 continents, 4

climatic zones and 3 different lithological contexts. Results from alpha and beta-diversity analyses on whole cave microbial communities provided some interesting perspectives on ecological preference for fungal and bacterial assemblage for caves placed in temperate-tropical and temperate-continental climatic regions respectively. Fungal diversity would be stimulated by higher organic matter contents, sustained by higher primary productivity rates of surrounding ecosystems across warmer and wetter climates (van Beynen et al., 2002; Marques et al., 2016; Paula et al., 2020). In contrast, bacterial diversity seemed to be favored in caves located in less warm and productive environments, probably due to lower competition for niches along less complex microbial communities (Reboleira et al., 2022). On the other hand, our results seemed to indicate a drastic reduction of fungal and bacterial alpha diversity along settings in arid climates, probably due to the more challenging environmental constraints. These results would suggest that, when climate variability drove the productivity and microbial diversity of surface soils worldwide (Maestre et al., 2015; Delgado‐Baquerizo et al., 2016), it could lead to microbiota structural changes even in spatially confined and buffered environments like caves, due to the close trophic connection between subterranean and surface donor environments. Furthermore, for the first time, we provided early evidence of a shared microbiome core among caves worldwide, including 50 fungal and 52 bacterial dominant phylotypes, identified in 19 fungal and 28 bacterial genera. Finally, climatic variables appear as stronger predictors of compositional and diversity patterns of microbial dominant phylotypes, selecting specific fungal (*Pseudogymnoascus*, *Acremonium*, *Gymnoascus*, *Cladosporium* and *Parengyodontium*) and bacterial (*Rubrobacter* and *Actinomycetospora*) dominant taxa across gradients of growing aridity conditions.

The main findings of this work were: i) Show-caves share common microbial traits in contrast to the natural one; ii) Novel insights into potential direct microbiome cave contamination by human-related bacteria and commensal/opportunistic human associated fungi and dermatophytes; iii) Bacteria was the most sensitive group to tourism pressure responding to both direct and indirect pressure, while fungi and archaea only responded to indirect pressure; iv) Tourism changes composition of microbial communities, with Drift as the dominant selective processes; v) We

provided, for the first time, early evidence of a shared microbiome core among caves worldwide; vi) Climatic variables appear as strong predictors of compositional and diversity patterns of global microbial dominant phylotypes in caves.

Future researches are needed: i) Expanding dataset of Italian caves by sampling additional underground environments, including both tourist and wild habitat, to confirm the trends of microbial diversity pattern found in anthropized caves with respect to the wild one; ii) Expanding dataset of Italian caves including both tourist and wild settings, to confirm microbial diversity ecological responses to tourism impact, possibly including the photosynthetic assemblage of microalgae; iii) Expanding global caves microbiome dataset, possibly including African, Arctic and Antarctic caves (mainly as volcanic ice-caves) encompassing a larger climatic and microbial diversity.

5.1 References

Borderie, F., Tête, N., Cailhol, D., Alaoui-Sehmer, L., Bousta, F., Rieffel, D., ... & Alaoui-Sossé, B. (2014). Factors driving epilithic algal colonization in show caves and new insights into combating biofilm development with UV-C treatments. Science of the Total Environment, 484, 43-52.

Burgoyne, J., Crepeau, R., Jensen, J., Smith, H., Baker, G., & Leavitt, S. D. (2021). Lampenflora in a show cave in the Great Basin is distinct from communities on naturally lit rock surfaces in nearby wild caves. Microorganisms, 9(6), 1188.

Carmichael, S. K., Bräuer, S. L., & Engel, A. S. (2015). Microbial diversity and manganese cycling: a review of manganese oxidizing microbial cave communities. Microbial life of cave systems, life in extreme environments, 3, 137-60.

Cuadros, J. (2017). Clay minerals interaction with microorganisms: a review. Clay Minerals, 52(2), 235-261.

Delgado‐Baquerizo, M., Maestre, F. T., Reich, P. B., Trivedi, P., Osanai, Y., Liu, Y. R., ... & Singh, B. K. (2016). Carbon content and climate variability drive global soil bacterial diversity patterns. Ecological Monographs, 86(3), 373-390.

Docampo, S., Trigo, M. M., Recio, M., Melgar, M., García-Sánchez, J., & Cabezudo, B. (2011). Fungal spore content of the atmosphere of the Cave of Nerja (southern Spain): diversity and origin. Science of the total environment, 409(4), 835-843.

Dominguez-Moñino, I., Jurado, V., Rogerio-Candelera, M. A., Hermosin, B., & Saiz-Jimenez, C. (2021). Airborne fungi in show caves from Southern Spain. Applied Sciences, 11(11), 5027.

Griffin, D. W., Gray, M. A., Lyles, M. B., & Northup, D. E. (2014). The transport of nonindigenous microorganisms into caves by human visitation: a case study at Carlsbad Caverns National Park. Geomicrobiology Journal, 31(3), 175-185.

Jurado, V., Del Rosal, Y., Liñan, C., Martin-Pozas, T., Gonzalez-Pimentel, J. L., & Saiz-Jimenez, C. (2021). Diversity and seasonal dynamics of airborne fungi in Nerja Cave, Spain. Applied Sciences, 11(13), 6236.

Madsen, A. M., Larsen, S. T., Koponen, I. K., Kling, K. I., Barooni, A., Karottki, D. G., ... & Wolkoff, P. (2016). Generation and characterization of indoor fungal aerosols for inhalation studies. Applied and Environmental Microbiology, 82(8), 2479-2493.

Mulec, J. (2014). Human impact on underground cultural and natural heritage sites, biological parameters of monitoring and remediation actions for insensitive surfaces: Case of Slovenian show caves. Journal for Nature Conservation, 22(2), 132-141.

Maestre, F. T., Delgado-Baquerizo, M., Jeffries, T. C., Eldridge, D. J., Ochoa, V., Gozalo, B., ... & Singh, B. K. (2015). Increasing aridity reduces soil microbial diversity and abundance in global drylands. Proceedings of the National Academy of Sciences, 112(51), 15684-15689.

Mulec, J., Oarga-Mulec, A., Šturm, S., Tomazin, R., & Matos, T. (2017). Spacio-temporal distribution and tourist impact on airborne bacteria in a cave (Škocjan Caves, Slovenia). Diversity, 9(3), 28.

Ogórek, R., Dyląg, M., Višňovska, Z., Tančinová, D., & Zalewski, D. (2016). Speleomycology of air and rock surfaces in Driny Cave (Lesser Carpathians, Slovakia). Journal of Cave and Karst Studies, 78(2), 119-127.

Saiz-Jimenez, C. (2012). Microbiological and environmental issues in show caves. World Journal of microbiology and biotechnology, 28, 2453-2464.

Stegen, J. C., Lin, X., Fredrickson, J. K., Chen, X., Kennedy, D. W., Murray, C. J., ... & Konopka, A. (2013). Quantifying community assembly processes and identifying features that impose them. The ISME journal, 7(11), 2069-2079.

Stegen, J. C., Lin, X., Fredrickson, J. K., & Konopka, A. E. (2015). Estimating and mapping ecological processes influencing microbial community assembly. Frontiers in microbiology, 6, 370.

Vellend, M. (2010). Conceptual synthesis in community ecology. The Quarterly review of biology, 85(2), 183-206.

Young, K. D. (2006). The selective value of bacterial shape. Microbiology and molecular biology reviews, 70(3), 660-703.

Zhelyazkova, V., Hubancheva, A., Radoslavov, G., Toshkova, N., & Puechmaille, S. J. (2020). Did you wash your caving suit? Cavers' role in the potential spread of Pseudogymnoascus destructans, the causative agent of White-Nose Disease. International Journal of Speleology, 49(2), 7.

Zhou, Z., Wang, C., & Luo, Y. (2020). Meta-analysis of the impacts of global change factors on soil microbial diversity and functionality. Nature communications, 11(1), 3072.

Zhu, H. Z., Zhang, Z. F., Zhou, N., Jiang, C. Y., Wang, B. J., Cai, L., & Liu, S. J. (2019). Diversity, distribution and co-occurrence patterns of bacterial communities in a karst cave system. Frontiers in microbiology, 10, 1726.