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Taxonomy and functional profile of microbial communities across the depths of the Alpine Cenote Abyss ice cave

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Investigating the geomicrobiology of the cryosphere offers insights into past climate dynamics and the potential impacts of ongoing climate change. Here, we present the characterization of the microbial communities inhabiting ice sediments collected at various depths within the Cenote Abyss cave, located in the Italian Alps. First explored in 1994 following the drainage of an overlying lake, this site harbours one of the largest cave glaciers in the Dolomites. Metabarcoding and metagenomic analyses revealed a dominance of cold-adapted bacterial taxa, primarily Actinomycetota, Bacteroidota, and Pseudomonadota, with functional genes linked to distinct steps of the nitrogen cycle varying by cave depth. From the shallowest to the deepest ice cave zones, microbial communities shifted from nitrogen-fixing bacterial genera, including *Parafrigobacterium*, *Polaromonas*, and *Pedobacter*, to a higher prevalence of nitrifying bacteria such as *Nitrospira*. Functional metagenomic analyses revealed that genes involved in nitrogen and carbon cycling are broadly distributed across the cave depth zones, with the inner samples displaying the highest potential for nitrogen transformations, including complete denitrification pathways. CO_2 fixation pathways, including the Calvin–Benson–Bassham and Wood–Ljungdahl cycles, were partially represented and taxonomically diverse across the cave depths. Culturable bacterial strains from all depths demonstrated enzymatic activities relevant to organic matter degradation, while phenotype microarray analysis highlighted the metabolic versatility of the inner microbial community in utilizing organic nitrogen substrates, supporting the higher diversity of the inner cave zone compared to the outer cave zone. These findings underscore the ecological complexity and functional potential of microbial life in subterranean ice, offering insights into biogeochemical processes in cold and nutrient-poor environments with implications for climate change studies.

Keywords Ice cave, Cenote Abyss, Psychrophilic, Metagenome, Nitrogen cycle, Carbon fixation

Microbial life in the cryosphere represents a unique archive of peculiar and slow biogeochemical processes in ancient environments at low temperatures. As ice melts, the inner layers become exposed, often releasing mineral particles and organic material, which can lead to the development of a new topsoil layer[1]. This phenomenon significantly alters the microbial ecology of ice-covered regions, influencing both biodiversity and broader ecological dynamics. Cave ice accumulations that have been preserved for centuries remain poorly studied and offer exceptional archives for investigating the impact of climate and anthropogenic activity on the diversity and viability of ice-trapped microbial communities[2].

In glacial environments, frozen carbon stores are typically inaccessible to microorganisms. However, with rising global temperatures and increased rainfall, these stores are becoming more available, triggering the microbial production of greenhouse gases such as carbon dioxide, methane, and nitrous oxide[3]. Nitrous oxide, a key intermediate in the denitrification process of the nitrogen cycle, has been extensively studied in agricultural

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soils[4]. However, its role in the geobiological nitrogen cycle in glacial environments remains understudied and is crucial to predict future climatic changes[5].

Recent studies have revealed complex microbial communities of Bacteria, Archaea, and Fungi inhabiting ice cave deposits in both carbonate and volcanic cave systems located in the Alps and Antarctica[2,6,7]. The presence of psychrophilic microorganisms in these environments also suggests their involvement in forming secondary mineral deposits, such as ice speleothems and cryogenic calcite[8]. However, due to the limited nutrients availability and persistently low temperatures, biogeochemical cycles in ice caves progress very slowly. Nonetheless, several recent studies have recorded measurable microbial activity in glacial settings, particularly nitrogen fixation, nitrification, and denitrification, highlighting the essential role of psychrophilic microbes in the cold biosphere's nutrient cycling[9].

Several karstic regions in the Alps contain caves formed in limestone or dolostone during warmer climatic periods, which are now partially or entirely filled with ice deposited during the Last Glacial Maximum or more recent cold episodes, such as the Little Ice Age[10]. These ice deposits are now rapidly melting due to ongoing regional warming. Over the past two decades, several caves previously known as lakes or dolines with icy bottoms have reopened, providing access to pristine environments sealed off by ice for centuries or millennia[11]. Exploring these environments presents an unprecedented opportunity to study the microbiome of subsurface ice deposits. However, due to the challenging access conditions and limited sampling possibilities of vertical ice caves, only a few studies provide microbiological characterizations of their different layers, including complete analysis of functional genes through metagenomics. These few studies conducted 16S rRNA amplicon sequencing across ice layers of varying ages in a Romanian ice cave (Scărișoara Ice Cave)[2,7]. They found that microbial communities shift primarly in response to ice age, organic carbon content, and exposure to light. A more recent study examined the microbial communities of a Pyrenean ice cave using metabarcoding and proteomic approaches[12]. Their results indicate that even sligth temperature increases may lead to shifts in metabolism and expression of stress or heat-response proteins in resident microbial communities. On the other hand, microbial communities inhabiting ice caves within Alpine Mountain systems remain largely unexplored, despite the ecological and scientific relevance of their glacier fields and geological formation.

This work investigates the microbiomes inhabiting the Cenote Abyss, a striking vertical carbonate ice cave in the Dolomites, which extends 285 m deep and hosts one of the region's most voluminous ice deposits[13]. Initially explored in 1994 by the Speleological Club Proteo of Vicenza after the draining of a lake at 2940 m a.s.l. in the Regional Park of Fanes-Sennes-Braies, the Cenote Abyss is a remote environment. As an alpine dolomitic ice cave, and given its persistent low temperatures, absence of light, and almost total confinement from the surface environment, Cenote Abyss can be considered an extreme ecosystem in terms of poor nutrient availability and low temperatures[12,14]. It therefore represents a unique model system for studying microbe-environment interactions within the subsurface biosphere. Furthermore, its massive ice deposits offer valuable insights into past climatic conditions and the ongoing evolution of climate-driven melting in the Dolomites.

Methods

Geographical settings and cave environment

The Cenote Abyss opens at 2940 m a.s.l. in the Natural Park of Fanes, Sennes and Braies. The area where the cave develops, between Conturines and the Lavarella Mountains, is characterized by a wide syncline structure comprising sedimentary rocks ranging from the Upper Triassic to the Lower Miocene and mainly composed of a succession of limestone and dolomite layers, with a total thickness of 1 km. The cave starts with a karst depression (the former lake named "Lago delle due forcelle"), about 20 m deep and 50 × 30 m wide. The lake suddenly disappeared during August 1994 due to the opening of a hole in the glaciated bottom. All the water drained inside cave passages below this peculiar ice plug. Therefore, it is probable that the cave environment has remained sealed and inaccessible due to the presence of ice plugs and deposits in the last centuries. The bottom of the former lake, a typical doline, is now occupied by an ice and snow cone, characterized by elongated fractures parallel to the depression's major axis. Entering the cave, it is possible to descend into a channel carved between the ice deposit and the rock wall. This conduit is characterised by ice walls sculptured with 1 m wide melting niches. Sample C13 was collected from the ice walls of this area, at 30 m of depth, and consists of an ice deposit with evidence of microbial colonisation in the form of black mud. From this area, it is possible to descend into a series of shafts developed at both the rock wall-ice contact and entirely inside the ice mass. Here, sample C16 was collected at a depth of 40 m and consists of ice containing brownish material. At a depth of 70 m the cave extends completely within the ice inside a tunnel carved by the airflow (The Wind Tunnel, Fig. 1). Here, the innermost sample C17 was collected and appeared as an ancient ice layer with brownish mats. The cave then descends 30 m via another shaft opening in a long NW-SE-oriented passage that progressively opens into a large underground room. From this point onward, the descent is along the ice deposit and the rock wall, with the first ending after about 60 m with a huge ice tongue suspended above the final chamber. The free-hanging depth of this deep chasm is 165 m (Paolo Verico Chasm, Fig. 1). The chamber at the bottom is 130 m long and about 40 m wide, progressively descending to the lowest point of the cave with a cone composed of a mixture of ice, sands, and boulders (at 285 m of depth).

The temperature along the cave changes depending on the depth and season. The first 50 m along the ice plug can reach temperatures as low as -1.5 °C during winter, and up to 0.3 °C during summer. Below the ice plug, temperatures are more constant with a fluctuation of 0.2 °C to 0.4 °C throughout the year.

Sample collection and total DNA extraction

Three expeditions were organised in October 2015, September 2016, and October 2018. While the first two expeditions aimed at geological and environmental characterization with a complete survey of the cave using laser scanning equipment[13], microbiological sampling from Cenote Abyss was performed during the most

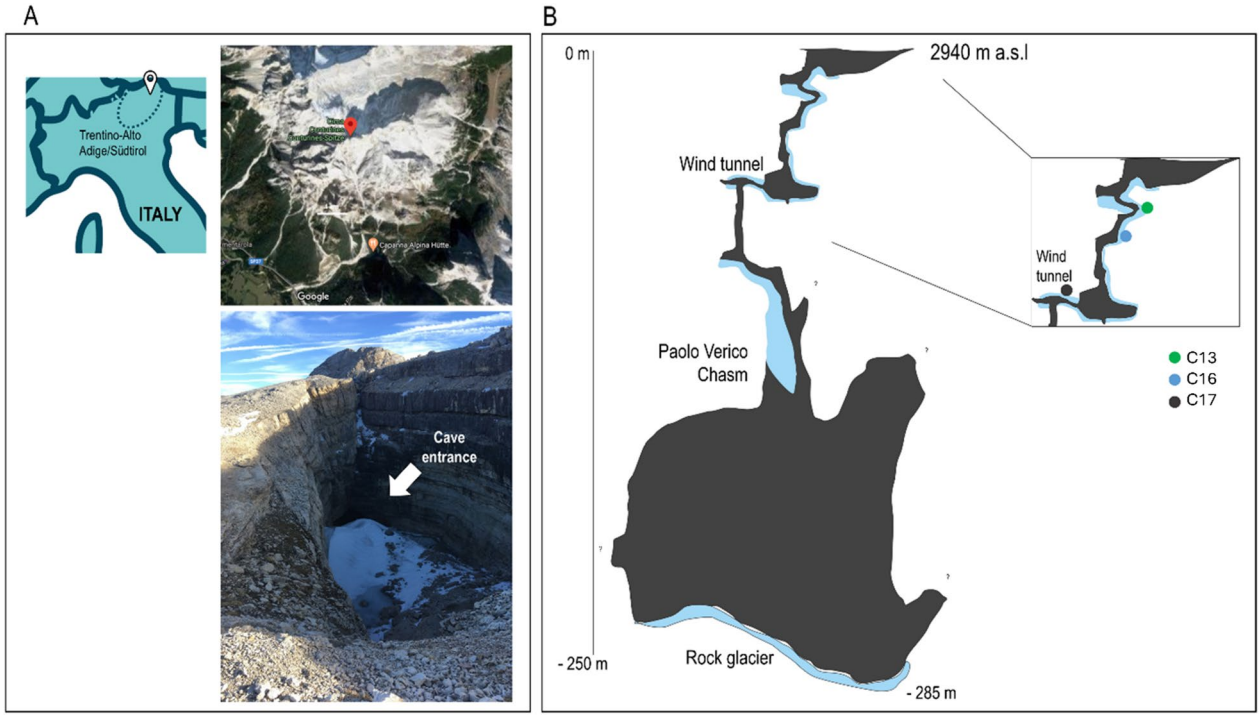

Fig. 1. (**A**) Geographical localization of the Cenote Abyss and cave entrance. (**B**) Schematic representation of the cave and sampling sites.

recent expedition. Due to limited accessibility, seasonal ice cover, and safety constraints, only a minimal amount of ice could be extracted from each site. Samples were collected after scraping with sterilized tools and stored in Eppendorf or Falcon tubes and transported on ice from the cave to the University of Bologna within 48 h. One part of each sample was stored at − 80 °C for DNA extraction, while the other part was maintained at + 4 °C for cultivation procedures.

Three samples were chosen for microbiological analyses, specifically the outermost samples C13 and C16, and the innermost sample C17 (Fig. 1). The cave samples were extracted for their total DNA using the PowerSoil DNA Isolation Kit (Qiagen), with slight modifications as previously described[15].

16S rRNA gene amplification, sequencing and data analysis

The extracted DNA was used as a template for PCR amplification targeting the V4 hypervariable region of the 16S rRNA gene using the primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′)[16] modified with an Illumina adaptor sequence at the 5′ end. 10 ng of total DNA was added to a 50 µL (final volume) PCR reaction mixture containing Takara Ex Taq buffer with $MgCl_2$ (10x; Takara Bio Inc., Tokyo, Japan), primers 200 nM each, dNTP mix 200 µM, Takara Ex Taq Polymerase 1.25 U. The thermocycling program included 1 cycle at 98 °C for 10 s, 30 cycles at 98 °C for 10 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 2 min. Amplicons were submitted for library preparation and Illumina MiSeq indexing and paired-end sequencing (2 × 250 bp; reagent kit, v2). The sequence analysis of 16S rRNA amplicons was performed by using Qiime2 version 2018.4[17] and DADA2 package version 1.5.0[18]. The demultiplexed and primer clipped reads were trimmed based on quality and length. Trimmed sequences were dereplicated, denoised, and merged, and chimeras were removed. The resulting 16S rRNA gene amplicon sequence variants (ASVs) were taxonomically classified using SILVA 138 SSU reference database[19]. Taxonomic attribution was manually corrected based on the latest NCBI taxonomy database.

Alpha diversity estimates were generated by using the Shannon, Inverse Simpson's, and Evenness indexes in Primer-E. Beta-diversity was assessed by using the Bray-Curtis dissimilarity and was plotted in a dendrogram in Calypso as previously described[20,21].

A phylogenetic tree was constructed using the ASVs resulting from the Illumina sequencing of V4 region of the 16S rRNA gene. For each sequence included in the tree, the most closely related sequences retrieved from the NCBI database (Best Blast Hits) were downloaded. MEGA11 was used to construct phylogenetic trees based on ClustalW sequence alignment and neighbor-joining clustering method with 1000 non-parametric bootstrap replicates[22].

Metagenomic sequencing and data analysis

Shotgun metagenomics were performed on 100 ng of total DNA extracted from the samples collected from the inner and the outer zones of the ice cave by HiSeq Illumina sequencing (paired end, 150 bp) at Novogene. Adapter and quality trimming of Illumina reads was performed with BBMap (https://sourceforge.net/projects/bbmap/).

The resulting reads were then subjected to taxonomy and functional profiling to derive a presence-absence profile of taxon-specific functions across the inner and outer samples. Specifically, the taxonomy profiling was performed using Kaiju[23] against the NCBI BLAST nr database, which contain Archaea, Bacteria, and Viruses (accessed in March 2023). The functional profiling was performed through mi-faser against a reference database of prokaryotic proteins[24]. To generate a unified dataset in which each read carried both a taxonomic classification and a functional annotation, Kaiju tabular output (read headers with NCBI taxon identifiers) was joined with a reformatted mi-faser output generated from files containing all reads mapped to a particular E.C. number.

Bacterial isolation, hydrolytic activity screening, and taxonomy attribution

Isolation of culturable microorganisms was carried out by resuspending 0.5 g of each sample in 1 mL of phosphate buffer. Serial dilutions were plated onto R2A plates (per 1 L: 0.5 g yeast extract, 0.5 g peptone, 0.5 g casamino acids, 0.5 g glucose, 0.5 g starch, 0.3 g sodium pyruvate, 0.3 g dipo tassium phosphate, 0.025 g magnesium sulfate, 15 g agar) and ISP2 plates (per 1 L: 4 g yeast extract, 10 g malt extract, 4 g dextrose, 20 g agar), which were incubated at 4 °C for two weeks. The grown colonies were purified and tested for their hydrolytic activities through amylolytic, proteolytic, ureolytic, lipolytc, and cellulolytic assays. To this purpose, bacterial biomass was plated respectively onto Starch Agar (per liter: 4 g glucose, 4 g yeast extract, 2 g starch and 15 g agar), Skim Milk Agar (per 1 L: 8 g nutrient broth, 15 g skim milk, 15 g agar), Christensen's urea agar (per 1 L: 1 g peptone, 1 g glucose, 5 g sodium chloride, 2 g monopotassium phosphate, 0.012 g phenol red, 20 g urea, 15 g agar), tween agar (per 1 L: 10 g peptone, 5 g sodium chloride, 0.125 g calcium chloride, 10 g Tween 80, 15 g agar) and CMC agar plates (per 1 L: 21 g glucose, 0.1 g yeast extract, 0.5 g peptone, 5 g carboxymethyl cellulose, 15 g agar). Positivity in the assays was evaluated after 4 days of incubation at 4 °C by observing either the formation of clear halos around the bacterial biomass or a color change from yellow to purple in the case of ureolytic activity. For starch and CMC agar plates, clear halos were visualized by adding Lugol's solution and 0.2% Congo Red, respectively, after the incubation period.

The bacterial 16S rRNA genes were amplified from the cave isolates' biomass by using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3')[25]. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's protocol. Sanger sequencing of samples was performed at Eurofins Genomics. Taxonomic affiliation was determined by comparing the sequencing data against the NCBI database.

Phenotype microarray

Phenotype Microarray was performed to investigate the metabolic activity of the microbiome inhabiting the innermost cave sample C17. A cell suspension was prepared by resuspending 1 g of sample in 20 mL of 0.1% $Na_2H_2P_2O_7$ at pH 7. The solution was gently shaken for 1 h to allow cell detachment and filtered under sterile conditions to eliminate sample particles that might interfere with assay. One EcoPlate (Biolog Inc.) was then inoculated with 150 µL of the suspension per well and incubated at 4°C for one month. The plate allows to assess in triplicate the utilization of 31 diverse carbon sources (one for each well) by employing a tetrazolium reporter dye. When microbes metabolize a substrate, NADH is produced, and the tetrazolium dye is reduced to form a visible colour. The metabolization of each substrate was assessed at time zero and after a month by reading the EcoPlate at 596 nm.

Results

Microbial diversity and taxonomy profile

A total of 11,893 reads were obtained, with a maximum of 4001 sequences for C17 and a minimum of 3922 for C16 (Table 1). DADA2 denoising resulted with 102 ASV for C13, 99 for C16, and 157 for C17. The evenness calculation was the highest in C13 and the lowest in C17. Shannon and Inverse Simpson indexes showed that C16 had the lowest values in both cases. C13 and C17 had the highest value for Inverse Simpson and Shannon indexes calculation respectively. Bray-Curtis similarity at genus level showed that C13 and C16 clustered together in the dendrogram (Supplementary Figure S1).

Twenty-two bacterial and three archaeal phyla were detected in the three samples under analysis. Archaeal phyla were found in traces in all samples except for C17, in which 2% of the total microbial community belonged to the Nanobdellota phylum (Fig. 2). In all samples, the two most abundant phyla were Pseudomonadota (28% in C13, 27% in C16, and 31% in C17) and Bacteroidota (26% in C13, 30% in C16, and 15% in C17). Pseudomonadota were mainly composed of Betaproteobacteria, followed by Gammaproteobacteria and Alphaproteobacteria (Fig. 2). In all samples, Betaproteobacteria were rich in Burkholderiales order that differed at family level. Indeed, C13 and C16 were rich in members of *Burkholderiaceae* family, whereas C17 were mainly composed of *Nitrosomonadaceae* (Supplementary Figure S2). The Bacteroidota phylum was mainly represented by *Sphingobacteriaceae* of Sphingobacteriales order in C16 (18%) and C13 (10%), the latter also presenting high abundance of *Prolixibacteraceae* of Bacteroidales order (11%) (Supplementary Figure S2). On the contrary,

Sample	Location	Depth (m)	Filtered Reads	ASVs	Shannon	Inverse Simpson's	Evenness
C13	Outermost	30	3970	102	4.33	65.12	0.94
C16	Middle	40	3922	99	4.22	49.15	0.92
C17	Innermost	70	4001	157	4.46	54.26	0.88

Table 1. Summary of illumina sequencing data analysis and alpha diversity indexes.

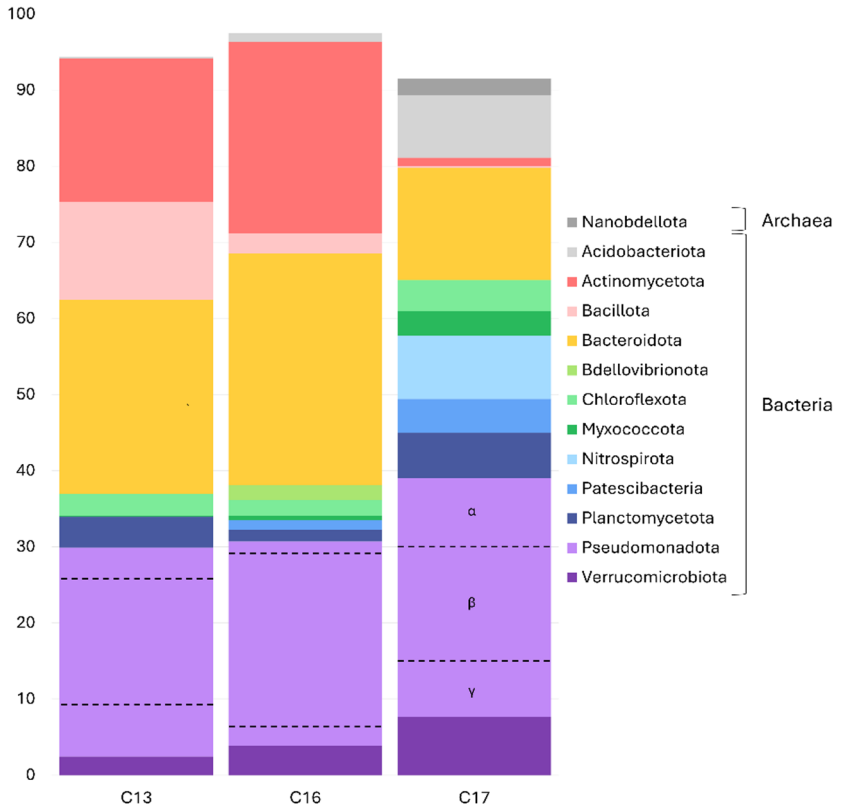

Fig. 2. Taxonomy composition of the three microbial communities at the phylum level. Only phyla with abundances > 2% in at least one sample are shown.

C17 was rich in *Chitinophagaceae* of Chitinophagales order (7%). When considering lower abundant phyla, the taxonomic profile resulted more diversified among the samples. Actinomycetota was the third most abundant phylum in C13 and C16 (19% and 25% respectively), whereas it was detected at low abundance (1%) in C17 (Fig. 2). Within Actinomycetota, Micrococcales was the most represented order (8% in C13 and 13% in C16), followed by Propionibacteriales (4% in C13 and 2% in C16). On the other hand, C17 was highly rich in *Nitrospiraceae* of Nitrospirota phylum, which accounted for 8% of the C17 microbial community and was absent in C13 and C16. Acidobacteriota, mainly of *Blastocatellaceae* family, were highly present in C17 (8%) and found in traces in C13 and C16 (< 1%). Bacillota (mainly of Clostridia class) was detected at 13% in C13, 3% in C16, and < 1% in C17. Verrucomicrobiota, Planctomycetota and Chloroflexota were retrieved in all samples with abundances varying between 2% and 6%.

In line with the clustering analysis, at the genus level the microbial community of C17 strongly differed from those belonging to C13 and C16 (Fig. 3). Sequences unclassified at the genus level amounted to 31% in C13, 46% in C16, and 72% in C17. *Pedobacter* was the most representative genus within the Bacteroidota phylum in C13 (8%) and in C16 (13%). Other abundant genera (2–6%) of Bacteroidota were BSV13, WCHBI-32 (*Prolixibacteraceae*), and *Ferruginibacter* (*Chitinophagaceae*) in C13. Within Gammaproteobacteria, *Polaromonas* was present in both C13 (2.5%) and C16 (4.3%). *Pseudoxanthomonas* (3.6%) and *Rhizobacter* (2.5%) were found in C13 only, while *Nitrosospira* (4.2%) was detected in C16. *Actinotalea* mostly represented actinobacterial Micrococcales were in C13 (3.6%) and *Parafrigobacterium* in both C13 (2.4%) and C16 (6.2%). In general, no abundant genera (> 1%) belonging to C17 were shared with C13 and C16 except for *Ferruginibacter* (1.8% in C17). *Nitrospira* dominated C17 (8.3%), MND1 and IS-44 of *Nitrosomonadaceae* family were detected at 6%, whereas *Terrimonas* of *Chitinophagaceae* represented 3% of C17 microbial community (Fig. 3).

Phylogenetic analysis based on 16S rRNA sequences revealed a relationship between the most abundant taxa from Cenote Abyss and bacterial species retrieved from cold environments, which are involved in the nitrogen cycle (**Supplementary Figure S3**).

Microbial functional profile analysis

The metagenome reads were annotated by comparing them to the Kyoto Encyclopedia of Genes and Genomes (KEGG)[26,27] to determine the microbial functional profiles of the outer cave zone (the merging of C13 and C16 samples) and the inner cave zone (the C17 sample). Metagenomic analysis revealed the main functions and taxa associated with metabolic pathways involved in nitrogen and carbon cycles to provide insights into the key players involved in two of the main biogeochemical cycles in Cenote Abyss.

KINGDOM	PHYLUM	CLASS	ORDER	FAMILY	GENUS	C13	C16	C17
Archaea	Nanobdellota	Ca. Nanoarchaeia	-	-	-	0.00	0.00	2.15
Bacteria	Acidobacteriota	Blastocatellia	Blastocatellales	Blastocatellaceae	-	0.18	0.00	5.62
	Actinomycetota	Actinomycetes	Micrococcales	Cellulomonadaceae	-	2.37	0.00	0.00
				Intrasporangiaceae	-	1.96	3.72	0.00
				Microbacteriaceae	Parafrigoribacterium	2.39	0.00	0.00
					-	0.00	8.87	0.00
			Propionibacteriales	Nocardioidaceae	Aeromicrobium	0.96	2.29	0.00
					Nocardioides	2.57	0.00	0.00
			Pseudonocardiales	Pseudonocardiaceae	Pseudonocardia	2.85	1.43	0.00
	Bacillota	Clostridia	Caldicoprobacterales	Caldicoprobacteraceae	Caldicoprobacter	2.19	0.00	0.00
			Eubacteriales	Oscillospiraceae	-	3.07	0.00	0.00
	Bacteroidota	Bacteroidia	Marinilabiliales	Prolixibacteraceae	BSV13	5.97	0.54	0.00
					WCHB1-32	5.01	0.23	0.00
		Chitinophagia	Chitinophagales	Chitinophagaceae	Ferruginibacter	2.32	0.00	1.82
					Terrimonas	0.00	0.00	2.85
					-	0.00	2.22	0.00
		Flavobacteriia	Flavobacteriales	Flavobacteriaceae	-	0.00	3.01	0.00
		Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Pedobacter	8.01	12.67	0.00
					-	0.00	2.17	0.00
	Chloroflexota	Chloroflexia	-	-	-	2.57	0.69	0.00
	Nitrospirota	Nitrospiria	Nitrospirales	Nitrospiraceae	Nitrospira	0.00	0.00	8.32
	Planctomycetota	vadinHA49	-	-	-	2.07	0.00	0.35
	Pseudomonadota	Alphaproteobacteria	Rhizobiales	A0839	-	0.00	0.00	3.37
				-	-	0.00	0.00	2.20
			Sphingomonadales	Sphingomonadaceae	Sphingomonas	2.59	0.25	0.00
		Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas	2.47	4.28	0.00
					Rhizobacter	2.49	0.00	0.00
				Oxalobacteraceae	-	2.09	4.90	0.85
			Nitrosomonadales	Nitrosomonadaceae	IS-44	0.00	0.00	3.72
					MND1	0.00	0.00	3.97
					Nitrosospira	0.00	4.23	0.00
		Gammaproteobacteria	-	-	-	2.90	0.00	2.00
			Lysobacterales	Lysobacteraceae	Pseudoxanthomonas	3.58	0.00	0.00
	Verrucomicrobiota	Verrucomicrobiia	Limnisphaerales	-	-	0.78	0.00	4.37

Fig. 3. Taxonomy composition of the three microbial communities at the lowest resolved taxonomic level. Shades of green represent the relative abundance of each taxon, with darker shades indicating higher abundance. Only taxa with a relative abundance > 2% in at least one sample are shown.

Functional genes involved in the nitrogen cycle

Nitrogen resources from drip water are considered highly relevant in oligotrophic environments such as Alpine ice caves[7]. Based on KEGG analysis, various genes of the nitrification process were identified in both outer and inner cave zones. In particular, the outer cave zone also showed the gene encoding hydroxylamine oxidase, a key enzyme in the nitrification process. In the inner zone, all denitrification genes were also identified, including the *nosZ* gene, which codes for the last enzyme of the metabolic pathway, nitrous oxide reductase. Some nitrate assimilatory and dissimilatory reduction genes were also identified in both samples. Finally, nitrogen fixation *nif* genes were present in both samples (Fig. 4).

In the outer zone, genes of both nitrification and denitrification (*narG*, *nirK*, *nirS*, *nosZ*, *norB*, *hao*, *nrxA*) mainly were affiliated with the classes Actinomycetes, Gammaproteobacteria, and Betaproteobacteria, whereas in the inner zone, the same genes were found mainly in the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Actinomycetes. In the outer zone, the *nirK* and *nirS* genes, both encoding nitrite reductases, were associated with the genera *Luteinomonas*, *Lysobacter*, and *Vulcaniibacterium*. In contrast, in the inner zone, the same genes were identified in the genera *Brucella*, *Chelatococcus*, *Comomonas*, *Lutibacter*, and *Thermomonas*. Again, in the outer zone, the B and C subunits of nitric oxide reductase were affiliated with the species *Rhodoferax ferrireducens* and the genus *Advenella*, whereas in the inner zone the same genes were found to belong to the genera *Rhodospirillum*, *Brevundimonas*, the family *Nitrobacteriaceae* and the order *Shingomonadales* (Supplementary Table S1). The nitrous oxide reductase encoded by the *nosZ* gene, which was only detected in the inner zone, was associated with the family *Nitrobacteriaceae* and the species *Competibacter denitrificans*. Finally, the nitrogen fixation *nif* genes were associated with the species *Geobacter chapellei* and the family *Nitrobacteriaceae* in the outer and inner zone, respectively.

Functional genes involved in the carbon cycle

Genes involved in all six known CO_2 fixation pathways were identified in both metagenomes analyzed (Supplementary Table S2, Fig. 5). However, none of the six cycles were fully represented. In the outer zone, RuBisCO genes (representing the Calvin–Benson–Bassham (CBB) cycle) were identified. Most of them were associated with the phylum Actinomycetota, especially the genus *Pseudonocardia*. A small proportion of RuBisco genes were also affiliated with Alphaproteobacteria members of the *Nitrobacteriaceae* family.

Two and three genes of the Arnon-Buchanan reverse tricarboxylic acid (rTCA) cycles were identified in the outer and inner zones, respectively. In regard to the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway), three genes were found in the metagenome of the inner sample. Two of the three genes (the anaerobic carbon-monoxide dehydrogenase catalytic subunit and the formate-tetrahydrofolate ligase) were also detected

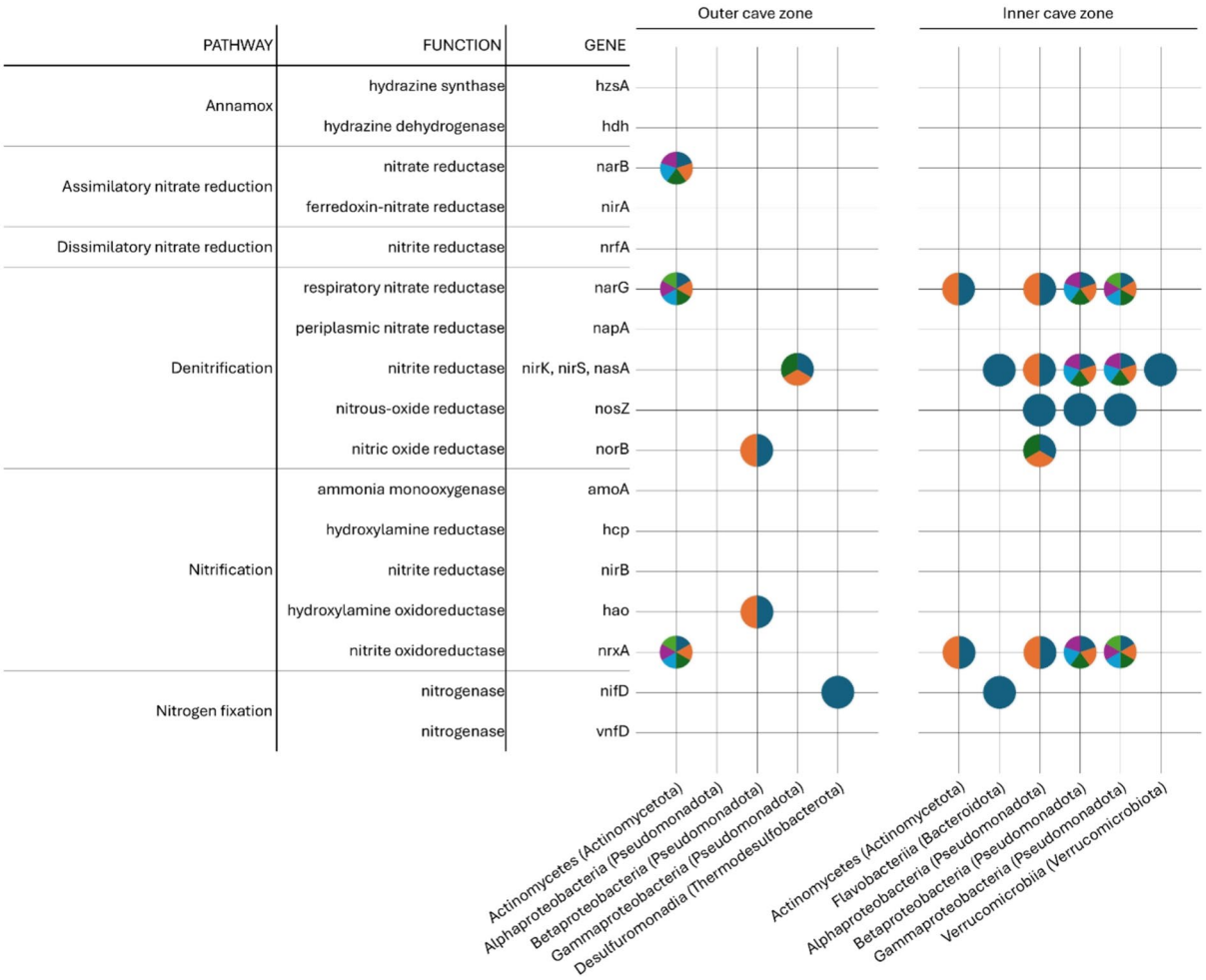

Fig. 4. Presence/absence and taxonomy association of key genes involved in the nitrogen cycle identified in the metagenomes under analysis. The number of colored segments in each pie chart corresponds to the number of the different lower-rank taxa contributing to the gene's presence.

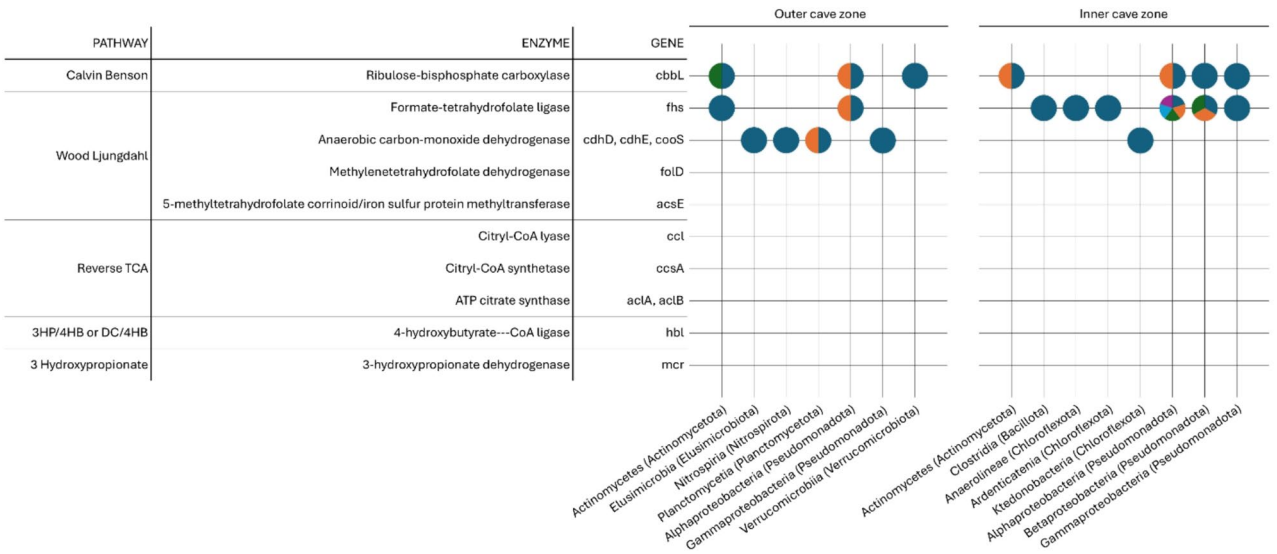

Fig. 5. Presence/absence and taxonomy association of key genes involved in the carbon cycle identified in the metagenomes under analysis. The number of colored segments in each pie chart corresponds to the number of different lower-rank taxa contributing to the gene's presence.

in the outer zone (Fig. 5). In the outer zone, the genes of the Wood-Ljungdahl pathway belonged to the class *Phycisphaerae* of the phylum Planctomycetota and to the phylum Nitrospirota in addition to members of the genus *Pseudonocardia* and the family *Sphingomonadaceae*. In the inner zone, the *cooS* gene of the anaerobic carbon-monoxide dehydrogenase was associated with the phylum Chloroflexota. For the other two genes encoding formate-tetrahydrofolate ligase and methylenetetrahydrofolate dehydrogenase, genus-level associations were obtained with *Sphingomonas*, *Phraetobacter*, and *Methylobacterium* (Supplementary Table S2).

The analysis of the two metagenomes also highlighted the presence of genes involved in the Fuchs-Holo pathway, the 3-hydroxy propionate/4-hydroxybutyrate pathway and the di-carboxylate 4-hydroxy butyrate pathway. Several orthologous genes were found to be potentially involved in carbon fixation pathways in the two cave zones, and these genes were shown to belong to different taxa, in line with the different taxonomic composition of the two samples. For example, the genes of the Fuchs-Holo metabolic pathway in the outer zone were mainly affiliated with the genera *Pseudonocardia*, *Polaromonas*, and *Pseudoxanthomonas* whereas in the inner zone the most represented genera were *Sphingomonas* and *Nitrosomonas* (Supplementary Table S2).

In addition to the genes involved in carbon fixation mechanisms, comparison of genome reads with the KEGG database highlighted the presence of several functional genes involved in the degradation metabolism of cellulose, starch and hemicellulose, such as, cellulose 1,4-beta-cellobiosidase, endoglucanase, alpha-amylase, endo-1,4-beta-mannanase, endo-1,4-beta-xylanase and non-reducing end alpha-L-arabinofuranosidase. Most of these genes in the outer zone were affiliated at the class level with Actinomycetes, Gammaproteobacteria and Betaproteobacteria, while in the inner zone the most represented classes were those of Alphaproteobacteria and Actinomycetes, but other taxonomic classes were also present. Conversely, no functional genes linked to pectin and lignin metabolism were found (Supplementary Table S2).

Some genes encoding enzymes for the degradation of aromatic compounds were also detected in both cave zones. In particular, some of the genes involved in the aerobic degradation of benzoate to catechol, as well as those required for the further oxidation of catechol, were identified. In the outer zone, the *benA* gene coding for the alpha subunit of benzoate/toluene 1,2-dioxygenase was identified, as well as the genes for 1,2 and 2,3-catechol dioxygenase necessary for the meta- or ortho-opening of the aromatic ring of catechol. The *benA* gene was affiliated with the Actinomycetes class, while the two catechol dioxygenases were correlated with the genus *Pseudonocardia*. The metagenome of the inner zone, on the other hand, contains the sequence of the catechol 2,3-dioxygenase gene as well as *catC* and *pcaD* genes, which encode two downstream steps in the ortho-metabolic pathway of catechol oxidation. These three genes were taxonomically assigned with the genera *Sphingomonas*, *Bradyrhizobium* and *Stenotrophomonas*, respectively (Supplementary Table S2).

Biodiversity and metabolic activities of culturable bacteria

Based on colony morphology and pigmentation on R2A plates, a total of 26 bacterial strains (6 from sample C13, 8 from sample C16, and 12 from sample C17) were isolated and taxonomically characterized by 16S rRNA sequencing (Table 2). Most of the isolates were affiliated with the phyla Actinomycetota (13 isolates) and Pseudomonadota (12 isolates), whereas only a single isolate was assigned to the phylum Bacillota (*Bacillus* sp. CA231). Actinobacterial strains mainly belonged to the genera *Pseudarthrobacter* (5 strains) and *Nocardia* (4 strains), followed by *Kokuria*, *Nocardioides*, *Rhodococcus,* and *Arthrobacter* (each with one strain). On the other hand, *Pseudomonas* was the most represented genus among Pseudomonadota, accounting for 8 different isolates, followed by *Ramlibacter*, *Brevundimonas*, *Phenylobacterium,* and *Hydrogenophaga* (one isolate each) (Table 2). The strains CA247 and CA253 showed a percentage of identity with their best blast hit below 98% (respectively *Nocardia coeliaca* NR_104776.1 and *Pseudomonas svalbardensis* NR_197749.1), indicating that they might belong to new species within the genera *Nocardia* and *Pseudomonas*.

The entire strain collection was then screened for the production of extracellular hydrolytic enzymes (i.e., amylase, cellulase, protease, lipase, and urease). As a result, 23 out of 26 isolates exhibited at least one hydrolytic activity. The most represented hydrolytic activities were protease and urease (12 isolates each), followed by cellulase (6 isolates), lipase (5 isolates) and amylase (2 isolates) (Table 2).

Phenotype microarray of the microbiota in the innermost part of the cave

Since the innermost sample C17 harbored the highest number of culturable strains and showed diverse metagenomic functional profiles, a Phenotype Microarray assay was performed using EcoPlates to investigate the functional diversity of the microbial community by evaluating its capacity to utilize 31 different energy sources. After a month of incubation in the dark at 4 °C, the C17 microbial community metabolized approximately 58% of the 31 substrates present in the EcoPlate (Fig. 6). In particular, most of the organic acids and aminoacids included in the plate were efficiently metabolized. Carbohydrates, amine and complex carbon compounds (except for D-cellobiose, D-Mannitol, and Tween-40) were generally used less. This result, together with the high number of bacterial strains from the inner cave zone (C17) showing ureolytic activity (Table 2), supports a high activity of this microbial community towards organic nitrogen compounds.

Discussion

Investigating the microbiological communities within glacier ice layers and ice deposits allows the study of distinct niches, providing valuable clues for reconstructing past climates and understanding the impact of climate change on ice ecosystems[2]. Cenote Abyss is a high-altitude ice cave that serves as a valuable model system for investigating the development of microbial life under psychrophilic conditions and for understanding the impacts of climate change and ice melting on microbial diversity.

In this study, we first provide a complete vertical characterization (taxonomic, metagenomic, and metabolic) of a high-altitude alpine ice cave, offering novel insights into microbial functional pathways, including carbon and nitrogen cycling under low-temperature and poor-nutrient conditions. This comprehensive taxonomic

ID	Sample	Hydrolytic activities					Best Blast Hit (NCBI)		
		Am	Pr	Li	Ce	Ur	Taxonomy	% identity	Accession number
CA230	C13	-	-	-	-	+	*Kokuria palustris*	99.49	NR_026451.1
CA231	C13	-	+	-	-	-	*Bacillus sp.*	99.91	NR_157731.1
CA232	C13	+	-	-	-	-	*Ramlibacter ginsenosidimutans*	98.25	NR_133836.1
CA233	C16	-	+	-	-	-	*Pseudarthrobacter polychromogenes*	99.57	NR_026192.1
CA234	C16	-	+	-	-	-	*Pseudarthrobacter quantipunctorum*	99.4	NR_197727.1
CA235	C16	-	+	-	-	-	*Pseudarthrobacter phenanthrenivorans*	99.77	NR_074770.2
CA236	C16	-	+	-	-	-	*Pseudarthrobacter quantipunctorum*	99.9	NR_197727.1
CA239	C16	-	+	-	-	-	*Pseudarthrobacter polychromogenes*	99.7	NR_026192.1
CA240	C17	-	-	-	+	++	*Nocardia coeliaca*	99.73	NR_104776.1
CA242	C17	-	-	-	-	++	*Nocardia coeliaca*	99.11	NR_104776.1
CA243	C17	+	-	-	+	++	*Brevundimonas bullata*	99.38	NR_113611.1
CA244	C17	-	-	-	+	++	*Nocardioides cavernae*	98.87	NR_156135.1
CA245	C17	-	-	-	+	++	*Rhodococcus qingshengii*	99.91	NR_145886.1
CA247	C17	-	-	-	+	++	*Nocardia coeliaca*	97.49	NR_104776.1
CA248	C17	-	-	-	+	++	*Nocardia coeliaca*	99.72	NR_104776.1
CA249	C17	-	-	-	-	-	*Phenylobacterium haematophilum*	98.51	NR_041991.1
CA251	C13	-	-	-	-	-	*Pseudomonas frederiksbergensis*	98.28	NR_117177.1
CA252	C13	-	-	-	-	-	*Hydrogenophaga laconesensis*	98.78	NR_149183.1
CA253	C13	-	+	+	-	-	*Pseudomonas svalbardensis*	97.28	NR_197749.1
CA254	C16	-	-	-	-	+	*Pseudomonas migulae*	99.57	NR_114223.1
CA256	C17	-	+	+	-	+	*Pseudomonas svalbardensis*	98.62	NR_114223.1
CA257	C16	-	+	-	-	+	*Pseudomonas caspiana*	99.23	NR_152639.1
CA258	C17	-	+	+	-	-	*Pseudomonas caspiana*	98.55	NR_152639.1
CA259	C16	-	-	+	-	+	*Pseudomonas fildesensis*	98.83	NR_170438.1
CA260	C17	-	+	+	-	-	*Pseudomonas caspiana*	99.32	NR_152639.1
CA261	C17	-	+	-	-	-	*Arthrobacter ginsengisoli*	99.6	NR_178602.1

Table 2. Hydrolytic activities and taxonomy of bacterial strains isolated from the cenote Abyss.

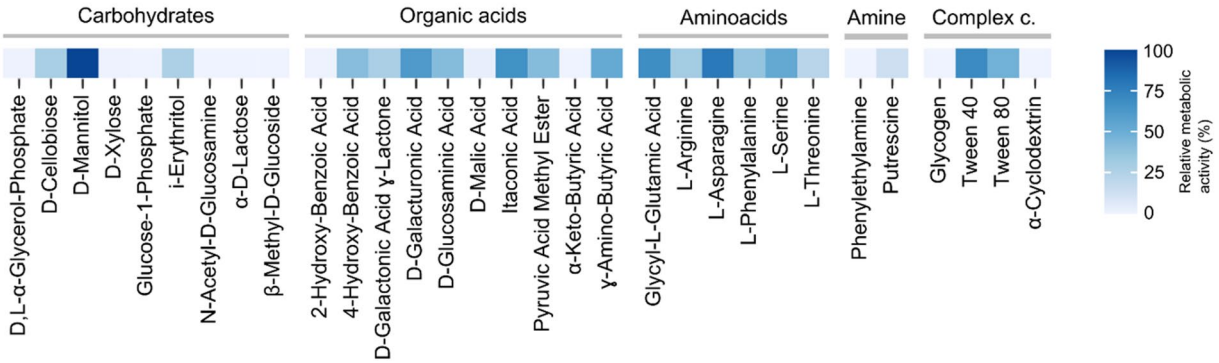

Fig. 6. Heatmap illustrating the relative metabolic activity of the innermost microbial community (C17) in in response to various organic compounds.

and functional characterization is particularly valuable given the challenges in reaching and accessing alpine ice caves, as well as the difficult sampling conditions, which allowed the collection of only small quantities of material.

The microbial community of the Cenote Abyss ice cave exhibited considerable taxonomic and functional diversity, revealing a dynamic microbial ecosystem adapted to oligotrophic and cold environments. The differences in microbial composition between the three samples analyzed, two collected at the outer cave zone (C13 and C16) and one collected at the inner cave zone (C17), underline the microhabitat heterogeneity present even within a relatively confined subterranean environment.

Amplicon sequencing analysis revealed comparable bacterial diversity across the three sampling points along the vertical profile of the ice core (Shannon Index 4.22–4.46). Similar diversity values were reported in other ice

caves, such as the Obstanser Eis-Höhle in the Carnic Alps and the Scărișoara Ice Cave in Romania[2,14]. In contrast, much higher diversity indices were observed in the Alaska Lemon Creek Glacier[28] and alpine permafrost on the Tibetan Plateau[29] (average Shannon index of 8.2 and 6.5, respectively). This might be at least partly due to lower amounts of nutrient influx and other environmental perturbances that are present in ice caves compared to glacier surfaces and permafrost soils.

Despite the similarity in diversity indexes between the samples collected from the Cenote Abyss cave, the microbial community in the inner ice sample C17 differed from those in the outermost zone both in taxonomic composition and in functional genes related to nitrogen and carbon cycling. On the other hand, the functional profile observed in C17 was similar to that described in other oligotrophic caves, such as Monte Cristo[30]. The observation of functionally redundant but taxonomically distinct microbial communities across ice layers highlights a key survival strategy in cold, energy-limited environments, that is, the decoupling of community structure from ecological function.

In terms of microbial composition and functions, Pseudomonadota and Bacteroidota were highly abundant in all the samples, reflecting patterns reported in other nutrient-poor polar and alpine cold environments[31,32]. However, there was a differentiation between the inner and outer samples at lower taxonomy levels of these phyla. Furthermore, C17 was characterized by the higher abundance of members of Nitrospirota and Acidobacteriota, while C13 and C16 showed a high abundance of members of Actinomycetota. This divergence could reflect micro-environmental differences, such as nutrient gradients, water availability, or oxygen levels[7,33,34]. At the genus level, the outermost samples (C13 and C16) were dominated by *Pedobacter* (Bacteroidota) and *Polaromonas* (Betaproteobacteria). *Pedobacter* dominance was previously reported in the Scărișoara and Obstanser caves, and includes psychrotolerant species capable of aerobic, heterotrophic growth across a wide temperature range[35,36]. Similarly, *Polaromonas* species are psychrophilic r-strategists with metabolic versatility, known to contribute to mineral weathering in glacial environments[37–39].

In line with the 16S rRNA sequencing data, the metagenomes of samples collected from the outer zone of the cave revealed that most genes associated with the degradation of complex plant polymers (e.g., cellulose and hemicellulose) were prevalent in members of the Actinomycetes class, particularly the genus *Actinotalea*. These findings underscore the role of Actinomycetota in the breakdown of organic material likely derived from surface plant inputs. *Actinotalea* species, previously detected in the Romanian Scarisoara Ice Cave[7], are well known for their ability to degrade cellulose and hemicellulose, thereby, contributing to nutrient cycling and soil carbon sequestration. Like many Actinomycetota, they are widely distributed in cold ecosystems, including Antarctic and Arctic permafrost, glacial lakes, and river systems[31,32,40]. The outer cave zone also showed a high abundance of the genera such as BSV13 and WCHB1-32 (*Prolixibacteraceae* family of Bacteroidota phylum). BSV13 might be involved in nitrate fermentation, while WCHB1-32 members might sustain organic matter degradation[41,42]. Metagenomic data confirmed the prominent role of Actinomycetes in nitrogen and carbon cycles in these samples. Indeed, most of the genes involved in denitrification (e.g., *narG*, *nirK*, *norB*, *norC*) were affiliated with Actinomycetota phylum. These findings support the hypothesis that in outer ice layers of Cenote Abyss, complex organic matter is metabolized via fermentation and denitrification mostly by Actinomycetota and Bacteroidota members. The metabolites deriving from these metabolic processes could be a source of nutrients for the heterotrophic microorganisms predominantly found in the outermost samples.

The inner cave zone (C17) was dominated by *Nitrospira*, with additional contributions from unclassified members of the *Blastocatellaceae* and *Pedosphaeraceae* families, as well as, the uncharacterized genera MND1 and IS-44 (*Nitrosomonadaceae*). These taxa play a crucial role in the nitrogen cycle. *Nitrospira* species are chemolithotrophic nitrifiers, efficient in nitrite oxidation even at low concentrations. They are commonly found in diverse environments, including biofilms, soil, and subsurface systems[43–45]. Recently, members of the *Nitrospira* genus have been found in caves in various natural environments including Alpine caves[46]. MND1 and IS-44 are ammonium oxidizers, with recent reports identifying them in volcanic and alpine cave environments[46,47]. Additional abundant families in C17 were *Blastocatellaceae* and *Pedosphaeraceae*, the first adapted to oligotrophic and drought-prone conditions[48,49], while the second was capable of degrading complex polysaccharides[50]. Other genera enriched in the C17 sample were *Terrimonas* and *Ferruginibacter*, which are commonly present in various cold natural habitats including a rapidly retreating glacier in the Austrian Alps[51]. These data, combined with metagenomic data from the main marker genes of the nitrogen cycle, including *nosZ* that encodes nitrous oxide reductase, indicate that the microbial community in the inner and most isolated ice layer of the Cenote Abyss is actively involved in the various oxidative and reductive steps of the nitrogen cycle. Efficient interactions can be hypothesized between heterotrophic members of the denitrifying genus *Terrimonas* and nitrifying autotrophs *Nitrospira*, together with members of *Ferruginibacter*, which are capable of hydrolyzing organic matter. Similar associations have previously been reported in studies of microbial communities from domestic water treatment systems[52]. Furthermore, the *fumB* gene (fumarate hydratase, class I) was identified in the metagenome of the inner cave zone. This gene belongs to the *Nitrospira* genus, plays a key role in the citric acid cycle and is involved in the reversible conversion of fumarate to malate.

Interestingly, different taxa contributed to similar nitrogen transformation functions in the outer and inner cave zones, indicating functional redundancy among phylogenetically diverse groups. This phenomenon is frequently reported in extreme environments, where ecological functions are often preserved despite shifts in community composition[53]. Notably, only the inner zone was featured by the presence of ammonia-oxidizing Archaea from the *Nitrosopumilaceae* family[54]. Members of the *Nitrosopumilaceae* family are known to use the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle for inorganic carbon fixation, and several marker genes for this pathway, including acetyl-CoA carboxylase, were found in the C17 metagenome. Additionally, genes for urease, which is required for metabolizing nitrogen-rich organic compounds such as urea, were identified in both metagenomes. Taken together, these findings indicate that microbial communities in the inner layers of Cenote Abyss can perform all the steps of the nitrogen cycle.

Carbon fixation was supported by the presence of genes encoding enzymes from the reverse TCA (rTCA) cycle, such as α-ketoglutarate synthase, and the nearly complete Calvin-Benson-Bassham pathway, including the RuBisCO gene. These findings are consistent with reports from other oligotrophic cave environments, such as the Kartchner Caverns (USA)[55] and Monte Cristo cave (Brazil)[30], highlighting the use of non-photosynthetic CO_2 fixation strategies by subsurface microbial communities.

The coexistence of several incomplete carbon fixation metabolic pathways has been observed in various cold oligotrophic natural environments. A possible explanation is that, in glacial environments with limited energy reserves, even partial metabolic pathways may play a significant role in carbon assimilation. Indeed, several microorganisms are able to utilize incomplete metabolic pathways to fix CO_2, albeit with low energy production[56]. Furthermore, the complexity of the microbial community could favor the association of incomplete metabolic pathways with other processes that can produce additional energy or utilize products generated by incomplete metabolic pathways. Finally, some of the incomplete metabolic pathways may provide the utilization of diverse carbon sources without resulting in complete carbon assimilation[57,58].

Culture-based analysis corroborated the high functional diversity observed in the metagenomic data. The predominance of Actinomycetota and Pseudomonadota among isolates, capable of producing extracellular hydrolytic enzymes such as proteases and cellulases, supports the hypothesis that enzymatic versatility is a critical survival strategy in nutrient-poor subterranean environments. Similar trends were observed in high-altitude glacier soils[59], where enzyme production was linked to the ability to access complex organic substrates. Genera such as *Arthrobacter*, *Pseudomonas*, *Flavobacterium*, and *Bacillus*, also reported in the Obstanser[14] and Scărișoara caves[7], demonstrate the consistent presence of aerobic, chemo-organoheterotrophic bacteria in cold environments. Furthermore, the cellulolytic and amylolytic activity detected in some of the twenty-six isolated strains is consistent with data obtained from metagenomic sequences indicating, the presence of numerous genes linked to cellulose and starch degradation. Similar results were observed in a recent metagenomics study analyzing the microbial metabolism of alpine permafrost on the Tibetan plateau[29]. The detection of genes and cultured strains capable of producing hydrolytic enzymes under cold conditions highlights their potential for biotechnology applications, particularly in bioremediation and industrial processes that require cold-active enzymes.

The diverse functional profile of the C17 microbial community was further supported by Phenotype Microarray analysis, which revealed its capacity to utilize complex carbon-based polymers, including Tween 40 and Tween 80, a range of amino acids and organic acids, and, among carbohydrates, mainly mannitol and D-cellobiose. Comparable patterns have been observed in other studies characterizing different cold habitats[60,61].

Finally, the combined dataset (16S rRNA amplicon sequencing, shotgun metagenomics, metabolic profiling by Phenotype Microarray, and characterization of culturable strains) provides the first comprehensive characterization of a vertical Alpine ice cave microbiome. The Cenote ice cave harbors a complex microbial community along the depth of the cave. Metagenomic data indicate that important metabolic processes related to carbon and nitrogen cycles are present in the different layers of the cave. In the most superficial part, in relation to the relative abundance of the different taxa identified, heterotrophic processes of organic plant polymer transformation (cellulose, hemicellulose) appear to prevail, in addition to mineralization and denitrification reactions. In the deepest part, on the other hand, autotrophic processes of carbon fixation and nitrification appear to prevail, as evidenced by the relative abundance of the genus *Nitrospira* and other microorganisms associated with the oxidation of ammonium to nitrite.

Conclusion

This study analyses the microbial communities inhabiting different depths in the Cenote Abyss ice cave, a unique subterranean habitat characterized by darkness, low temperatures, and limited nutrient availability. Despite these unfavorable life conditions, microbial communities exhibited high taxonomic diversity and metabolic potential. Our results indicate that the microbial ecosystem in this ice cave is driven by aerobic chemo-organoheterotrophy and supported by chemoautotrophic processes and nitrogen cycling. Denitrification predominates in the outer cave regions, while nitrification and carbon fixation appear to be more pronounced in the innermost, oligotrophic zones. The analysis of culturable bacteria and the presence of hydrolytic activities further emphasize the metabolic potential of these ice cave microbes. Although the number of samples analyzed was limited due to logistical constraints, the integration of multiple approaches allowed a comprehensive characterization of microbial communities across three distinct depths of the Cenote Abyss ice cave. This analysis provides first insights into the vertical microbial composition and functional potential of this unique Alpine ice cave, expanding our understanding of microbial survival strategies in subterranean cold environments.

Data availability

The 16S rRNA amplicon sequencing and shotgun metagenomic raw data were deposited in the Sequence Read Archive of NCBI under accession number PRJNA716835.

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Author contributions

SF and DG analysed the data and wrote the manuscript. DG performed DNA extractions, bacterial strains isolation, and 16S rRNA gene sequencing data processing. AF performed shotgun metagenomic data processing. EL performed functional metabolic assays. AR and FS collected the samples. FS organized and funded the scientific expedition to collect and analyse the ice cave samples. MC provided economic resources, supervised the work and revised the manuscript. All authors edited the manuscript and approved the final version.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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