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The World of Cave Microbiomes: Biodiversity, Ecological Interactions, Chemistry, and the Multi-Omics Integration

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Caves are ecosystems and natural heritage sites with magnificent biological diversity, from microorganisms to animals, despite their selective environment for development and survival. Until now, studies on cave microbiomes have included taxonomic classification through metagenomics analysis, demonstrating microbiological heterogeneity mainly composed of prokaryotic organisms or bacteria species. The generally oligotrophic environment, with limited energy input and dark zone, is the main feature differentiating the cave microorganisms from the other microbiomes and resulting in a unique habitat, which has unexplored regard to biology and chemical diversity. Multi-omics integration is a key strategy for scanning and integrating the macro and micromolecular universe, providing a better comprehension of processes and answers of the microbiome in natural cavities. Here, we highlight the strategies employed to study the cave microbiome, from the microbial genomes to their ability to secondary metabolites production, and also the multi-omics integration to explore the chemical and biological diversity in caves.

Keywords: caves, metagenomics, metabolomics, prokaryotes, metabolites, multi-omics

1. Introduction

The cave ecosystem provides rough environmental conditions for microbial survival;[1] however, the substrates from the caves host heterogeneous microbiota with ecological properties.[2,3] Even more, some studies highlighted the importance of the microbial community as key organisms at the base of the subterranean food web and in decomposition processes.[4,5] The microbial groups most relevant in caves are the Fungi kingdom, *Archaea*, and *Bacteria* domain, the last one is more representative and widely reported in the literature.

Once the cave physicochemical properties and environmental variables act on the ecosystem biodiversity, the trophic levels or the light intensity are relevant to determining the division inside a cave and the distribution of microorganisms in the substrates.[6] The oligotrophy and absence of light are the main characteristics of a cave extreme habitat to a living organism.[7] Conversely, recent research shows that some subterranean habitats may not be limited by the energy input[5] since their substrate have a high availability of organic matter through floods and animal excreta (e.g., bat guano). Autochthonous microorganisms from caves have metabolic strategies enabling survival in the highly selective environment for life, hence may be a source of unprecedented microbial bioproducts.

The integration of the system of biology provides different ecological answers to living organisms in the cave environment. Likewise, the cave microbiome may be a potential source for novel organic compounds,[8] especially bacterial species, which are already known for secondary metabolite production. For this reason, the multi-omics integration (genome, transcriptome, proteome, and metabolome) is a strategy to achieve new insights and perspectives about the biology and chemistry of the cave ecosystem. In this perspective, our group has been working

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with different omics models of ecological integration of microorganisms in the environment or among other species[9] and the isolation of metabolites produced by the endophytic microorganism.[10] Recently, we related for the first time the occurrence of a symbiosis mechanism between amphibians and microorganisms.[11,12] Within the framework of cellular adhesion, our study combines the strategy using different omics techniques and biological assay, which allow us to highlight the inhibition of adhesion mechanisms, decreasing bacterial colonization.[13]

Also, we evaluated the chemistry of marine microorganisms,[14] combining the transcriptomics and metabolomics approaches to comprehend the interactions between marine organisms and their microbiota,[15] also the quencher effects of singlet oxygen species by microalgae.[16] In advanced technologies, we develop images to evaluate the interactions between plants and microorganisms.[17] Regarding the ecological interactions of microbiomes in the environment, we began the metabolomics and transcriptomics studies of microorganisms living in substrates with high concentrations of iron ore and dark zones in Brazilian caves.

This literature review brings an overview of the *Bacteria* diversity associated with the cave ecosystem. As well as the high-throughput genomic sequencing to the identification of the microbial taxonomic profile and ecological functions in the caves. On the other hand, metabolomics brings to light microbiome interactions as well as the opportunity to unveil novel metabolic pathways. Other important aspects, like characteristics and variables of the caves, are correlated with the diversity, abundance, and distribution of microorganisms in substrates. At last, the integration of advanced tools may improve our comprehension of the cave microbiome.

2. Cave Microbiome Diversity

Environmental variables and trophic conditions contribute to the uniqueness of the habitat and shape the diversity and abundance of the microbiota in an ecosystem. Regarding the metabolism requirement, the caves microorganisms express functional enzymes as a driving force for catalyzing reactions using minerals or inorganic matter to survive in low nutrient input.[18] Chemolithoautotrophic microorganisms oxidize inorganic molecules such as iron,[19] manganese,[20,21] ammonia,[22,23] methane,[24,25] and sulfur[26,27] which are the most abundant in caves substrates. The metabolic trait of these organisms varies according to the inorganic matter availability and the ecological role in the ecosystem as the biogeochemical process,[28,29] which is involved in a cave's physical structuration maintenance.

Caves as a sink of methane favor the high diversity of methanotrophic microorganisms. The ability of the methanotrophic species to survive and develop in poor nutritional substrate consists of its preference to use carbon from methane molecules as an oxidative energy source.[30] The major microbial groups in caves are the methanotrophs belonging to the *Archaea* and *Bacteria*, as α- and γ-proteobacteria species,[31,32] shown in Figure 1. Both microbial groups have a significant part of unculturable species thus, metagenomic sequencing determines the taxonomic profile and the genes expressing enzymes with ecological functions in caves.

The nitrogen cycle involves essential reactions mediated by the microorganisms, catalyzing a series of reactions in which nitrogen from N_2 atmospheric and ammonia are fixated through its oxidized form as NO_2^- and NO_3^- in the subterranean habitats.[22] In the oligotrophic environment,

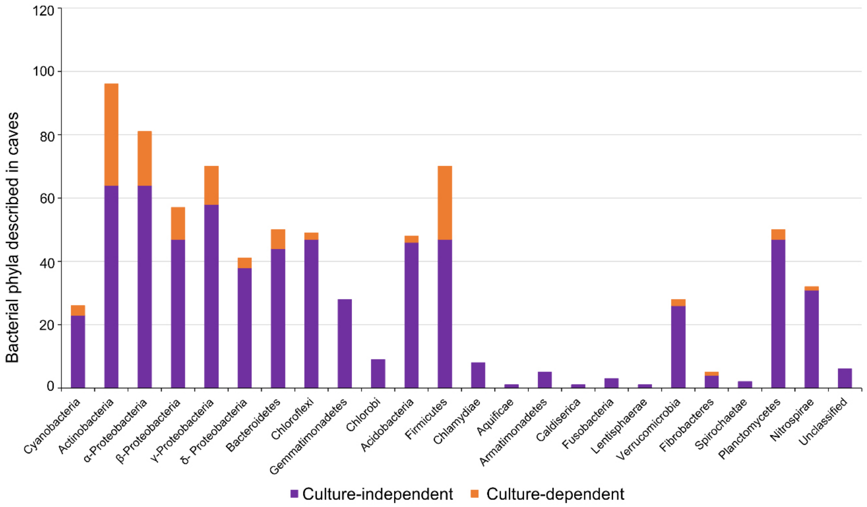


Figure 1. Bacterial phylogenetic profile in caves, the correlation between the culture-independent (violet) and dependent (orange), both using targeted and untargeted metagenomics analysis.

there are biological and ecological markers common in soil and sediment, represented by the specific microbial species with nitrogen-fixing and ammonia-oxidizing capacity,[33] i.e., they use inorganic nitrogen for the development of extremophilic microorganisms.[34-36] The metagenomic analysis using caves samples emphasizes the presence of these microorganisms by the functional enzymes of nitrogen cycle metabolism (Table 1).

Heterotroph microorganisms in caves represent some bacterial groups and the fungi kingdom, which profit from the high organic matter biomass in the substrates, e.g., guano, using organic carbon as an energy source.[37] In the underground system, the heterotrophic species are found in the entrance and twilight zones or according to the trophic levels schematized by Ghosh *et al*.,[6] also confirming the relevance of division into the cave for microbiome studies or the distinction of microbial groups. Heterotrophic metabolism requires high organic input and more mechanisms to sustain the metabolic reactions, such as inorganic sources or light, making them more selective.

In an underground system with extremophile organisms, uncultivable protocols are employed for the determination of widespread microorganisms in nature, nurturing in different mechanisms and energy sources. Next-generation sequencing (NGS) technology is a high-throughput way to identify microorganism taxonomy and differentiate living organisms, and genes prediction involved in ecological functions in a low-cost and fast procedure.

3. Molecular Methodologies to Microbial Taxonomic Classification in Caves

The microbial "dark matter" challenges the awareness of all genomes of living organisms composing an ecosystem. Over the years, NGS methodologies enabled the barcode of the native microbiome constituents through deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) sequences to distinguish among bacteria, archaea, fungi, and algae. The overwhelming majority of living microorganisms in an ecosystem are uncultivable in controlled conditions, somehow affecting the exact taxonomic classification.[38] Thus, the high-throughput sequencing in the genomic field has advanced a significative step to bring to light the microbial "dark matter" and is a breakthrough in cave microbiome studies.

Genomic sequencing involves two strategies using environmental DNA fragments: untargeted and targeted analysis. The shotgun is an untargeted technique that identifies a complex microbial sample without choosing a specific set of microorganisms.[39] The targeted or metabarcoding analysis focuses on the conservative regions of ribosomal RNA, making a more selective sequencing method to determine taxonomic microbial domains.[40] Besides that, the taxonomic profile classification requires a representative database to compare genomic sequences, which is still growing to deposit new genetic sequences of microbial strains.

Metagenomics distinguishes each domain by DNA extraction of all living organisms in the environmental samples to gene sequencing.[41] There are many targets of conserved regions of ribosomal RNA utilized in environmental metabarcoding studies, but the majority of rRNA used in the classification of microbial domains at genus and species levels are 16S-23S rRNA to prokaryotes[42] and 18S rRNA to eukaryotes.[43] And, for fungal DNA barcoding is sequenced the internal transcribed spacer (ITS) regions,[44] revealing the fungi diversity in the environment.

However, we focused on the methodologies using RNA sequencing involving: DNA extraction from samples, followed by polymerase chain reaction (PCR) amplification with good-quality DNA, and the comparison between hypervariable regions of ribosomal RNA and the genomic

Table 1. Enzymes and genes correlated with microbial ecological functions in caves

Gene functional marker	Enzyme	Predictive functions in cave ecosystem
pmoA- amoA, *pmoB- amoB*, *pmoC-amoC*, *mmoX*, *mmoY*, *mmoZ*, *mmoB, mmoC*, *mmoD*, *mxaF/xoxF*, *gmaS*, *mauA*, *mcrA*,	methane monooxygenase (MMO)	oxidation of methane for metabolic reactions
pmoA- amoA, *pmoB- amoB*, *pmoC-amoC*		nitrogen cycle metabolism ammonia assimilation
anfG, *vnfD*, *vnfE*, *vnfK*, *vnfG*, *vnfH, nifH*, *nifD*, and *nifK*		nitrogen cycle assimilation
	RuBisCO	CO_2 fixation
	ATP-citrate lyase	carbon assimilation
	sulfide-quinone reductase (SQR) and Sox system	sulfide oxidation
Quorum sensing		antimicrobial resistance

ATP: adenosine triphosphate.

database.[38] The ribosomal RNA is most used in samples directly collected from caves to analyze the biodiversity in the niche and is applied to the identification of the isolated strain.

The identification of bacteria in caves is successfully processed by the molecular systematic using 16S rRNA or 23S rRNA genomic sequencing[45] through culture-dependent and independent protocols.[46] The 16S rRNA gene sequencing presents high coverage of bacterial groups at the genus and species level in environmental samples widely used in caves phylogenetic investigations in culture-dependent and independent protocols.[47] Figures 1 and 2 show the comparison of bacterial richness using different methodologies. These data were obtained through the original articles contained in the "Clarivate Analytics Web of Science", covering from 2010 to 2023 and the keywords used to research were "cave microorganisms" and "cave microbiomes".

Even the most common phyla in caves-the Actinomycetes-reveals that three different substrates analyzed by both techniques show a decrease in the variability of cultivation methods.[46] In this way, reinforcing the effectiveness of the culture-independent by offering a magnificent overview of free-living organisms widespread in the cave environment and the hugeness of unculturable-or unknown-microorganisms to encourage biological studies. On the other hand, the cultivated-based methods of bacterial species are essential to understanding the entire microbial genome and biology on high throughput and also as a requirement for chemical investigation.

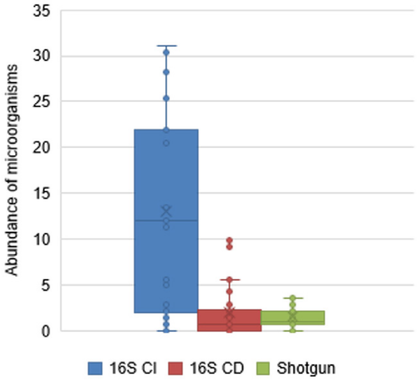


Figure 2. Correlation among techniques to *Bacteria* domain identification in cavities microbiomes, using Next-Generation sequencing. 16S CI refers to the microorganisms identified by the culture-independent method; 16S CD for culture-dependent, and the shotgun for untargeted metagenomics.

Shotgun metagenomics is an untargeted analysis to identification of the metagenomes of all living organisms from an ecosystem, such as bacteria, viruses, fungi, and protozoa. Cave substrates presented a high efficacy to microbial identification in sub-species level resolution without the cultivation and isolation steps and the functional genes shown in Table 1.[48] The drawbacks of the shotgun analysis are the requirement of a large genomic databank, high-biomass samples, and expensive costs.[39] Besides, the library has a limited cover of environmental samples but is better for pathogenic microorganisms and the human microbiome.[48]

The main difference between 16S rRNA and shotgun metagenomic sequencing is the resolution of the taxonomic level in the bacterial community; shotgun provides species-strain level data while the 16S rRNA covers genus and species.[49] The advantage of shotgun metagenomic sequencing over the 16S rRNA analysis, and other metabarcoding targets, is the determination of the ecological functions through the specific genes without any specific microbial target.[34,50]

4. The Bacterial Community in the Ecosystem of the Caves

Prokaryotic cells are highly adapted to inhospitable environments through biochemical reactions enabling survival despite the adversities herein, bacteria domain is widely distributed in oligotrophic substrates and aphotic zone in caves. The main groups described in the cave ecosystem are *Actinobacteria*, α-*Proteobacteria*, γ-*Proteobacteria*, β-*Proteobacteria*, δ-*Proteobacteria*, *Bacteriodetes*, *Acidobacteria*, *Firmicutes*, and others in smaller proportion, shown in Figure 1. Cave features and factors shape the diversity and abundance of bacterial groups, which is related to their genotype, and the phenotype implies metabolic responses.[29] In this way, microbial metabolism is essential to determine the development of particular bacterial species and the ecological interactions, despite the selective environment for life.

The Actinobacteria phylum is ubiquitously and well-adapted to several environmental variables or adversities, besides that, it is a filamentous bacteria which have ecological importance in nature.[51] Buresova-Faitova *et al.*[52] reported autochthonous actinobacterial species in pristine caves, evidencing that the intact and conservative niche is capable of hosting new bacterial strains. Rare microorganisms in caves are indicators of undiscovered genomes to expand the knowledge about the biodiversity in the poorly exploited ecosystem.

The metabolic pathways prediction, through the NGS methods, indicates that actinobacterial species participate in ecologic functions in the niche, e.g., the biogeochemical processes[53] and energy flow equilibrium.[50] These metabolic features make the actinobacteria the most relevant group in the underground ecosystem; besides that, there is biotechnological and pharmacological interest due to their extensive secondary metabolism. The *Streptomyces* and *Nocardia* species are present in the unfavorable environment of caves, which are isolated for chemical studies signaling biological properties in different targets.[54]

The second largest phyla in caves, α-, β-, δ-, and γ-*Proteobacteria*, are Gram-negative bacteria, and it is related to being a possible biomarker in the extremophilic environment because of their metabolism. Autotrophic *Proteobacteria* are widespread on sediment, water, and rock in caves[28,55] since their metabolism enables them to thrive in unfavorable conditions.[56] The methanogenic proteobacteria oxidize methane as a sole carbon and energy source from substrates in caves.[57] Thus, the cave microbiome contributes to the high diversity of proteobacteria species, but there are no studies about the bioproducts, and the cultivation protocols still challenge to deepen the chemical investigations.

The heterotrophs bacteria in caves need high organic matter content, and many of them are pathogenic for humans and may be emerging new diseases. The substrates with high amounts of organic content host heterotrophic species belonging to the *Firmicutes* phylum,[58] which also may be an outcome of local anthropization with the introduction of waste disposal or human microbiota. Otherwise, the *Firmicutes* species, in scarcity of nutrients, use strategies to get energy by interacting with other microorganisms, thus, the result of this mechanism is the identification of enzymes related to antibiotic resistance.[59,60] Despite the pathogenic properties, *Bacillus licheniformis* isolated from caves provide peptides with antibacterial and antifungal effects,[61] showing that *Firmicutes* species also produce bioactive compounds.

In the entrance or twilight zone in caves, the cyanobacteria species with photoautotrophic metabolism may develop,[62] but they require a high trophy level to survive, as well as the terrestrial microorganisms. In addition, groups found in low proportions in caves substrates: *Acidobacteria*, *Bacteriodetes*, *Chloroflexi*, and *Planctomycetes* are well-characterized by shotgun metagenomics and 16S rRNA gene sequencing; on the other hand, there are limited data regarding their culturable bacteria species. Reinforcing that the taxonomic diversity and abundance of the microorganisms are correlated strongly with the environmental variables.

The interactions among microorganisms are favorable to their survival in any niche, as in the cave substrates. Heterotroph species need interaction with autotroph groups to provide energy and survive in scarcity conditions.[63] In other cases, the complex interspecies interaction leads to the biofilm formation and adhesion to the surface, like in walls and rocks, sustained by an extracellular polymeric matrix.[64] The biofilm is a bacterial association showing a high degree of communication among the cells via quorum sensing.[49,65] Due to the high humidity of the cave environment, the biofilm is sampled to identify the bacterial taxonomic diversity and the ecological interaction among other specimens in the microbial network.

The synergism of microorganisms is critical to promote adaptation in oligotrophic conditions and darkness. Therefore, the co-existence of some bacterial species in the community is related to the presence of others, predicted by the functional genes responsible for both metabolic cooperations.[18,28] The interactions among microorganisms in any ecological niche are challenging in controlled conditions. Also, the cultivation protocols commonly involve monoculture to isolate each strain, which may override the existence of other bacteria species. In an extreme environment, such as the caves, the obtention of these microorganisms is even harder.

5. How do Environmental Variables Influence the Cave Microbiome?

The environmental variables affecting the cave ecosystem are divided into two groups: the biotic and abiotic factors. The biotic factors are related to the interactions among living organisms, such as fauna, flora, and microbiota, or biological interactions. The abiotic factors concern the temperature, humidity, pH, or geological composition, which means the physical properties,[66,67] besides the localization and seasons.[68,69] And then, each cave has particular characteristics to provide specific conditions for microbiome development. These variations can, directly and indirectly, affect the diversity and abundance of microbiome constituents. In general, microorganisms have features or acquire mechanisms in answer to the environmental properties of the cave.

The microorganisms are widespread on different substrates such as sediment, guano, wall, rock, wood, water, soil, moonmilk, and others according to environmental conditions. The total organic carbon (TOC) parameter measures the organic content from the substrate, which enables the correlation with the taxonomic profile and their metabolic responses.[70-72] The availability of organic matter offers an appropriate requirement for heterotroph or chemoorganotroph microorganisms' development.[28] In other words, the energy input defines the microbial biomass and composition given the metabolic demand.

The human presence and the fauna habituating caves, mainly bats, deposit organic content in this ecosystem, stimulating the development of selective microbial groups. Guano is an excellent substrate for heterotrophic microbial proliferation, which also recycles the organic content in the substrate[73] despite reports about pathogenicity of guano and promotion of high proliferation of pathogenic microorganisms.[37] Otherwise, the presence of animals is essential to the biogeochemical process and the equilibrium of the environment.

The anthropization process affects the native microbial composition with the insertion of human microbiota, resulting in decreased local biodiversity.[74,75] These microorganisms can modify the cave microbiome, which may interact negatively and cause geological deterioration.[18,76] For this, preservative measures are necessary to avoid the degradation of cave structures or even the loss of genetic heritage.[77] The human intervention modifies the pristine cave microbiome for the presence of pathogenic microorganisms.

Regarding the physical aspects of the ecosystem of the caves, the atmospheric gases inside caves, usually composed of O_2, CO_2, NO_2, N_2O, and NH_3, play a crucial role as an inorganic energy source.[22] As a requirement of microbial metabolism, they catch specific gases to obtain inorganic carbon, nitrogen, and oxygen as a starter for each metabolic cycle. The composition and concentration of atmospheric gases influence the variability of microorganisms along the cave and reinforce their importance for ecosystem maintenance.

The pH variability in substrates influences the distribution of microorganisms, ranging by the microbial affinity to acid or alkaline substrate.[78] Acidic caves favor the development of specific microbial strains in low-pH substrates. This ability is related to the genetic modifications of specific strains belonging to the same genus and directly influences the microbial distribution inside caves.[26] These modifications affect microbial metabolism, showing differences among the populations according to the capacity to resist different pH conditions.[71] The mineral precipitations, such as $CaCO_3$, by the microbiome in caves, depending on the metabolism and pH.[53,79]

Whether the samples are collected in sediment from a wall, ceiling, or rock, there is another parameter commonly measured in a cave environment reflecting the composition and biomass.[72,80,81] Mineralogical composition varies according to the cave formation and directly influences the microbial composition due to the availability of substrate to the metabolic cycle.[82,83] The concentration and variability of minerals may illustrate the distribution of microorganisms along the cave and their capacity to catalyze reactions and get the energy to survive in cave substrates.

The biological and physical parameters are mensurable in caves and are possible to correlate with NGS techniques to identify taxonomy patterns distributed in the ecosystem.[28,84] Despite the ecological contributions of the microbial "dark matter" remaining unknown, Figure 3 summarizes the correlation between cave environmental properties, such as the incidence of light and trophic levels, in the microbiome and their ecological interactions in nature.

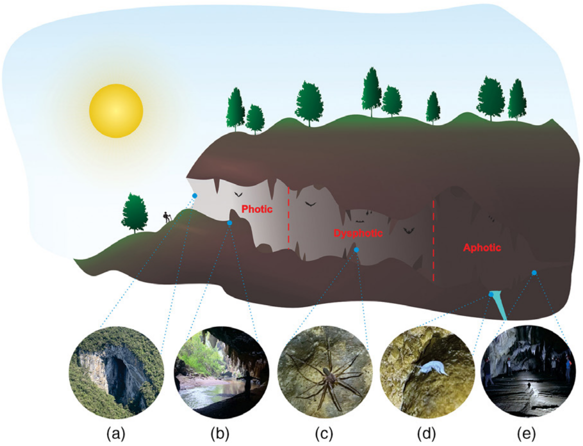


Figure 3. Schematic view inside a cave according to the three zones: the photic (high incidence of light); the dysphotic (transition between the entrance and the dark); and the aphotic (absence of light). (a) Entrance, outside view; (b) entrance, inside view; (c) and (d) fauna hosting in caves ecosystem; (e) deep zone.

6. Ecological Functions of Cave Microbiome

The microbiota is omnipresent in ecosystems, but each microorganism composing the environment has its complexity and dynamic, challenging the comprehension of the microbial network in an ecosystem.[85] Microbial enzymes catalyze reactions to microbiome maintenance, interactions among the organisms, or response to environmental variations,[50] of which are predicted using NGS approaches. The ecological functions of the microbiome in caves depend on the genotype and phenotype of each microorganism, influencing their transcriptome, proteome, and metabolome.

The influences of environmental variables in caves microorganisms are evidenced by the mechanisms of resistance to antibiotics enzyme-mediated, even in locations preserved from the anthropization process.[86] These mechanisms of cave-dwelling microorganisms to survive in the environment reflect their antibiotic resistance, even when compared to terrestrial ones.[59] Enzymatic metabolic changes of microbial population in caves are related to competitive interaction among microorganisms in oligotrophic substrates, which also justify their ecological role in the niche.

The genomic analysis and bioinformatic tools allow the prediction of enzymes with ecological importance in nature, which is widely applied in ubiquitous microorganisms.[28] The functions of bacteria widely discussed and established in natural cavities are geochemical reactions[19,81,87] and atmospheric gas transformation.[22,88] Most pathways prediction in the caves microbiome focused on energy metabolism or mechanism of defense in competition with other microbial groups to assure survival in extreme conditions.[7,89] Table 1 lists specific functional genes and enzymes responsible for the maintenance of cave ecosystems identified by DNA sequencing in metagenomics analysis.

Key metabolic genes (*pmoA*, *mmoX*, *mxaF/xoxF*, and *gmaS*) belonging to methane oxidation, with *pmoA* and *mmoX* are mainly involved in coding methane monooxygenase (MMO) family enzymes, catalyzing methane from the environment in order to get energy.[56] The *pmoA* enzyme is a functional biomarker widely described in methanotrophic microorganisms, able to oxidize the methane from the environment. Over the years, the modulation of the physical structuration of the caves is linked to the methanotrophic activity, being relevant to the ecological role in the ecosystem.

The *Bacteria* and *Archaea* domains biologically mediate the reactions of the nitrogen cycle along the cave, and the abundance and diversity depend on the atmospheric N_2 availability.[90] The main functional biomarkers, *amoA* and *nifDKH* genes, indicate the expression of enzymes capable of converting atmospheric nitrogen into ammonia or nitrogen fixed to profit the living organisms in the cave substrate. In extreme environmental conditions, such as oligotrophy, lack of light, and pH variations, the nitrogen availability in the substrate is the main source of nutrition to the microbiome,[33] justifying the prevalence of the ammonia-oxidizing and nitrogen-oxidizing biomarkers in cave ecosystem.

The *vnf* and *anf* genes are "alternative" nitrogenases in *Bacteria*, helping in the fixation of N_2 in environments with an absence of molybdenum (Mo), but the presence of vanadium (V; *vnf*) or iron (Fe; *anf*).[91] Mo is the main cofactor of *nif* gene and a scarcer and limiting micronutrient in terrestrial environments.[92] Although both nitrogenases are structurally similar to *nif* produced, separate gene clusters encode the *vnf* and *anf*. Also, *vnf* and *anf* have different catalytic properties and perform a more efficient conversion of N_2 in higher temperatures.[92,93] Although *nif* genes are the predominate form in nature, it is common to find microorganisms that have more than one type of nitrogenase, such as *Anabaena variabilis*, a heterotrophic, heterocyst-forming and nitrogen-fixing cyanobacterium (*nif* and *vnf*) *Nostoc* spp.[94] and some other diazotrophs such *Azotobacter vinelandii* (*nif*, *vnf*, and *anf*).[91]

Several pathways and mechanisms correlate to microbe adaptation and survival as a dynamic response of microorganisms in caves, either by quorum sensing genes as a competition among microorganisms, and an interesting side of these events is the formation of bioproducts. Microbial metabolism uses organic and inorganic matter to incorporate the components in their biomass through functional genes and specific enzymes,[70] which also is favorable to studies of the chemical diversity in this ecosystem.

The production of complex metabolites is still untapped due to the limitation of culture-dependent methodologies to procedure the chemical analysis. The main bottleneck is the environmental conditions supplied to microorganism development in oligotrophic substrates. Metagenomics advances have led to determining the taxonomy and their genes capable of producing high-complexity compounds: e.g., Polyketide Synthase (PKS) and Nonribosomal Peptide Synthetases (NRPS) pathways.[6,95,96] Although the chemical analyses of cave-dwelling bacteria are limited, the NGS and transcriptomics research on underground ecosystems indicates metabolic pathways to the production of secondary metabolites.

7. Chemical Studies and Biological Properties of the Isolated Microorganisms

Natural products are the primary source of bioactive metabolites or a scaffold to develop new drugs with regulatory agencies approval and widely commercialized.[97,98] As mentioned before, environmental factors and occurrence influence the microbial phenotype, and cultivation protocols enable the evaluation of metabolite production in reason of their metabolism, such as the PKS and NRPS pathways.[96] The occurrence of new compounds in the cave-dwelling microorganisms leads to the investigation of how microorganisms living in the inhospitable conditions of caves and isolated from substrates far from their origin (e.g., soil and marine environment) may influence the biosynthetic pathway to the production of secondary metabolites.

As an example of other ecosystems, terrestrial or surface environments started with the bacteria as a source for the discovery of bioactive compounds, in which the species from *Actinobacteria* are the common group producer of antibiotics molecules, using a sensing strategy to survive with a complex of microorganisms. The marine microbiome is widely explored for the isolation of bacteria to investigate the promissory source of compounds. Also, the hypogean and inhospitable ecosystem contribute to the research of new microbial genotypes.

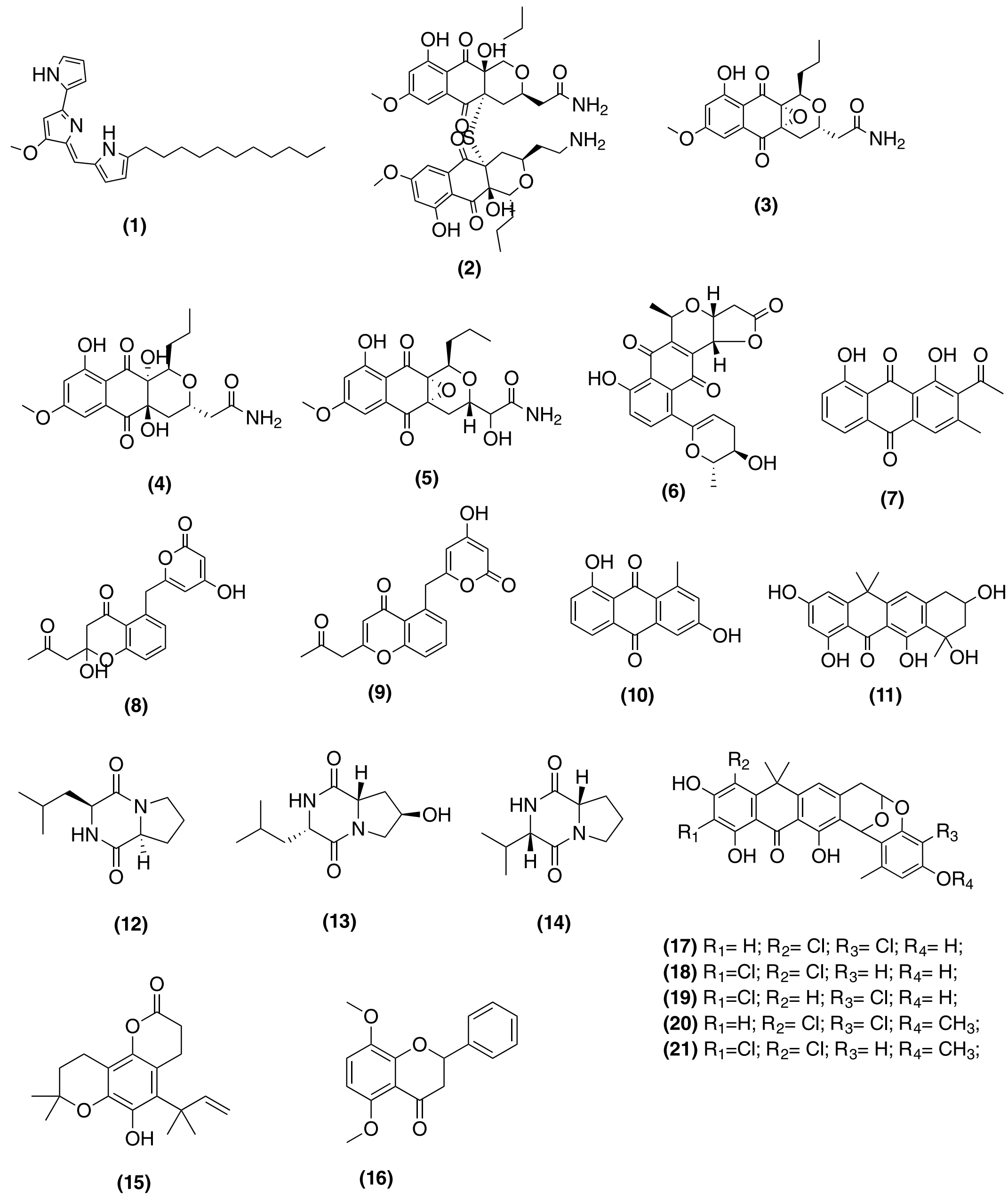
As an innovative source, the cave's extreme substrates host bacteria species able to be cultivated for evaluation of secondary metabolites with biotechnological and pharmacological properties.[8,99] Ghosh *et al*.[6] report the antibacterial activity from *Actinobacteria*, *Proteobacteria*, and *Firmicutes* and summarize the biological effects of another microbial domain. On this wise, the oligotrophic and aphotic environment entails strategies to thrive in caves, as the inter-species interactions commit their metabolic pathway to enzyme expression and metabolites biosynthesis.

As the most predominant and abundant phyla in the cave ecosystem, the *Actinobacteria* species are targets in studies involving bioactive compounds and are considered a source of new antibiotics.[100] The cave-derived actinomycete produces high-complexity compounds with biological activity and varies according to the culture media and methodology.[54] The response of *Actinobacteria* strains under different stimuli, such as co-cultivation, exposure to sub-inhibitory concentrations of antibiotics, and metal supplementation, are strategies to explore their chemical diversity.[101] Thus, actinobacterial species were susceptible to different stimuli in the growing medium that influence the biosynthetic pathways.

Table 2 and Figure 4 list the secondary metabolites from cave *Actinobacteria* according to their biological activity. As well known, antibiotic metabolites have been reported in soil-terrestrial bacteria as the inter-species competition; in this way, specific species produce enzymes belonging to the PKS and NRPS, which in cultivation protocols activate the biosynthesis of secondary metabolites.[96] Besides the antimicrobial compounds, the cytotoxic effects are related to the naphthoquinone derivatives against colon (TCT-1 and Caco-2), lung (A549), ovary (SKOV3), and cervix (Hela) cancer cell lines.[102-104] Researching new scaffolds for cancer treatment is very challenging, and the extreme environment may host distinct microorganisms as a source of bioactive compounds.

Table 2. Secondary metabolites isolated from caves microorganisms

Substrate	Taxonomic classification	Identified compound	Biological activity	Reference
Bacteria domain				
Soil	*Streptomyces* sp.	undecylprodigiosin (**1**)	antioxidant	105
Soil	*Nonomuraea* sp.	hypogeamicin A (**2**) hypogeamicin B (**3**) hypogeamicin C (**4**) hypogeamicin D (**5**)	cytotoxic to colon cell line (TCT-1)	106
Soil	*Streptomyces* sp.	xiakemycin A (**6**)	cytotoxic to tumoral cell lines (A549, MCF-7, HepG-2, HeLa, HCT-116, SH-SY5Y, and PC-3) and antibiotic	104
Moonmilk	*Streptomyces* sp.	cyclodysidin D chaxalactin B		107
Soil	*Streptomyces* sp.	huanglongmycin A (**7**) huanglongmycin B (**8**) huanglongmycin C (**9**)	antibiotic and cytotoxic to tumoral cell lines (A549, SKOV3, Hela, and Caco-2)	103
Sediment	*Micromonospora* sp. *Nonomuraea* sp. *Microbispora* sp. *Streptosporangium* sp.	aloesaponarin II (**10**) hypogeamicin B (**3**) tetarimycin B (**11**) funisamine		101
Sediment	*Streptomyces* sp. ICC1 *Streptomyces* sp. ICC4	diketopiperazines (**12**), (**13**), (**14**) nordentatin (**15**) 2', 5'-dimethoxyflavone (**16**) diazepinomicin	antimicrobial activity	108
Water, moonmilk	*Streptomyces* isolates M2_9, M4_24, and M5_8	4,10-dichloroanthrabenzoxocinone (**17**) 10,12-dichloroanthrabenzoxocinone (**18**) 4,12-dichloroanthrabenzoxocinone (**19**) 4,10-dichloro-3-*O*-methylanthrabenzoxocinone (**20**) 10,12-dichloro-3-*O*-methylanthrabenzoxocinone (**21**)	antibacterial, antifungal, and anticancer activities	109
Moonmilk	*Streptomyces lunaelactis* MM109^{T}	lunaemycin A and derivatives	antibacterial	110



(17) R_1= H; R_2= Cl; R_3= Cl; R_4= H;
(18) R_1=Cl; R_2= Cl; R_3= H; R_4= H;
(19) R_1=Cl; R_2= H; R_3= Cl; R_4= H;
(20) R_1=H; R_2= Cl; R_3= Cl; R_4= CH_3;
(21) R_1=Cl; R_2= Cl; R_3= H; R_4= CH_3;

Figure 4. Selected secondary metabolites produced by cave microorganisms. Table 2 contains each compound associated with the bacterial species; substrates, where the microorganisms were isolated, and their biological effects.

The first step to the prospection of bioactive compounds in caves microbiome depends on the cultivated-based techniques, focusing on the best choice for isolation and fermentation in selective culture medium. The cultivation of cave microorganisms and sampling are challenging and vary according to growth metabolism and culture medium composition. The slow-growing species requires poor culture media, such as the R2A (Reasoner's 2A agar) culture medium,[102] since the fast-growing species may suppress its development in the cultivation. In contrast, microorganisms with rapid metabolism grow in a medium with a high concentration of nutrients.[104] In other cases, when there is a microbial target, the selective medium is employed to prevail the growth of a specific bacteria group, which is applied to studies of *Streptomyces* genera using ISP2 culture medium, a yeast malt extract medium.[111] The strategies to get a high diversity of microorganisms from environmental samples are variations in the culture medium compositions, the incubation time optimization, temperature, and rotation in shaker equipment. All these parameters are essential to reach a good quality of isolated microorganisms in chemical investigations.

After the isolation and fermentation of microorganisms in the liquid medium, the secondary metabolites are extracted from the supernatant through different conditions, such as the liquid-liquid partition between an organic solvent, e.g., ethyl acetate, obtaining the medium polarity compounds,[103,106] or by drying the supernatant and suspension in methanol.[111] The next step is the determination of the composition of the extracts obtained from isolated strains.

Aiming the knowledge of the high diversity of metabolites produced by cave microorganisms, mass spectrometry allows us the annotation compounds based on spectral data or fragmentation patterns.[112,113] For the metabolomics data acquisition, the ionization step is crucial for the detection of analytes, thus, electrospray ionization is widely employed for low molecular weight organic compounds detection,[114] which in turn is highly suitable for the characterization of metabolites produced by microorganisms. The spectrometry data are processed for the annotation of compounds or discovery of new metabolites, comparing in the platform, as the Global Natural Products Social Molecular Networking (GNPS).[112] Lastly, the metabolomics advancements and statistical analysis promote an overview of the microbiome, distinguishing or grouping microorganisms conforming to their metabolomes in different biological and ecological contexts.

8. The Omics Sciences in the Underground Ecosystem

Omics sciences allow the measurement and analysis of biological structure, involving micro to macromolecular analysis, or from genotypes to phenotypes, of a specific individual in the population. Here, we highlighted the biology system for comprehension of a particular microbiome, which consists of the four main areas of the omics field: genomic, transcriptomic, proteomic, and metabolomic. Environmental features affect different degrees in the system of biology. The multi-omics integration provides an overview of a whole ecosystem, even in particular conditions, resulting in comprehension improvement of the biology in the extreme microbiome.[115]

A high-throughput DNA sequencing approach provides taxonomic classification and functional genomes. Metagenomics successfully determines the entire genome of the microbial population, even the uncultivated ones.[39,45] These analyses may provide the taxonomic classification of caves microorganisms and their functional genes involving the metabolic cycle.[50] The section about methodologies for microbial identification, using culture-dependent and independent, reveals the importance of metagenomics to evaluate the impact of microbial cave groups on the local biodiversity and their ecological function in the hypogean ecosystem.

Transcriptomic science enables the assessment of active genes in microbial cells correlating the functions in a particular ecosystem via high-throughput methods. NGS for transcriptome studies provides valuable data about each gene responsible for biological reactions in the metabolic cycle of a set of organisms in the ecosystem at a given time point.[116] Moreover, these active genes are responsible for expressing enzymes to biomolecule production, like the PKS and NRPS pathways to the biosynthesis of secondary metabolites.[95,96] Table 2 shows the functional genes and the corresponding enzymes of their metabolic cycle.

Proteomics evaluates a set of proteins of the organisms and correlates them with metabolic functions and maintenance of cells. Specific genes are responsible for transcript expression to produce intracellular biomolecules.[117,118] The proteomic analysis identifies a microbial strain based on its structural proteins or intrinsic metabolites.[119] In the caves, the application of mass spectrometry using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) for protein characterization provided the taxonomic profile of airborne biomass to compare with the tourism season, reporting the impact of human intervention on the shape of the natural microbiome. These variables assessment with proteomics studies give more information about biodiversity distribution in caves ecosystem, metabolic flux, and ecological function.[120] The genomic, transcriptomic, and proteomic unravel the ecosystem particularities or interactions in the microbial community.

The untapped underground ecosystem converges to research for autochthonous microorganism and their chemical and biological potential in an entire ecosystem. Top-down strategies show the response of the cave microbiome under environmental stressors and the influence on chemical diversity. The chemical profile data enable the distinction of microbiome constituents, outlier identification, and prediction of the relevant chemical markers related to environmental variables. The research of bioactive metabolites in caves microbiome may be assessed by the metabolomics statistical analysis and avoid isolation steps of each compound.[121]

Metabolomics methodologies are employed in natural products research, bringing different answers about the metabolism.[122] In this regard, metabolomics is a magnificent approach to correlating environmental factors with microbial metabolome based on the chemical profile of each individual in the microbiome.[123] The response of cave microorganisms was observed through the biosynthesis of secondary metabolites according to the exposure to stressor stimuli in the cultivable protocols, in which the multivariate statistical analysis pointed out the specific metabolites produced by different conditions.[101,124] The cave conditions shape the microbiome biodiversity and metabolism; therefore, they can provide unprecedented metabolites or new occurrences for drug discovery and offer valuable insights considering the ecology relationships.

The omics sciences are the way to understand the knowledge gap of the microbial community associated with the cave environment.[125] The integration of data multi-omics explores a set of statistical strategies to get information on a specific ecosystem and its effects on the organisms.[126] The multi-omics integration is the pathway to explore in different biological levels the living organisms of the microbiome in natural cavities, also may be the tool for discovering new genomes and bringing to light the challenging world of microbial "dark matter".

9. Multi-Omics Integration as a Way to Illuminate the Cave Bacteria Biology

One of the great challenges when dealing with non-model microorganisms, especially in the case of extremophile specimens such as bacteria living in caves, relies on how to access them from a biological point of view and unravel the molecular potential contained in their secondary metabolism. The identification of this molecular framework is one of the current bottlenecks in this context.[127] In this sense, a systemic approach offers the possibility of considering a new model of study from a holistic point of view. In other words, this type of approach considers the complex interrelationship between the different degrees of organization at the molecular level, offering a solid basis for explaining certain traits observed. The combination of different data sources, especially those obtained on a large scale, such as genomics, transcriptomics, proteomics, and metabolomics, has been used in technological advances and the discovery of new molecules, their respective metabolic pathways of origin, and ecological interactions.[9] The increase in the availability of this type of data is one of the main reasons for the cheapening of omics analyses, especially regarding sequencing techniques and, gradually, the use of mass spectrometry.[113] Of the advantages of using this type of integrative multi-omic approach, in addition to holistic considerations on the study organism, Chauvel *et al*.[128] also highlight the decreased risks of false positives, since different data sources point in the same direction and the possibility of new insights regarding the relationship between molecular interplay and its phenotypic response for a given trait.

Of the possible ways of performing integration, multi-staged analysis and meta-dimensional analysis are the two main approaches to data integration (Figure 5).[129] Multi-staged analysis basically integrates a dataset from a sequential or hierarchical approach. That is, in a linear or hierarchical way, in a sequence of steps, the analysis is divided into several axes where two different scales inscribed in a given time are used to build an explanatory model. In such a way, the association between the different types of data is first sought and, then, the result of this association with the phenomenon of interest. Meta-dimensional analysis is based on the multiple integration of different types of data simultaneously, building a multivariate model associated with a given result. That is, constructing an explanatory model is based on combining results from different scales and merging them. This type of approach, in turn, can be divided into three different strategies: (*i*) concatenation-based integration, which involves the concatenation of all omic data obtained in a given experimental set into a single matrix; (*ii*) transformation-based integration, where appropriate transformations are applied to the data in such a way that they lead to a uniform set of information, allowing the combination into merged datasets; (*iii*) model-based integration, where machine learning models are obtained separately for each dataset before the actual combination.[129-131]

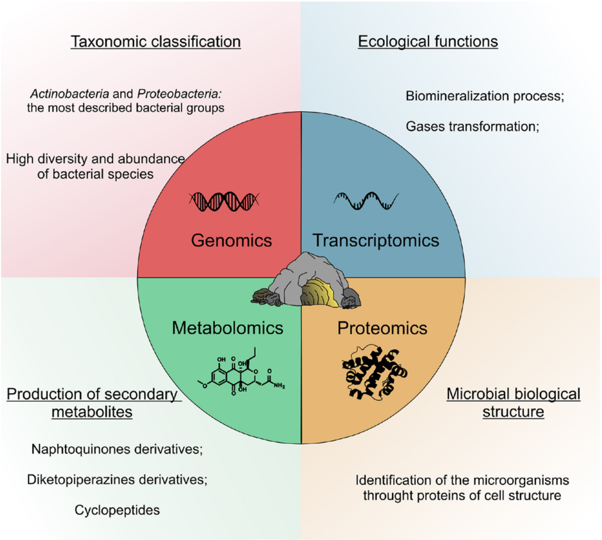


Figure 5. The integration of omics approaches illuminates different aspects of the biology of the cave microorganisms.

The integration of omics data has shown an outstanding potential in the examination of molecular complex underpinnings.[132] Maansson *et al.*,[127] using an integrative approach where they combined metabolomics and genomics data obtained from 13 species closely related to *Pseudoalteromonas luteoviolacea*, discovered new antimicrobial bioactive compounds belonging to the thiomarinols class, which until now had not been described, as well as the identification of the biosynthetic gene cluster for indolmycin, which traditional methods could not predict. In addition to the discovery of new bioactive compounds, as well as their biosynthetic gene clusters, data integration is also a good strategy for the study of microbial interaction.[133,134] Rieusset *et al*.[135] in order to assess the impact of the environment on the secondary metabolism of *Pseudomonas* spp., using cross-data metabolomics integration, were able to show that the production of several secondary metabolites produced by *Pseudomonas* spp. were significantly modulated when such bacteria were cultivated when in contact with plant roots, where this difference favored root growth. Despite such advances in the use of multi-omics approaches in the study of new models, it is noted that a significant part of the papers focuses on two sets of omics data, generally opting for the integration between genomic and metabolomics data.[9] In this sense, it would be interesting not only to use other omics data platforms but also to integrate them in order to present more robust and reliable models for the biological phenomenon of interest. This is because, as was very well presented by Zaramela *et al.*[134] genomics presents what is possible to be happening, transcriptomics presents what is possibly happening, proteomics what makes a given process happen and, finally, metabolomics presents what is actually happening.

10. Conclusions

Since the crescent advances in science and the challenges for the treatment of diseases, new strategies in hard-to-reach environments and integration of omics sciences contribute to investigations of new metabolites biologically active. The exploration of caves microbiome is the source of rare microorganisms or microbial phenotypes correlated to the environment adversities leading to metabolites discovery. The advances in metagenomic analysis led to taxonomic classification in cave ecosystems and correlation with the active functional genes. However, the microbial richness in caves still represents unexplored biodiversity, and it is necessary to improve the genomic field to increase the knowledge about possibly extremophile microorganisms.

There are some bottlenecks to the comprehension of caves microorganisms, and the integration of multi-omics provides new insights into caves studies. The investigation of microorganisms in extreme conditions successfully started with the metagenomic approach to characterize genes and their ecological role. The integration of genomic, transcriptomic, proteomic, and metabolomic is a potential strategy to fulfill and overcome the knowledge gap about cave microbiomes.

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