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Tourism affects microbial assemblages in show caves

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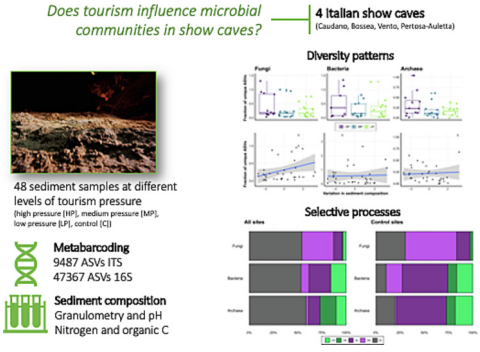
HIGHLIGHTS

- Effects of tourism on microbial communities in show caves have been rarely investigated.
- We investigated sediment communities of Fungi, Bacteria and Archaea in four Italian show caves.
- We tested if tourism influences underlying mechanisms of composition of microbial communities.
- Tourism changes composition of microbial communities, with Drift as the dominant mechanism.
- We provide new perspectives on the dynamics of microbial communities under human disturbance.

GRAPHICAL ABSTRACT



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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.

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

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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 (Leibold and Chase, 2017).

In this context, the operational framework proposed by Stegen et al. (2013, 2015), based on the conceptual synthesis delineated 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 that may evolve either in a scenario of *Variable Selection* (e.g. Graham et al., 2017), when the species composition is determined by multiple variables, or in a scenario of *Homogeneous Selection* (e.g. Allen et al., 2020), 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 and 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 and Pipan, 2019; Poulson and 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 air (Martin-Sanchez et al., 2014; Porca et al., 2011), water (Ando and Murakami, 2020; Moldovan et al., 2020), soil (Kukla et al., 2018; Mammola et al., 2017) and speleothems (Bercea et al., 2019; Pfendler et al., 2018), 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 and Pringle, 2010) or contrasting effects (Bercea et al., 2019; Mulec and Oarga-Mulec, 2016). 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.

2. Materials and methods

2.1. Sampling design

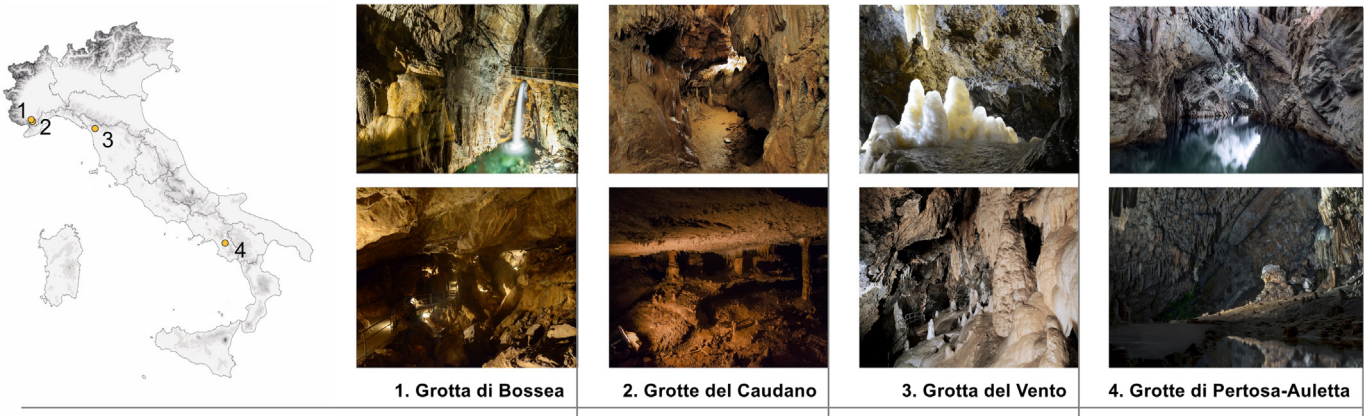
We performed our study in four Italian show caves (Fig. 1). In each cave, we adopted a replicated factorial sampling design wherein sediment samples were collected in three areas placed at progressive lateral distance from the tourist path, which was intended as proxy of tourism pressure. We focused 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. 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, for a total of 9 replicates for each sampling sector (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. Overall, we obtained 48 samples (4 show caves × 4 areas × 3 sampling sectors) (see Appendix A 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.

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 collected 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.

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 and



	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).

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 and 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).

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).

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., 2020). 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 and Mundt, 2020) packages.

2.5.1. 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; Zhu et al., 2019), 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.

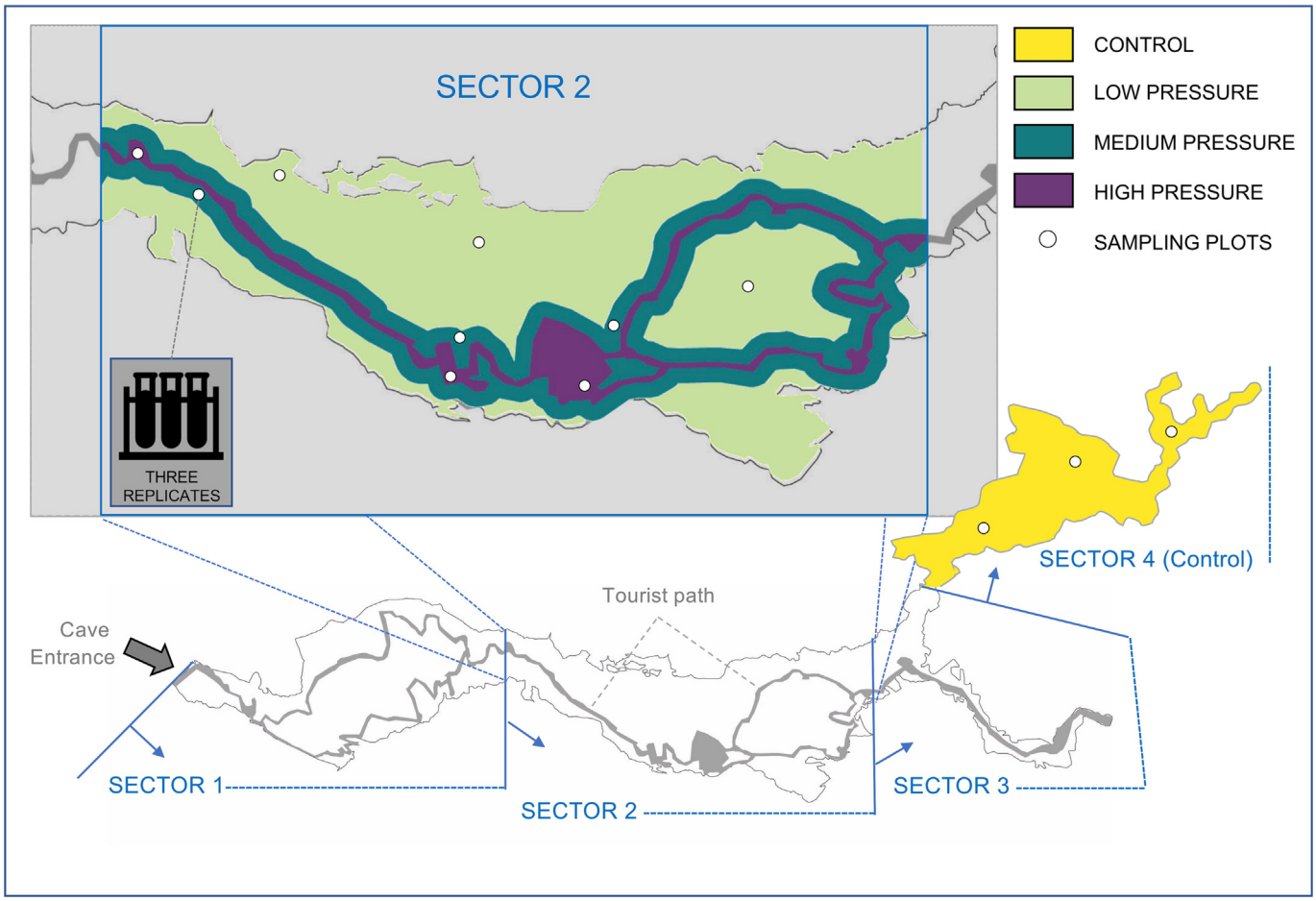


Fig. 2. Schematic representation of the sampling design. The cave was subdivided in four sectors: Sectors 1 to 3 included the area open to tourism and Sector 4 encompassed the Control area, closed to the public. Within each touristic sector, three areas of tourism pressure were identified: the High Pressure 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).

2.5.2. 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).

2.5.3. 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 by visually inspecting the distribution of the residuals (Zuur et al., 2010). Results were retained significant with a p-value < 0.05.

2.5.4. 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. 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., 2013). Values of $\beta\text{-NTI} < -2$ indicated significantly less turnover than expected, i.e. *Homogeneous Selection*, while values of $\beta\text{-NTI} > 2$ 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. 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 Table 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 (Table 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).

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 1252 up to 540,803 per sample. After singletons and rare taxa (<5 reads) removal (1108 out of 10,595 ASVs total), a total of 9487 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 2066 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). 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 (Table 1).

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 (Table S2). The results of the models (Table 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.

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 (Table 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.

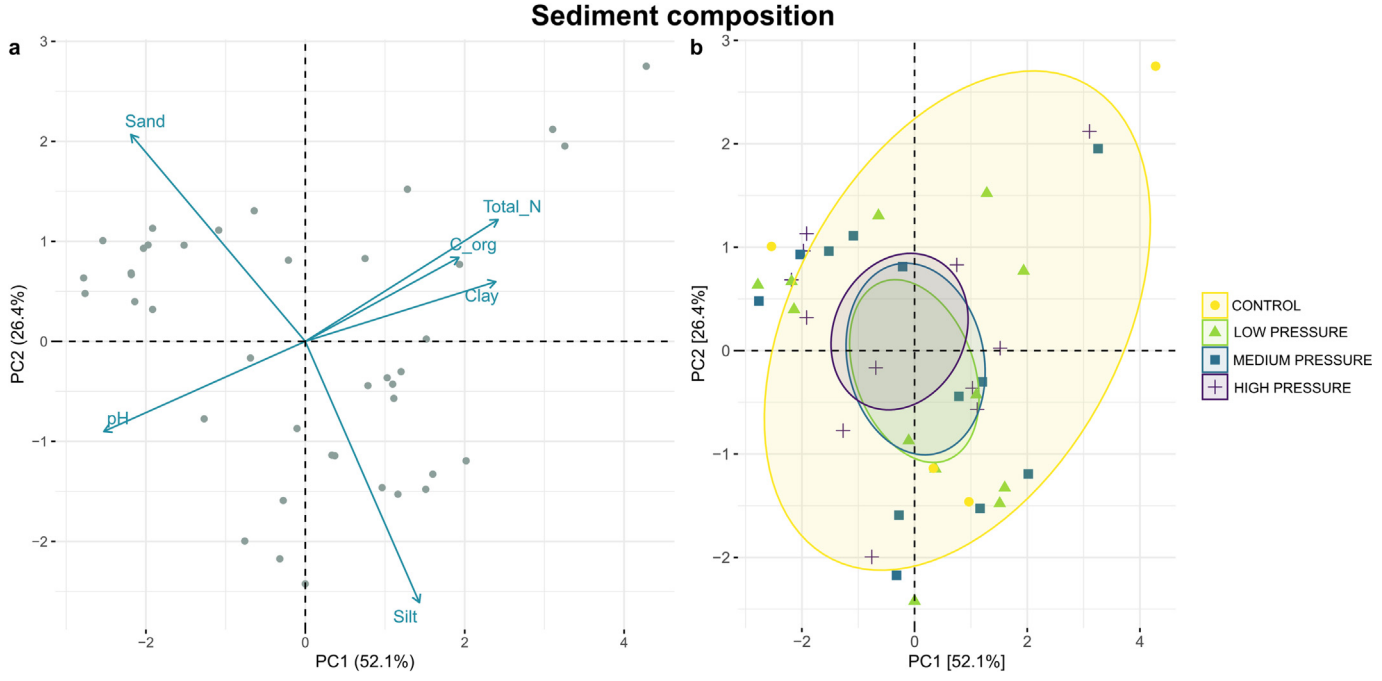


Fig. 3. Results of PCA performed on sediment parameters: a) eigenvectors of environmental variables; b) samples, grouped by tourism pressure. Ellipses represent standard deviations around the centroid of each group.

Table 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.

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**

Table 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.

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**

3.4. Identification of selective processes

When considering all sites, the analysis of the different contribution of selective processes (Table 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.

When analysing control samples separately, he contribution of the Drift component was drastically reduced, ranging from 10 % in Bacteria to 30 % in Fungi. Conversely, Dispersal resulted 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,

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 that 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.

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, and spread around

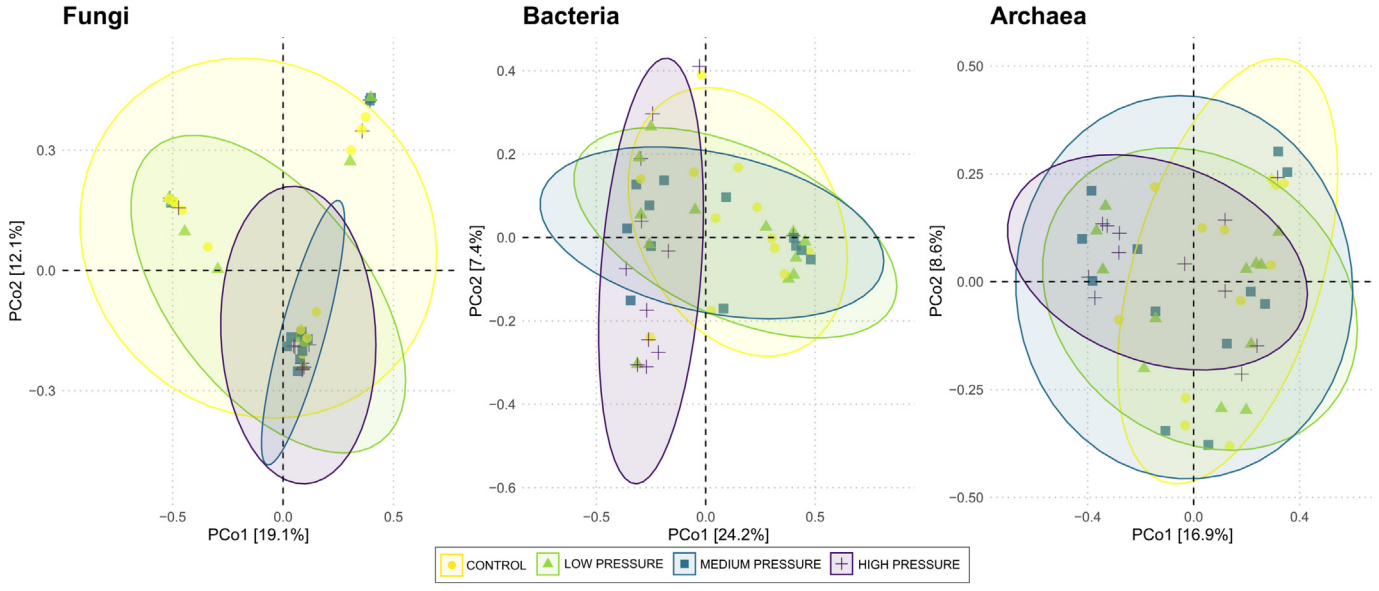


Fig. 4. Ordination according to the first two PcoA axes of the communities of Fungi, Bacteria and Archaea (from left to right). Samples are grouped by levels of tourism pressure. Ellipses represent standard deviations around the centroids of each group.

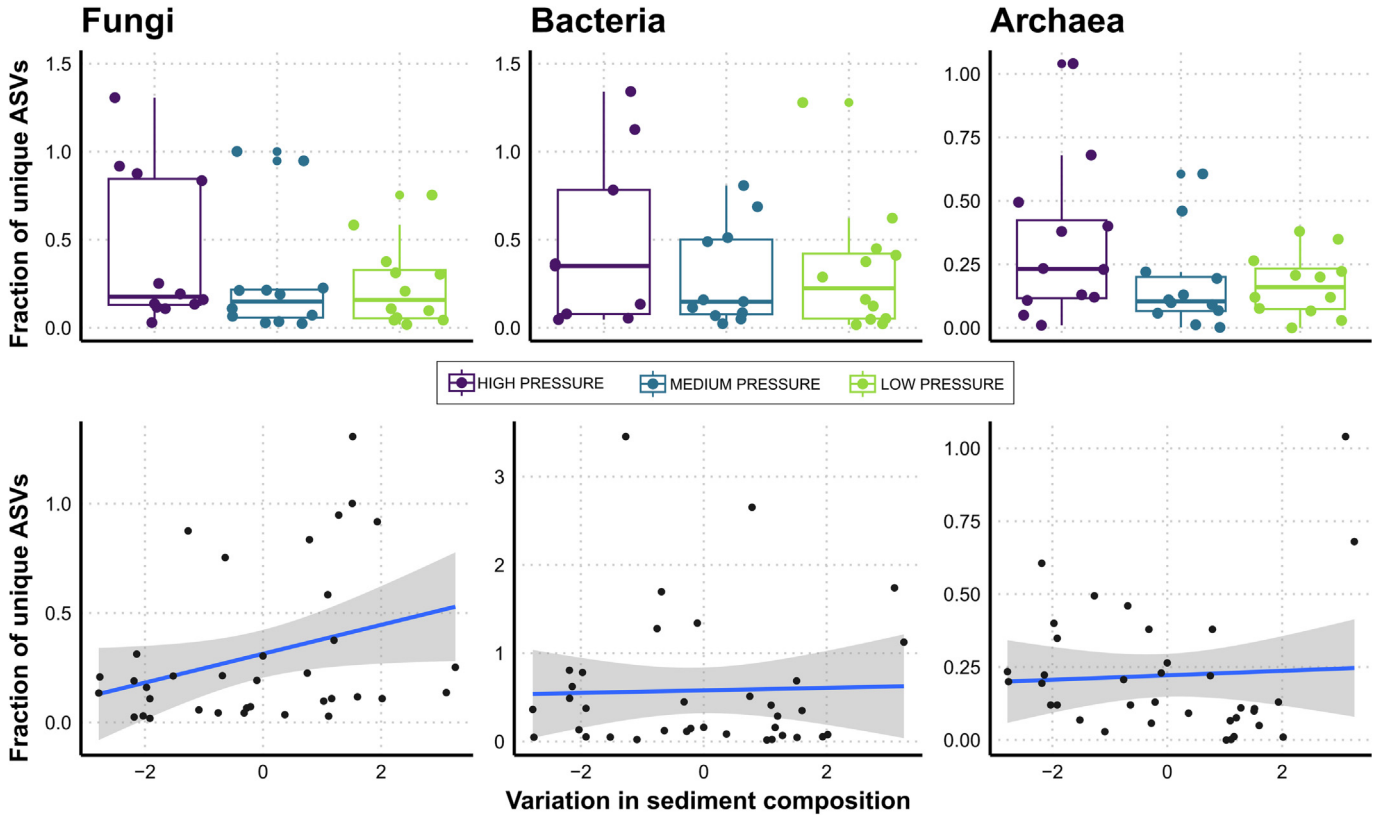


Fig. 5. Boxplots representing the fraction of unique ASVs in the three levels of tourism pressure (purple = High Pressure; light blue = Medium Pressure; green = Low Pressure) with respect to control areas of the three examined microbial groups (upper panel). Regression lines with confidence intervals 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).

during visits (Mulec, 2014; Saiz-Jimenez, 2012; Zhelyazkova et al., 2020), as observed by other authors (Alonso et al., 2019; Dong et al., 2020; Rachid and Güngör, 2021). 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; Zhou et al., 2020), 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 (Alonso et al., 2022; Marques 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; Rachid and Güngör, 2021), 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

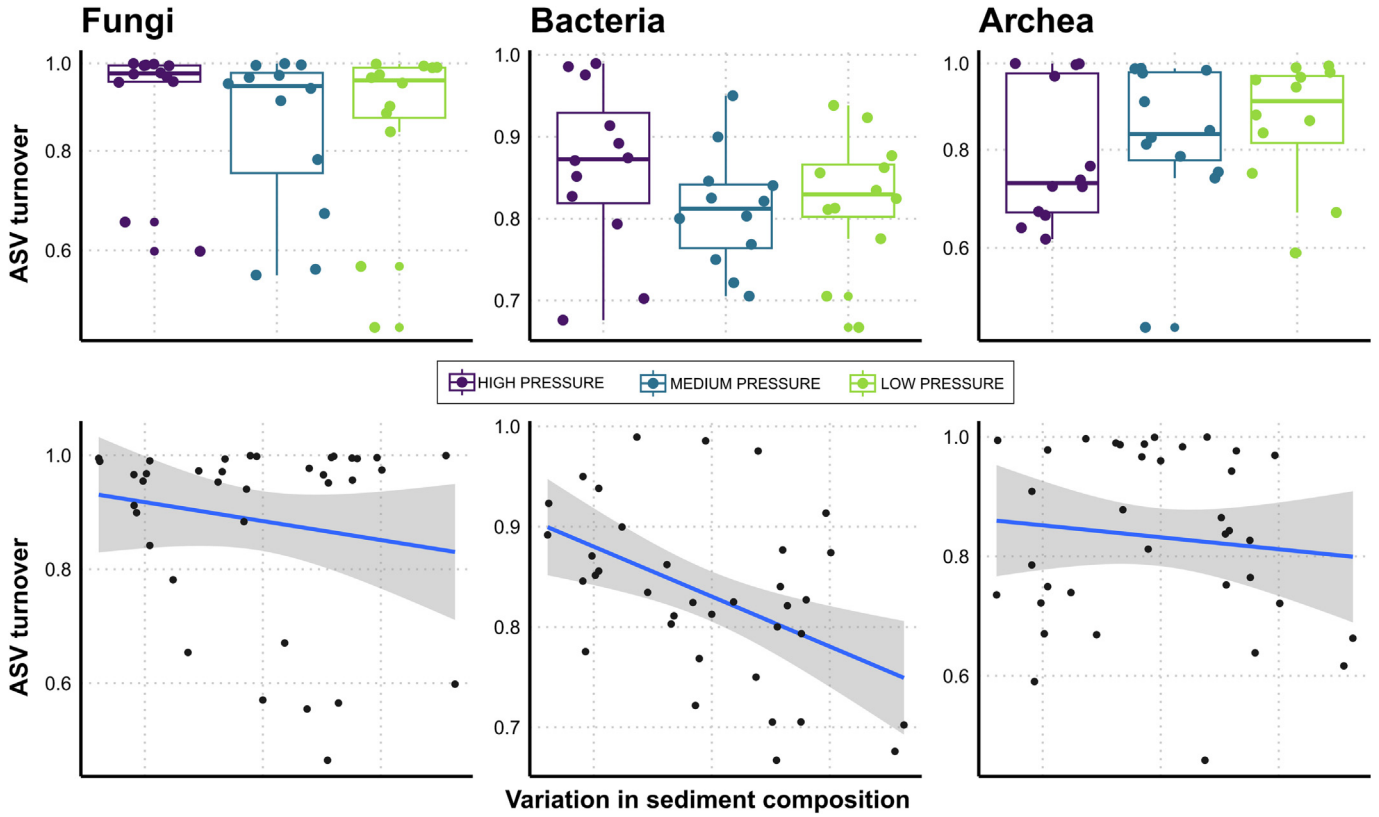


Fig. 6. Upper panel reporting boxplots representing the ASV turnover in the three levels of tourism pressure (purple = High Pressure; light blue = Medium Pressure; green = Low Pressure) with respect to control areas of the three examined microbial groups (upper panel). Lower panel reporting regression 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).

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 (Madsen et al., 2016) 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162106.

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.

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

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CRediT authorship contribution statement

Conceptualization: MI and EP; field work: EP, GN, RA and MI; laboratory analyses: FB, CC, LS, GCV, VP, AP and AZ; data analysis: EP and MI; writing - original draft: EP; writing - review & editing: all coauthors; funding acquisition: MI.

Data availability

Data will be made available on request.

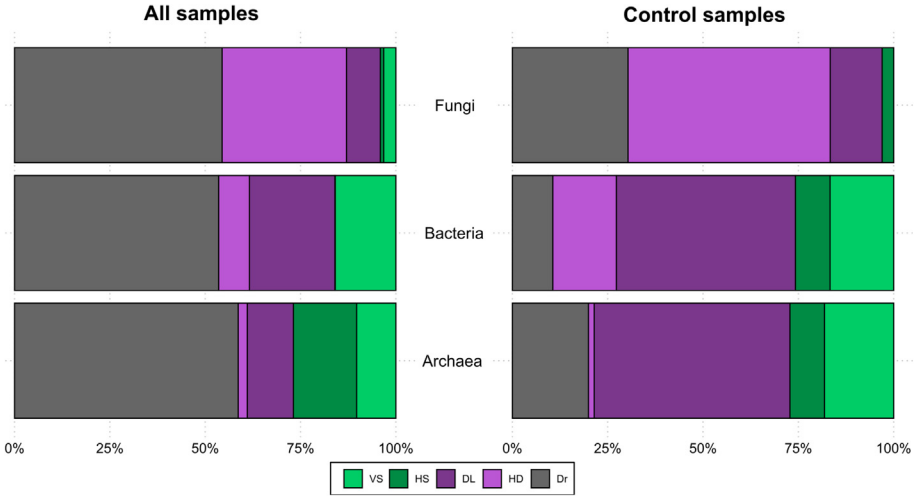

Fig. 7. Barplots representing the different contribution of selective processes to the community composition of the three examined microbial groups calculated by considering: a) all samples; and b) control samples (VS = Variable Selection; HS = Homogeneous Selection; DL = Dispersal Limitation; HD = Homogenizing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

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marco Isaia reports financial support was provided by Government of Italy Ministry of Education University and Research. Elena Piano reports financial support was provided by PON Research and Innovation.

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Statement of authorship

MI and EP set the lines of enquiry and designed the study; GCV, LS, VP, AP, AZ, FB and CC provided important advice in the study design and on sampling methodology; EP, GN, RA and MI performed the field work; FB, CC, LS, GCV, VP, AP and AZ coordinated and/or performed the lab work; EP and MI planned the statistics methodology and EP analysed the data; EP led the writing of the paper; FB provided arguments of microbial ecology for the discussion. All authors reviewed the first draft of the paper and provided improvements to the original text.

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