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Review

Prokaryotic Communities from Pristine Cave Environments: Biotechnological Potential with Sustainable Production

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Abstract: Pristine environments, such as caves, are unique habitats that are isolated from human activity and are exposed to extreme environmental conditions. These environments are rich sources of microbial diversity, and the microorganisms that thrive in these conditions have developed unique survival skills. One such skill is the biosynthesis of secondary metabolites with potential bioactivities, which provide the organisms with a competitive advantage in these extreme environments. The isolation and characterization of microbial strains from the surfaces of pristine cave environments are important for exploring the biotechnological potential of these organisms. These studies can reveal new products with antibacterial, antifungal, anti-inflammatory, antioxidant, and anticancer activities, among others. In addition, the identification of specific compounds responsible for these biological activities can contribute to the development of new drugs and products for sustainable biotechnological applications. Recent developments in genomics, bioinformatics, chemoinformatics, metabolic engineering, and synthetic biology have opened new possibilities for drug discovery, making the exploration of bacterial secondary metabolites more promising. In recent years, several bacteria with bioactive potential have been described, and several compounds with bioactivity have been identified. These findings are essential for the development of new drugs and products for the benefit of society. This paper discusses the potential of microorganisms found in pristine cave surfaces as a source of new metabolites with bioactivity that could have sustainable biotechnological applications. The authors suggest that more research should be conducted in these environments to better understand the microorganisms and the biosynthesis of these metabolites and to identify new compounds and metabolic pathways that could be of interest for the development of new drugs and products. The aim is to highlight the importance of these habitats as a potential source of new bioactive compounds that could be used for sustainable biotechnological applications.

Keywords: anticancer activity; antimicrobial activity; bioactive compounds; microorganisms; pristine environments; sustainable resources

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1. Introduction

Pristine environments can be defined as places with limited or no connections to anthropogenic activities [1]. Sometimes these environments are exposed to one or more extreme environmental parameters, such as temperature, salinity, osmolarity, UV radiation, pressure, or pH, with values close to the limit of life [2]. These are conditions that make survival impossible for most life forms. However, there are microorganisms that have adapted their metabolisms to live in these environments. These ecosystems can be marine or terrestrial, for example, deserts [3,4], arctic sea ice [5,6], and deep sea [4,7,8], where different studies have been conducted to understand how microbial life is possible under these hostile conditions.

Microorganisms living in unique and extreme environments, such as caves, often develop specialized traits and metabolic pathways due to the selective pressures of their environments. These microorganisms have evolved to survive in nutrient-limited conditions, which often results in the production of unique secondary metabolites. These compounds can have a wide range of bioactivities, including antimicrobial, antifungal, antiviral, and anticancer properties [9], and they have the potential to be used in various fields, such as the agriculture, medicine, and food industries [10,11].

The aim of this review is to provide an overview of the various studies conducted in recent years on the identification of microorganisms in primitive environments and the study of potential bioactive compounds produced by these microorganisms, as well as to review the various methodologies used for these studies.

Our ultimate goal is to study microorganisms isolated from marine, Paleolithic, and volcanic caves, with the main objective of finding compounds that have an activity against multi-resistant pathogenic microorganisms that are a public health problem so that they can be an alternative to antibiotics. We are also analyzing the ability of these compounds to inhibit the proliferation of different tumor cells so that in the near future, their use as nutraceuticals and/or adjuvants in tumor therapies can be considered. In addition, we are looking at the antioxidant activity of the compounds, which could be beneficial for health in nutrition, pharmacology, cosmetics, or even used in the food sector. In the area of cultural heritage, we are trying to find sustainable alternatives to produce biocides with activity against microorganisms that degrade cultural heritage in order to apply environmentally friendly products to heritage. Thus, this review allows us to have a broad view of all the studies already conducted and to decide which are the best directions to continue the bioprospecting of compounds for sustainable biotechnological application.

2. Methodology

This study aims to consolidate and review the research results of microorganisms in pristine environments, to show the potential that exists in caves, and to highlight the importance of further studies in these sites. To facilitate future investigations, we will present the methods currently used to identify microorganisms and their potential activities, as well as the identified bioactive molecules produced by microorganisms from primitive environments. The literature published on the subject throughout the 21st century was thoroughly searched in Scopus to find data for this study. The search terms used were “antimicrobial activity”, “anticancer activity”, “bioactive compounds”, “cave”, “microorganisms”, and “primitive environments”. The search was limited to articles written in English and to years of publication of 2000–2023. This allowed the selection of the most interesting articles from the non-specific articles, without omitting those relevant to the review.

3. Pristine Cave Environments

Caves are the most studied pristine environments, which are not uniform environments in terms of geological and geochemical characteristics [12]. Caves are present

worldwide [9], with different origins and characteristics [13]. Caves can be formed by mechanical processes (tectonic caves), differential erosion and scouring (marine and aeolian caves), volcanic processes (volcanic caves and lava tubes), glaciers melting (glacial caves), or rock dissolution (solution caves) [9,14].

In general, caves are divided into four zones on the basis of light penetration: entrance zone, twilight zone, transition zone, and dark zone (Figure 1). The entrance zone is similar to the outside environment and receives full sunlight, allowing photosynthetic life. The temperature varies depending on the outside environment. In the twilight zone, the penetration of light is low, so the activity of photosynthesis stops, and no plant survives in this zone. The temperature remains constant but changes from time to time depending on the weather on the ground. In the transition zone, there is no light, but surface environmental fluxes, such as temperature and humidity, exist. The dark zone is completely dark, and the temperature and humidity remain constant throughout the year [9,15]. The transition zone and the dark zone are oligotrophic (limited in nutrients), although some may be rich in certain minerals either naturally or due to exposure to a nutrient-rich source [16], for example, organic matter present in surface waters or in streams, such as debris, microorganisms, feces, and dead animals [17,18]. These zones are dark ecosystems or have low levels of light and are characterized by low stable temperature, relatively high humidity, low pressure, and low oxygen concentration [19]. In addition, they contain limited materials and have little energy exchange with the environment [20].

Terrestrial caves have microhabitats, such as water, ceilings, floor, moonmilk (deposits of carbonate minerals that occurs within various subterranean systems [20]), and speleothems (stalagmites and stalactites) [21]. In contrast to terrestrial caves, marine caves are not completely isolated from the external environment due to the continuity of the aqueous medium, allowing movement into and out of caves. The mechanical action of the waves may also be an important factor of change [22]. On the other hand, sea caves are much less explored due to access difficulties [23].

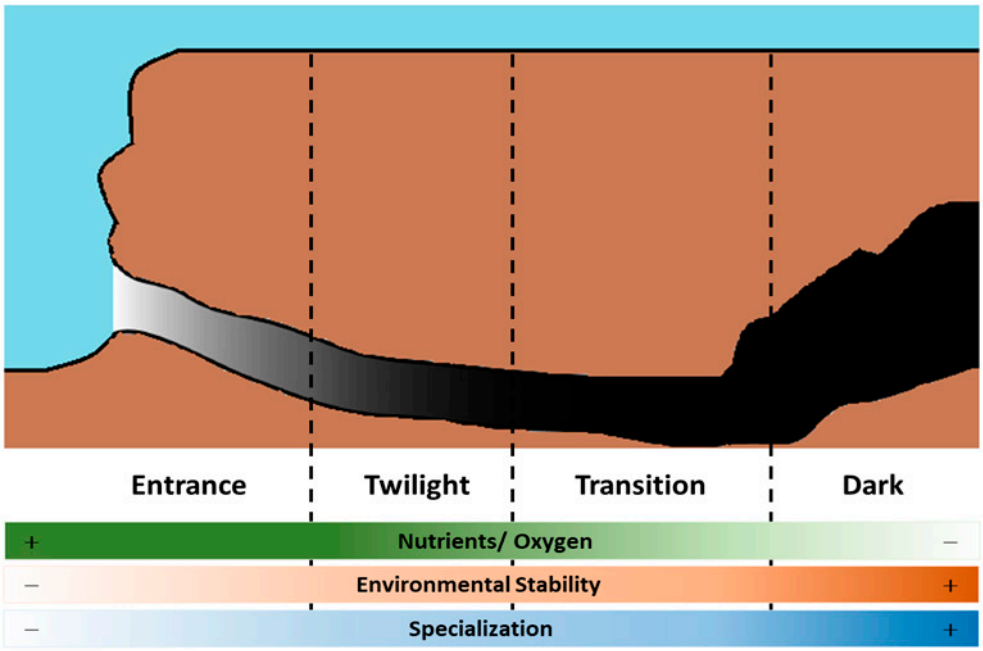

Figure 1. Schematic representation of the different zones in the cave ecosystem (Adapted from [24,25]).

4. Microorganisms in Pristine Cave Environments

Microorganisms are essential to life on Earth and can be found almost everywhere. In pristine environments, through evolutionary change, microorganisms have developed

strategies adapted to such hostile conditions [26]. They have adapted their metabolism to survive in extreme conditions with low levels of elements, such as carbon, nitrogen, and phosphorus, as well as the chemical composition of the surfaces, which directly affect community diversity. This occurs specifically in caves, which are natural geological formations formed by cavities in the rock and are considered extreme and unfavorable living environments due to severe abiotic conditions [27]. Colonization of substrates in caves is not homogeneous [28]. Different groups of microorganisms occupy different ecological niches in different caves, and together with cave fauna and environmental factors, such as carbon dioxide, temperature, and organic matter content, they determine the biotic functions of caves [16]. Microorganisms colonize in host rock and detrital sediments with different compositions and/or structures [28], where minerals act as environmental filters that provide specific microhabitats for metabolically similar microorganisms [29]. Microbial colonization is ultimately a complex and dynamic process determined and controlled by physicochemical characteristics and biochemical factors [28]. Studies on the species composition of microbial populations in pristine environments have revealed a high biodiversity within the Bacteria domain [27].

4.1. Identification of Microorganisms

Identification of microorganisms can be performed using classical microbiology techniques, including culture-dependent [21] techniques. These methods involve the use of normal, oligotrophic, or specialized culture media to count, purify, and identify microbial isolates [16]. However, a major challenge in culture-dependent studies is to find suitable culture conditions for the cultivation of different bacterial species [21]; in addition, this method provides limited information on community structure [30]. Molecular biology techniques have been successfully used in the detection of microorganisms in their environment. Such techniques, based on the detection of nucleic acids, allows the differentiation of microorganisms within complex microbial communities [31]. Detection of microorganisms is usually based on the sequences of the small subunit ribosomal RNA (rRNA) genes (16S for prokaryotes) because rRNA genes are highly conserved and contain a level of variability that allows the identification of microorganisms detected by their sequences and the possibility of performing phylogenetic analyses with their closest relatives. A variety of methods have been used to analyze these sequences, including polymerase chain reaction (PCR)-based fingerprinting methods, such as DGGE (denaturing gradient gel electrophoresis) [32,33], T-RFLP (terminal restriction fragment length polymorphism) [33,34], clone library construction [35], quantitative PCR assays (including those targeting functional genes of interest), sequencing, and the use of stable isotope probing methods [36,37]. DNA sequencing approaches are very useful for phylogenetic identification [38], and more recently, next-generation sequencing (NGS) tools on a variety of platforms, such as Roche FLX 454 pyrosequencing [39], Illumina [40–42], and SOLiD and Ion Torrent PGM [43,44], have been applied to the study of cave microorganisms [16].

New tools for understanding the microbial world have been provided by culture-independent methods [45], such as metagenomics, metaproteomics, metatranscriptomics, and metabolomics, which are fundamental for fully identifying microbial diversity and recognizing its interactions with biotic and abiotic factors [38]. Metagenomics approaches, as functional sequence-based analyses of the collective microbial genomes contained in an environmental sample [46], have also evolved in recent years. In the classical metagenomic approach, environmental DNA was cloned into vectors using ultracompetent host strains. The resulting clone libraries were then screened for either specific marker genes (sequence-driven approach) or metabolic functions (function-driven approach) [47]. Currently, metagenomics typically involves two specific sequencing strategies: amplicon sequencing, most commonly of the 16S rRNA gene as a phylogenetic markers, or shotgun sequencing, which captures the full range of DNA in a sample [41,43,48]. Typically, 16S rRNA gene amplicon sequencing is limited to taxonomic classification at the genus level,

depending on the database and classifiers used, and provides limited functional information [49]. Shotgun metagenomics provides a more robust and reliable assessment of microbial diversity and has the advantage of classifying bacteria at the species and strain level. It also allows the functional relationships between hosts and bacteria to be studied by directly determining the functional content of samples and allows the exploration of previously unknown microbial life that would otherwise remain unclassified. However, the relatively high cost of shotgun metagenomics and more challenging bioinformatics have prevented its widespread use for microbiome analysis [41,43].

Rausch et al. 2019 [41] presented a study to systematically compare the experimental and analytical aspects of the two main technical approaches for microbial community characterization: 16S rRNA gene amplicon (variable regions V1, V2 and V3, V4) and shotgun sequencing. In addition, for each region, a one-step fusion PCR was compared with a two-step procedure, resulting in five different sequence profiles for each sample. The many aspects of bacterial community characterization are consistent when analyzed by different methods.

In another case, an investigation of the taxonomic composition of microorganisms in the Manao Pee Cave soil using high throughput metagenomic sequencing showed results consistent with the 16S rRNA study based on community structure. The shotgun metagenomic sequencing confirmed that Actinobacteria and Proteobacteria were the dominant bacterial phyla in the Manao Pee Cave community. Shotgun metagenomic sequencing provided higher resolution, allowing the detection of more microbial taxonomic profiles than 16S rRNA sequencing, especially of rare microorganisms. For example, at the family level, 123 bacterial families were identified by shotgun sequencing, but only 55 families were detected by amplicon sequencing [43].

To study the biodiversity, activity, and biodeterioration of the microbial populations thriving in the Escoural Cave (Portugal), NGS analyses were performed in different areas of the cave, revealing a predominant distribution of Proteobacteria (58%), Actinobacteria (19%), Firmicutes (7%), Acidobacteria (4%), Bacteroidetes (2%), Gemmatimonadetes (2%), Planctomycetes (2%), and Chloroflexi (1%) [50]. Miller et al. (2022) [4] analyzed DNA samples isolated from the Atacama Desert (Chile). The first (preliminary) microbiological results confirmed the presence of halophilic microorganisms, such as *Salinisphaera* sp. and *Haloparvum* sp., as well as other genera commonly found in saline environments, including *Acinetobacter* and *Pseudomonas*.

The identification and study of microorganisms in caves allows us to understand which species are in the majority and what roles they play in the diversity of these environments. Culture-independent methodologies are very useful in this identification, as they allow for a screening of all microorganisms present in a sample. The culture-dependent methods are interesting in the individual study of microorganisms, allowing their isolation for studies of metabolism and the production of compounds with bioactivity.

4.2. Microorganisms with Bioactivity

Pristine environments preserve large numbers of unstudied bacterial strains with specific metabolic pathways [20]. The unique characteristics of these environments give microorganisms the capacity to develop specific metabolisms and to produce new bioactive compounds with potential activities, such as antimicrobial, antifungal, antiviral, and anticancer [9].

In recent years, several strains isolated from this ecosystems have been proposed as new species based on a polyphasic taxonomic approach comprising chemotaxonomic, phylogenetic morphological, and physiological characterization, such as strain PO-11 (*Arthrobacter cavernae* sp. nov.*)* isolated from Karst cave sediments, Guizhou Province, China [51], strain MM109 (*Streptomyces lunaelactis* sp. nov.) isolated from moonmilk deposit from the cave 'Grotte des Collemboles', Belgium [52], strain SG1 (*Streptosporangium becharense* sp. nov.) isolated in a Saharan soil sample collected from Algeria [3], strain AG31 (*Arthrobacter psychrophenolicus* sp. nov.) isolated in an Alpine ice cave, Austria [53], among many

others. For instance, other bacteria showing important biological activities have also been proposed as new species, such as strains LM 036 and LM044 (*Saccharothrix violacea* sp. nov.), identified for the first time in a gold mine cave, Kongju, Korea. These strains exhibit antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Streptomyces murinus* and exhibit antifungal activity against *Aspergillus niger*, *Candida albicans*, and *Saccharomyces cerevisiae* [54]. Additionally, in Pha Tup Cave Forest Park, Nan province, Thailand, *Nonomuraea monospora* sp. nov. [55] was identified for the first time, exhibiting antibacterial activity against *Bacillus cereus*, methicillin-resistant *Staphylococcus aureus*, and *Paenibacillus lavae* and exhibiting antitumoral activity against KB (human oral epidermoid carcinoma) and NCI-H187 (human small cell lung) cell lines [56]. The strain MBRL 251 (*Streptomyces hundungensis* sp. nov.) isolated from limestone deposit sites, Manipur, India [57], showed antibacterial activity against *Curvularia oryzae*, *Fusarium oxysporum*, *Helminthosporum oryzae*, *Pyricularia oryzae*, *Rhizoctonia oryzae-sativae*, and *Rhizoctonia solani* [58].

NGS and field emission scanning electron microscopy were often used for bacterial characterization. For example, in the identification and the functional and morphological characterization in the lava tube cave, Fuente de la Canaria Cave, La Palma Island, was revealed a predominant abundance of *Proteobacteria* (37–89%), followed by *Actinobacteria*, *Acidobacteria*, and *Candidatus Rokubacteria*. In this study, the ecological role of the microbial communities was also predicted using bioinformatics software that estimated the functional profile from the 16S rRNA gene data, which obtained and predicted the metabolic pathways and enzymes involved in nitrogen, sulfur, methane cycles, and CO_2 fixation [59].

There are many other studies that report microorganisms from pristine environments with important biological activity. Table 1 outlines the bioactivity of various bacterial species from pristine environments. Most of these studies describe bacteria of the phylum Proteobacteria, Bacteroidetes, Firmicutes, and mainly, Actinobacteria.

4.2.1. Proteobacteria

Proteobacteria, the largest and most phenotypically diverse phylum, are Gram-negative bacteria [60] and one of the most abundant phyla in caves [19,61].

4.2.2. Bacteroidetes

Microorganisms of this type are phenotypically diverse, aerobic or facultatively anaerobic chemoorganotrophs, often producing carotenoids and/or flexirubin, which confer yellow or orange colony coloration [27].

4.2.3. Firmicutes

The most members of Firmicutes Phylum are Gram-positive, with low content of guanine and cytosine (G + C) in their genome [62,63]. The phylum is phenotypically diverse [64] and is one of the least robust taxonomic groups, with the taxonomic hierarchy of this phylum remaining weak [62]. Cells may be spherical, may have straight, curved, and helical rods or filaments, and may be with or without flagella and with or without heat-resistant endospores [64]. *Firmicutes* are abundant in soil and aquatic environments, where they participate in the decomposition and recycling of organic matter [62].

4.2.4. Actinobacteria

Actinobacteria are Gram-positive filamentous bacteria with a high content of guanine and cytosine (G + C) in their genome. They grow by a combination of tip elongation and hyphal branching [65]. Most Actinobacteria are saprophytic soil-dwelling organisms that spend most of their life cycle as semidormant spores, especially under nutrient-limited conditions. They are more abundant in soil than in other environments, especially in alkaline and organic soils, where they form a significant part of the microbial population

and are found both on the surface and more than 2 m underground. However, the family has adapted to very different ecological environments: actinomycetes are also found in fresh and salt water and in air [65]. Actinobacteria are particularly known for their potential to produce bioactive compounds, such as antibiotics, antimetabolites, and antitumor agents, with the genus *Streptomyces* having the greatest potential [15]. Approximately 45% of known bioactive compounds are secreted by Actinobacteria, of which 85% originate from the *Streptomyces* genus [66]. Actinobacteria are the most studied in the search for bioactive compounds. Members of Actinobacteria are reported to be a dominant microbial population in several cave ecosystems.

In Shuanghe Cave, China, the dominant phylum was *Actinobacteria* (42.13–48.03%) [30]. In Helmcken Falls cave in Canada (volcanic cave), 400 sample were collected from rocks, wall, sediment, and speleothems inside of cave. Isolates were screened, and most of the tested cave actinomycetes demonstrated antimicrobial activities. The results show that bacteria can be the source of novel compounds that provide precursors of new drugs to combat Gram-negative antibiotic resistant bacteria. This study also suggests a high possibility of finding new antimicrobial agents from previously unknown actinomycetes in volcanic cave habitats [11]. Yücel and Yamaç (2010) [67] isolated 180 actinomycete from Turkish karstic and tested for antimicrobial activity, where 27% exhibited activity only against Gram-negative bacteria and 33% against Gram-positive bacteria. Active cave isolate ratios against overall bacteria, yeasts, and filamentous fungi were determined as 15%, 19%, and 15%, respectively. In another case, in Belgium, different genera (*Agromyces*, *Amycolatopsis*, *Kocuria*, *Micrococcus*, *Micromonospora*, *Nocardia*, *Streptomyces*, and *Rhodococcus*) were isolated from cave milk deposits, and 87% of the bacteria showed activity against Gram-positive and 59% against Gram-negative bacteria [68].

Gonzalez-Pimentel et al. (2022) [42] reported the isolation of two strains of the genus *Crossiella*, likely representing a new species, isolated from the Altamira Cave, Spain. In vitro and in silico analyses showed the inhibition of pathogenic Gram-positive and Gram-negative bacteria and fungi, as well as the taxonomic distance of both strains from their closest relative, *Crossiella cryophile.*

Table 1. Studies of bioactivity of bacteria from pristine environments.

Phylum	Microorganism	Activity	Source	Reference
Actinobacteria	*Actinocorallia aurantiaca*	Antibacterial (*Paenibacillus lavae*)	Phanangkoi Cave, Thailand	[69]
	Actinoplanes brasiliensis	Antibacterial (*Staphylococcus aureus*)	Shuanghe Cave, China	[30]
	Actinoplanes friuliensis	Antibacterial (*Escherichia coli* and *S. aureus*) and Antifungal (*Botrytis cinerea*)	Shuanghe Cave, China	[30]
	Agromyces subbeticus	Antibacterial (*E. coli* and *S. aureus*) and Antifungal (*B. Cinerea*)	Shuanghe Cave, China	[30]
	Arthrobacter psychrolactophilus B7	Antibacterial (*S. aureus*, *E. coli*, *Enterobacter cloacae*, *Pseudomonas CN11*, *Pseudomonas aeruginosa*, and *MRSA)*	Scarisoara Ice Cave, Romania	[70]
	Arthrobacter sp. R-36193	Antibacterial (*P. aeruginosa*) and Antifungal *(Rhodotorula. mucilaginosa)*	Magura Cave, Bulgaria	[19]
	Arthrobacter sp. R4	Antibacterial (*P. Aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Crossiella sp.	Antibacterial (*B. cereus*, *S. aureus*, *E. coli*, *P. aeruginosa*, and *Acinetobacter baumannii*) and Antifungal	Altamira Cave, Spain	[42]

	(*Aspergillus versicolor*, *Penicillium chrysogenum*, *Cladosporium cladosporioides*, *Fusarium solani*, and *Ochroconis lascauxensis*)		
Dietzia natronolimnaea 44860	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *Psudomonas CN11*, *MRSA*, *Enterococcus falcium*, and *Klebsiella 19094*)	Scarisoara Ice Cave, Romania	[70]
Microbacterium ginsengiterrae DCY37	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *Psudomonas CN11*, *MRSA*, and *E. falcium*)	Scarisoara Ice Cave, Romania	[70]
Microbacterium pygmaeum KV-490	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *Psudomonas CN11*, *MRSA*, *E. falcium*, and *Klebsiella 19094*)	Scarisoara Ice Cave, Romania	[70]
Micrococcus luteus CJ-G-TSA7	Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
Micromonospora carbonacea	Antibacterial (*E. coli* and *S. aureus*) and Antifungal (*B. cinerea*)	Shuanghe Cave, China	[30]
Micromonospora chersinia	Antibacterial (*B. cereus* and *P. lavae*) and Anticancer (MCF7 and NCI-H187 cell lines)	Phanangkoi Cave, Thailand	[69]
Micromonospora sagamiensis	Antibacterial (*E. coli* and *S. aureus*)	Shuanghe Cave, China	[30]
Nocardia sungurluensis	Antifungal (*B. cinerea*)	Shuanghe Cave, China	[30]
Nocardioides albus	Antibacterial (*S. aureus*)	Shuanghe Cave, China	[30]
Nonomuraea roseola	Antibacterial (*B cereus*, *MRSA,* and *P. lavae*) and Anticancer (NCI-H187 and KB cell lines)	Phanangkoi Cave, Thailand	[69]
Pseudarthrobacter polychromogenes 20136	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *P. aeruginosa*, *Pseudomonas CN11*, and *MRSA*)	Scarisoara Ice Cave, Romania	[70]
Saccharothrix texasensis	Anticancer (NCI-H187 and KB cell lines)	Phanangkoi Cave, Thailand	[69]
Spirillospora albida	Antibacterial (*B. cereus*, *MRSA*, and *P. lavae*) and Anticancer (NCI-H187 cell line)	Phanangkoi Cave, Thailand	[69]
Streptomyces alboflavus	Antifungal (*B. cinerea*)	Shuanghe Cave, China	[30]
Streptomyces albogriseolus	Antibacterial (*S. aureus*)	Shuanghe Cave, China	[30]
Streptomyces albus	Antibacterial (*E. coli*)	Shuanghe Cave, China	[30]
Streptomyces anulatus	Antibacterial (*S. aureus*)	Shuanghe Cave, China	[30]
Streptomyces aurantiacus	Antibacterial (*E.coli, S. aureus*, and *P. aeruginosa*)	Kotumsar Cave, India	[71]
Streptomyces avidinii	Antibacterial (*Salmonella typhimurium*, *S. aureus*, *E. coli*, *P. aeruginosa*, *Listeria monocytogenes*, and *Listeria innocua*)	12 Portuguese volcanic caves, Terceira Island, Azores	[72]
Streptomyces flavofungini	Antibacterial (*S. aureus*) and Antifungal (*B. cinerea*)	Shuanghe Cave, China	[30]
Streptomyces longisporoflavus	Antibacterial (*E. coli, S. aureus*, and *P. aeruginosa*)	Kotumsar Cave, India	[71]
Streptomyces luridus	Antibacterial (*E. coli* and *S. aureus*)	Kotumsar Cave, India	[71]

	Streptomyces mauvecolor	Antibacterial (*Proteus* sp., *S. typhimurium*, *S. aureus*, *E. coli*, *P. aeruginosa*, *L. monocytogenes*, and *L. innocua*)	12 Portuguese volcanic caves, Terceira Island, Azores	[72]
	Streptomyces nojiriensis	Antibacterial (*Proteus* sp. and *E. coli*)	12 Portuguese volcanic caves, Terceira Island, Azores	[72]
	Streptomyces olivaceus	Antibacterial (*E. coli* and *S. aureus*) and Antifungal (*B. cinerea*)	Shuanghe Cave, China	[30]
	Streptomyces prasinosporus	Antibacterial (*E. coli* and *P. aeruginosa*)	Kotumsar Cave, India	[71]
	Streptomyces roseus	Antibacterial (*E. coli, S. aureus*, and *P. aeruginosa*)	Kotumsar Cave, India	[71]
	Streptomyces sp. 82293	Antibacterial (*M. luteus* and *S. aureus*)	Volcanic cave, Canada	[73]
	Streptomyces spiroverticillatus	Antibacterial (*S. typhimurium*, *S. aureus*, *E. coli*, *P. aeruginosa*, *L. monocytogenes*, and *L. innocua*)	12 Portuguese volcanic caves, Terceira Island, Azores	[72]
	Streptomyces yanii	Antibacterial (*S. aureus*) and Antifungal (*B. cinerea*)	Shuanghe Cave, China	[30]
Proteobacterias	*Acinetobacter* sp. CJ-S-PYD4	Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Candidimonas bauzanensis BZ59	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *Pseudomonas CN11*, *P. aeruginosa*, and *MRSA*)	Scarisoara Ice Cave, Romania	[70]
	Caulobacter henricii 15253	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *Pseudomonas CN11*, *P. aeruginosa*, and *MRSA*)	Scarisoara Ice Cave, Romania	[70]
	Comamonas sp. BM-9_6	Antibacterial (*B. subtilis*, *X. oryzae*, and *P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Delftia acidovorans 14950	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *Pseudomonas CN11*, *P. aeruginosa*, *MRSA*, and *Klebsiella 19094*)	Scarisoara Ice Cave, Romania	[70]
	Micrococcus luteus	Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Obesumbacterium proteus	Antibacterial (*P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas brenneri 97-391	Antibacterial (*S. aureus*, *E. cloacae*, *Pseudomonas CN11*, *P. aeruginosa*, and *MRSA*)	Scarisoara Ice Cave, Romania	[70]
	Pseudomonas fluorescens	Antibacterial (*P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas fluorescens 15834	Antibacterial (*Xanthomonas oryzae* and *P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas fluorescens LMG 14576	Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas fragi	Antibacterial (*P. aeruginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas grimontii 97-514	Antibacterial (*S. aureus*, *E. coli*, *E. cloacae*, *Pseudomonas CN11*, *P.*	Scarisoara Ice Cave, Romania	[70]

		aeruginosa, *MRSA*, *Klebsiella 19094*, and *E. falcium*)		
	Pseudomonas kilonensis DSM 13647	Antibacterial (*B. subtilis*)	Yumugi River cave, New Guinea	[61]
	Pseudomonas migulae NBRC 103157	Antibacterial (*B. subtilis* and *P. aeruginosa*)	Yumugi River cave, New Guinea	[61]
	Pseudomonas plecoglossicida	Antibacterial (*B. subtilis*, *X. oryzae*, and *P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas putida	Antibacteral (*B. subtilis* and *X. oryzae*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas resinovorans ATCC 14235	Antibacterial (*B. subtilis*)	Yumugi River cave, New Guinea	[61]
	Pseudomonas sp.	Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Serratia proteamaculans	Antibacterial (*P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Serratia sp.	Antibacterial (*B. subtilis*, *X. oryzae*, and *P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Serratia sp. 136-2	Antibacterial (*P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Serratia sp. L0305	Antibacterial (*P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Stenotrophomonas sp.	Antibacterial (*P. aeruginosa*)	Magura Cave, Bulgaria	[19]
	Stenotrophomonas sp. DIC6JA	Antibacterial (*P. aeruginosa*)	Magura Cave, Bulgaria	[19]
	Pseudomonas sp.	Antibacterial (*S. aureus*)	Kadıini Cave, Turkey	[13]
Bacteroidetes	*Myroides* sp. IT-2012	Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Sphingobacterium sp.	Antibacterial (*X. oryzae* and *P. aeruginosa*) and Antifungal (*R. mucilaginosa*)	Magura Cave, Bulgaria	[19]
	Sphingobacterium sp. Ag8	Antibacterial (*P. aeruginosa*)	Magura Cave, Bulgaria	[19]
Firmicutes	*Bacillus amyloliquefaciens*	Antibacterial (*P. aeruginosa*)	Magura Cave, Bulgaria	[19]
	Bacillus cereus	Antibacterial (*S. epidermidis*, *B. subtilis*)	Kadıini Cave, Turkey	[13]
	Bacillus eiseniae	Antibacterial (*S. aureus*)	Cave in the Hindu Kush Mountain, Pakistain	[39]
	Bacillus humi	Antibacterial (*S. typhi*)	Cave in the Hindu Kush Mountain, Pakistain	[39]
	Bacillus sp.	Antibacterial (*S. aureus* JE2 and *S. aureus* SH1000)	Rogers Belmont Cave, USA	[74]
	Bacillus sp.	Antibacterial (*B. subtilis*)	Kadıini Cave, Turkey	[13]
	Bacillus thuringiensis	Antibacterial (*S. epidermidis* and *B. subtilis*)	Kadıini Cave, Turkey	[13]
	Bacillus toyonensis BCT-7112	Antibacterial (*S. aureus*, *E. cloacae*, *Pseudomonas CN11*, *P. aeruginosa*, and MRSA)	Scarisoara Ice Cave, Romania	[70]
	Bacillus weihenstephanensis	Antibacterial (*S. epidermidis* and *B. subtilis*)	Kadıini Cave, Turkey	[13]

Brevibacillus borstelensis	Antifungal (*C. albicans*)	Cave in the Hindu Kush Mountain, Pakistain	[39]
Brevibacterium frigoritolerans	Antibacterial (*S. epidermidis* and *B. subtilis*)	Kadıini Cave, Turkey	[13]
Fictibacillus nanhaiensis	Antibacterial (*S. typhi* and *S. aureus*)	Cave in the Hindu Kush Mountain, Pakistain	[39]

While many studies have been conducted to identify bioactivity in cave bacteria, some of them do not identify the compounds that have activity. This can be due to a variety of reasons, such as the complexity of the microbial community in caves or the limitations of the analytical techniques used. However, identifying the specific compounds that have activity is crucial for further research and development of potential applications. It would be beneficial for future studies to focus on identifying and characterizing the bioactive compounds produced by cave bacteria, as this can lead to a better understanding of their potential uses and applications.

5. Potential for Bioactive Compounds Production

Microorganisms in complex ecological niches with limited nutrients biosynthesize secondary metabolites with activity to give them an advantage over others [75,76]. Secondary metabolites are adaptive molecules that have evolved for purposes other than primary metabolism [77]. Unlike primary metabolites, they are produced by individual species or genera for specific physiological, social, or predatory reasons; therefore, these compounds are closely related to the ecology of the producing organism [78]. These molecules are structurally and chemically diverse and can have different activities [10]. This competition among microorganisms in pristine environments may favor the synthesis of bioactive compounds that inhibit the growth of competitors (or predators), which can have a possible action on the growth of other cell types, such as cancer cells. In caves, where there are animals (such as rodents, reptiles, birds, arthropods, amphibians, and especially bats) that are the reservoirs of viruses in these ecosystems, the contact among microorganisms and animal excrements, which could be composed of some pathogenic virus, promotes the production of antiviral agents by microorganisms [9]. Many of these compounds have no terrestrial analogues and are unique in terms of chemical structure and biological activity [79]. Elucidating the nature of molecular signals, their targets, and the pathways underlying their production is an essential prerequisite for interpreting inter-kingdom communication, adaptive responses, and systems biology [26].

Traditionally, novel compounds produced by bacteria have been discovered through conventional bioprospection based on isolation of potential producers and screening their extracts in a variety of bioassays [80]. However the culture medium used affects the production of secondary metabolites [15]. Therefore, depending on the medium used and the culture conditions, the synthesized metabolites can be different. Strains need to be grown on different media and under different conditions, including formulations that mimic environmental conditions [81]. Axenov-Gribanov et al. (2016) [20] conducted a study in which they analyzed the antimicrobial activity of strains isolated when grown on different media, and the results showed differences in the bioactivity of the extracts, indicating that different media give rise to the production of different compounds.

Bérdy (2005) [66] conducted a comprehensive review of how bioactive compounds are isolated and identified, describing that the methods used can be robotic and automated, such as chromatographic methods (LC-MS (Liquid Chromatography–Mass Spectrometry), LC-MS-ELSD (Liquid Chromatography–Mass Spectrometry with Evaporative Light Scattering Detector), LC-NMR (Liquid Chromatography Nuclear Magnetic Resonance), HPLC-UV-Vis (High Performance Liquid Chromatography UV-Vis), HPLC-ELSD (High Performance Liquid Chromatography with Evaporative Light Scattering Detector),

HPLC-PDA (High Performance Liquid Chromatography with Photodiode Array Detection), and HPCCC (High Performance Countercurrent Chromatography)) and spectroscopic techniques (Multi-dimensional NMR (Nuclear Magnetic Resonance), X-Ray Crystallography, NOESY (Nuclear Overhauser Enhancement Spectroscopy), Electrospray MS (mass spectrometry), and HRMS (High-Resolution Mass Spectrometry)).Alternative methods for the detection of secondary metabolites include target assays based on enzyme or receptor inhibition. These are based on the strong correlations among metabolites, biological activity, and the target [81].

Isolation and characterization of microbial strains from pristine environments, including their antibacterial, antifungal, anti-inflammatory, antioxidative, and anticancer activities, and identification of some specific compounds responsible for such biological actions are essential to envision new products and explore the biotechnological potential of these organisms. Table 2 systematizes a list of identified compounds produced by bacterial species isolated in pristine environments. The molecular structures of the compounds are shown in Figure 2. These new compounds can be applied as bioprotective agents in heritage assets and civil construction, preventing the biocolonization of surfaces and in several other features of biotechnology in the food, medical, and pharmaceutical industries.

Table 2. Compounds produced by bacteria isolated from pristine environments.

Compound	Isolation and Identification Technique	Activity	Microorganism	Source	Reference
Antibiotic R2 (**1**)	Exclusion chromatography; RP-HPLC NMR HMBC	Antibacterial (*Bacillus subtilis*, *Escherichia coli*, *Listeria monocytogens*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Pseudomonas fluorescens*, and *Staphylococcus aureus*) and Antifungal (*Aspergillus carbonarius*, *Candida albicans*, *Mucor ramannianus*, and *Saccharomyces cerevisiae*)	*Streptosporangium* sp. *Sg3*	Saharan soil sample collected from Béni-Abbès, Béchar [3]	[82]
Atacamycins A-C (**2**)	HPLC-UV/Vis RP-HPLC LC-MS MS NMR	Anticancer (BXF 1218L, DIFI, LXFL 529L, MAXF 401 NL, MEXF 462NL, 22Rv1, UXF 1138L, and RKO cell lines)	*Streptomyces* sp. *C38*	Hyper-arid soil collected from Atacama Desert, Chile	[83]
Cervimycin A-D (**3**)	RP-HPLC NMR HPLC-UV/Vis HRMS	Antibacterial (*B. Subtilis*, *S. aureus*, and *Enterococcus faecalis*)	*Streptomyces tendae*	Cave Grotta dei Cervi, Italy	[84]
Chaxamycins A-C (**4**)	NMR HR-MS HPLC-MS	Antibacterial (*B. Subtilis*, *L. monocytogenes*, and *S. aureus*)	*Streptomyces* sp. C34	Hyper-arid soil collected from Atacama Desert, Chile	[85]
Chaxamycin D (**5**)	NMR UV/vis spectrometer HPLC-MS	Antibacterial (*E. coli* and *S. aureus*)	*Streptomyces* sp. C34	Hyper-arid soil collected from Atacama Desert, Chile	[86]

	RP-HPLC X-ray				
Huanglongmycin A (**6**)	HRMS NMR RP-HPLC HPLC-UV/Vis	Anticancer (SKOV3, HeLa, and Caco-2 cell lines)	*Streptomyces* sp. CB09001	Soil of karstic cave in Xiangxi, China.	[87]
Hypogeamicin A (**7**)	NMR HRMS RP-HPLC X-ray	Anticancer (TCT-1 cell line)	*Nonomuraea specus*	Soil of Hardin's cave, Ashland, Tennessee	[88]
Hypogeamicins B–D (**8**)	NMR HRMS RP-HPLC X-ray	Antibacterial (*B. subtilis*)	*Nonomuraea specus*	Soil of Hardin's cave, Ashland, Tennessee	[88]
Napyradiomycins (A1, 18-hydroxynapyradiomycin A1; A2; 16-oxonapyradiomycin A2; 4-dehydro-4a-dechloro-16-oxonapyradiomycin A2; B3; 4-dehydro-4a-dechloro-napyradiomycin B3) (**9**)	RP-HPLC HR-MS NMR	Antibacterial (*Cobetia marina*, *Phaeobacter inhibens*, *Pseusooceanicola batsensis*, and *M. luteus.)*	*Streptomyces aculeolatus* PTM-420	Desertas Island in Madeira, Portugal	[89]
Napyradiomycins (SF2415B3, 4-dehydro-4a-dechloro-napyradiomycin SF2415B3; A80915A; A80915C; 4-dehydro-4a-dechloro- napyradiomycin A80915A) (**9**)	RP-HPLC HR-MS NMR	Antibiofilm (*Marinobacter hydrocarbonoclasticus*, and *C. marina*)	*Streptomyces aculeolatus* PTM-029	Desertas Island in Madeira, Portugal	[89]
Undecylprodigiosin (**10**)	LC-MS HPLC-	Antimicrobial (*M. luteus*, *B. subtilis*, and *C. albicans*) and Antioxidant	*Streptomyces* sp. JS520	Soil Cave on mountain Miroc in Serbia.	[90]
Xenocylion B (**11**)	NMR X-ray	Antioxidant	*Streptomyces* sp. CB09001	Karstic cave in Xiangxi, China	[91]
Xiakemycin A (**12**)	HR-ESI-MS NMR HPLC-UV/Vis	Antibacterial (*S. aureus* (MSSA and MRSA), *Staphylococcus epidermidis* (MSSE and MRSE), and *E. faecalis* (VSE and VRE)), and Anticancer (A549, MCF-7, HepG-2, HeLa, HCT-116, SHSY5Y, and PC-3 cell lines)	*Streptomyces* sp. CC8-201	Soil of Karst cave, Chongqing, China	[92]
Mixture of compounds (4,10-dichloroanthrabenzoxocinone; 10,12-dichloroanthrabenzoxocinone; 4,12-dichloroanthrabenzoxocinone; 4,10-dichloro-3-*O*-methylanthrabenzoxocinone; and	LC-MS HPLC	Antibacterial (*B. subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* (MRSA), and *Salmonella enterica*), Antifungal (*Candida*	*Streptomyces* sp. M4_24, and M5_8	Caves Tatra Mountains, Poland	[93]

10,12-dichloro-3-*O*-methylanthrabenzoxocinone) (**13**)		*glabrata*, *Candida dubliniensis*, *C. albicans*, and *Candida guilliermondii*), and Anticancer (T47D cell line)			
Mixture of compounds (Cyclodysiden D; Chaxalactin B 14-Deoxy; Stylissazole B; Gyrophoric acid (4-Me ether; L-alanine amide)) (**14**)	HPLC LC-MS	Antibacterial (*B. subtilis*, *E. coli*, and *Pseudomonas putida*) and Antifungal (*C. albicans*)	*Streptomyces* sp. IB 2014/I/78-8	Moonmilk from Karstic Cave in Siberia, Russia	[20]

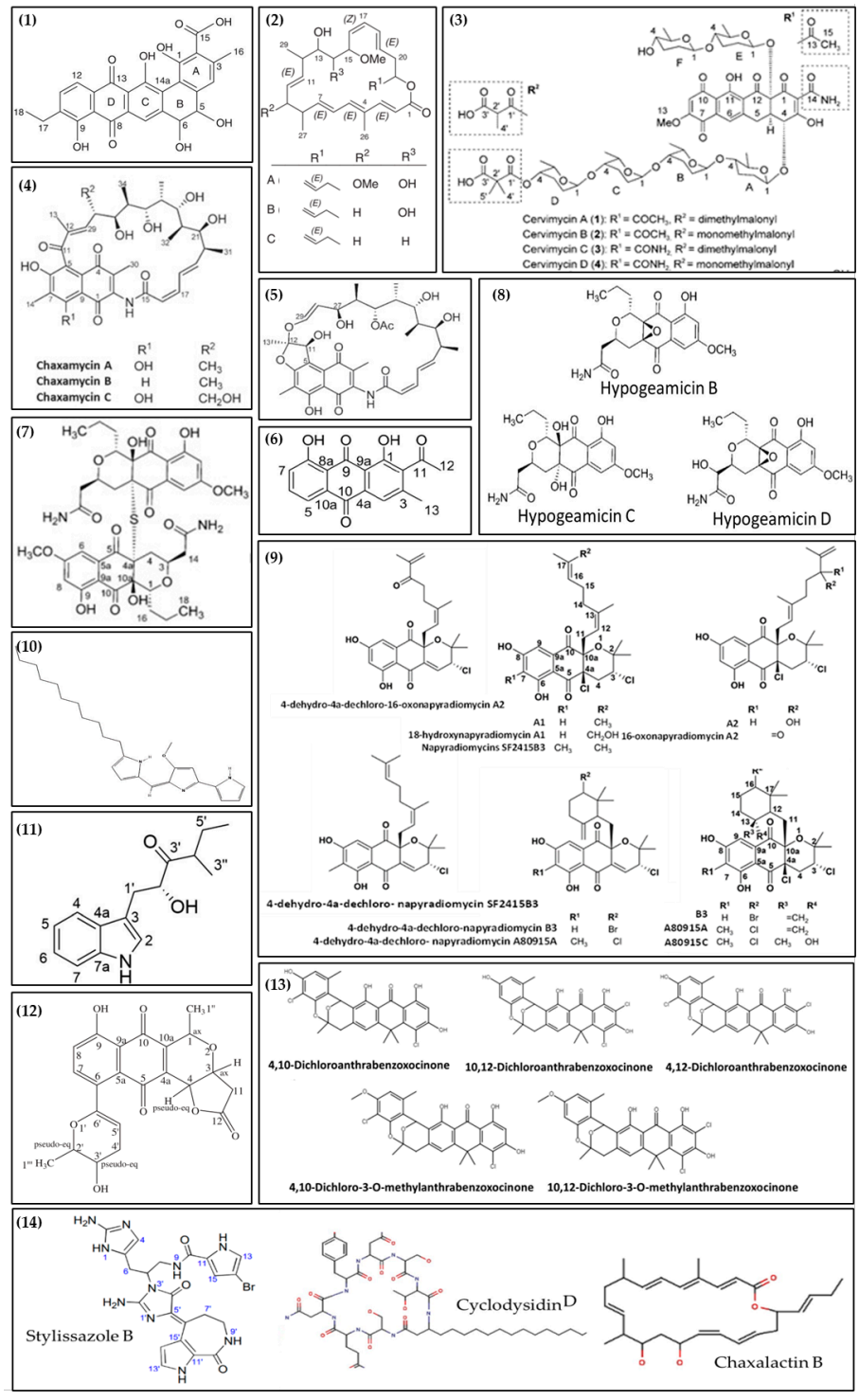

Figure 2. Molecular structure of the compounds in Table 2: (**1**)—Antibiotic R2 [82]; (**2**)—Atacamycins A-C [83]; (**3**)—Cervimycin A-D [84]; (**4**)—Chaxamycins A-C [86]; (**5**)—Chaxamycin D [86]; (**6**)—Huanglongmycin A [87]; (**7**)—Hypogeamicin A [88]; (**8**)—Hypogeamicins B–D [88]; (**9**)—Napyradiomycins (A1, 18-hydroxynapyradiomycin A1; A2; 16-oxonapyradiomycin A2; 4-dehydro-4a-dechloro-16-oxonapyradiomycin A2; B3; 4-dehydro-4a-dechloro-napyradiomycin B3; SF2415B3, 4-dehydro-4a-dechloro- napyradiomycin SF2415B3; A80915A; A80915C; 4-dehydro-4a-dechloro- napyradiomycin A80915A) [89]; (**10**)—Undecylprodigiosin [94]; (**11**)—Xenocylion B [91]; (**12**)—Xiakemycin A [92]; (**13**)—4,10-dichloroanthrabenzoxocinone; 10,12-dichloroanthrabenzoxocinone; 4,12-dichloroanthrabenzoxocinone; 4,10-dichloro-3-O-methylanthrabenzoxocinone; and 10,12-dichloro-3-O-methylanthrabenzoxocinone [93]; **14**—Cyclodysiden D; Chaxalactin B 14-Deoxy; Stylissazole B [20,95].

New Technological Advances

Recent developments in genomics, bioinformatics, chemoinformatics, metabolic engineering, and synthetic biology have opened up entirely new possibilities for drug discovery and have revived interest in bacterial secondary metabolites [80]. The potential for natural product discovery has increased drastically due to improvements in genome sequencing technologies and the power of advanced computational analyses of DNA sequences, allowing the development of new techniques, such as metagenomics, metatranscriptomics, metabolomics, and metaproteomics [38]. Combined with novel methods to isolate rare, previously uncultured bacteria, metagenomics can provide important insights into the metabolic/nutritional requirements of specific bacteria by analyzing biosynthetic genes that are thought to specify the biosynthesis of novel secondary metabolites; this information can be used to design specific growth media and conditions that allow bacteria to grow and synthesize secondary compounds of interest [80].

Metagenomics, which generates large amounts of sequencing data, makes it possible to reconstruct whole genomes, allowing not only the sequencing of complete genes and pathways but also the construction of evolutionary trees. The combination of deep sequencing and bioinformatics approaches allows metagenome-based genome reconstruction of even very complex systems [96]. In this way, numerous new biosynthetic gene clusters (BGCs) and enzyme domain sequences have been identified [48]. For instance, a study in two different coastal mangrove ecosystems in southern China revealed a total of 3622 BGC secondary metabolites in only 761 gene clusters (21.01%) encoding 174 different bioactive compounds. This finding indicated the existence of many new unknown bioactive compounds to be discovered. Most of these compounds, such as carotenoids, flexirubin, ectoin, and rhizomide, have been reported to have pharmacological functions, such as antioxidant, antimicrobial, enzyme stabilizer, or anticancer activities. This BGC data set may be of interest in exploring candidate gene clusters for antibiotic and antitumor activity [97]. In another study, the taxonomic composition and metabolic potential of microorganisms from Manao Pee Cave, an underground limestone cave in the western part of Thailand, was investigated using high-throughput shotgun metagenomic sequencing. *Actinobacteria* (51.2%) and *Proteobacteria* (32.9%) were the most abundant phyla in the cave soil community. Other bacterial phyla were also identified, but they were much less abundant, namely *Bacteroidetes* (3.9%), *Fimicutes* (3.7%), *Acidobacteria* (1.8%), *Planctomycetes* (1.6%), *Chloroflexi* (1.1%), *Gemmatimonadetes* (0.6%), and *Cyanobacteria* (0.5%). Metabolic potential analysis was performed by mapping reads to the Kyoto Encyclopedia of Genes and Genomes (KEGG), and deeper analysis of the metabolism function module revealed the relative number of genes involved in biosynthesis of secondary metabolites corresponds to 1.6% (e.g., streptomycin, novobiocin, and isoquinoline alkaloid biosynthesis). This study suggested that unique bioactive molecules with promising activity in medical and industrial processes may be obtained from Manao Pee Cave [43].

Metaproteomics, which involve the large-scale identification and quantification of proteins from microbial communities, provides direct insight into microbial phenotypes at the molecular level [98]. Through metaproteomics, it is possible to identify the function and expression of different proteins present in the community [99]. The main challenges

in metaproteomics include low protein yields, low peptide identifications, and database problems [100]. On the other hand, metatranscriptomics focuses on the global expression of RNA in the microbiome and can be employed to study the regulation of gene expression at the transcription level, which makes it possible to further study the function and metabolic pathway. Metatranscriptomics not only identifies the genetic content of the microbiota but also reveals details about transcriptionally active genes [100]. However, metatranscriptomics is faced with some problems caused by the short half-life of mRNA, enzymatic degradation of mRNA, and difficulty in detecting responses to environmental stimuli. The presence of mRNA is not always synonymous with the presence of protein. Metabolomics provides a complete image of microbial metabolism. Metabolomics can be used to assess the global metabolite profile (untargeted approach) or to measure specific metabolites (targeted approach). Targeted metabolomics analysis detects a predetermined set of metabolites, usually selected by proximity to the biological sample being analyzed or from metabolite libraries in software databases [100]. The main analytical techniques used to collect metabolomics datasets are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) because of their ability to detect small molecules and the unique advantages of each analytical instrument [101].

The evolution of analytical methods in recent years has allowed significant advances in knowledge of pristine environments, the microbial life that inhabits them, their relationships to the ecosystem, and their potential. The discovery of new compounds produced by microorganisms in this environment represents a unique opportunity to produce new, valuable compounds that are safe, environmentally friendly, cost effective, and sustainable. It is expected that the potential of pristine environments will be better understood in the near future.

6. Conclusions and Future Directions

The caves represent a unique ecosystem in terms of physical, chemical, geological, and biological characteristics. The conditions found in these ecosystems are close to the limits of life, so the microorganisms living there have adapted metabolism. This adaptation, which originated thousands of years ago, gives rise to the production of unique secondary metabolites that can be of interest today. Advances in techniques for identifying microorganisms and analyzing the compounds they produce have revolutionized our understanding of microbial diversity and metabolism. In the past, many microorganisms and their metabolites went undetected because they were difficult to isolate and culture in the laboratory. However, with the development of new technologies, it is now possible to identify microorganisms and their metabolites without the need for cultivation.

These approaches allow analysis of genetic material, gene expression, and protein function, providing a more comprehensive understanding of microbial diversity and metabolism. As a result, new bacterial species and new metabolic pathways that were previously unknown have been discovered. These discoveries have important implications in fields, such as biotechnology, medicine, and environmental science. The discovery of new bacterial strains in caves shows that these environments are important unexplored microbial reservoirs and that their study may give rise to knowledge not only in the area of biotechnology but also in the discovery of new species, thus increasing phylogenetic understanding and a possible new understanding of the origin of life on Earth. These discoveries will allow us to better understand the possibilities of life on other planets, as the microorganisms living in these environments are subject to events possibly similar to those on other planets.

There are many studies associated with actinobacteria, as compared with the other phyla; in many of the articles reviewed, activity screening was performed only on isolates identified as belonging to the phylum actinobacteria because they are described as having activities. Studies should be carried out in the remaining phylum, and these should not be discarded, as they have already demonstrated potential antimicrobial and antitumor activities.

Although there are several studies on the bioactivity of microorganisms isolated from caves, there is little information on the chemical structure of the active compounds and the metabolic pathways on which these compounds act and are produced. This situation could be overcome with more frequent use of high-resolution methods, such as metagenomics, metatranscriptomics, metabolomics, and metaproteomics, which would allow analysis of more information regarding the microorganisms and the biosynthesis of bioactive metabolites produced. Furthermore, by understanding the mechanisms behind the production of compounds with activity by bacteria, it may be possible to increase their production through genetic engineering techniques. This would allow for the sustainable production of these compounds, which could have important applications in fields such as medicine, biotechnology, and agriculture. Genetic engineering techniques can be used to manipulate the genes responsible for the biosynthesis of these compounds, increasing their expression and production. By combining advances in analytical methods with genetic engineering techniques, it may be possible to unlock the full potential of pristine environments and the microbial life that inhabits them.

It would also be interesting to broaden the spectrum of potential bioactivities of cave isolates, allowing the study of anti-inflammatory activity, for example, as well as extending the activity of antimicrobials applied to different areas, such as heritage biodegradation.

Continuing research in pristine environments is essential for a better understanding of their ecological functioning and for the discovery of new compounds with potential applications. Bioprospecting and the discovery of new compounds in these environments offer a unique opportunity to study and valorize these natural and cultural heritage habitats while also developing new green, safe, and sustainable solutions. By using low-cost and fast biotechnological processes, it is possible to obtain new products from microorganisms found in these environments. These products can have a wide range of applications, including in the pharmaceutical, agricultural, and environmental industries. They can also be used as more sustainable alternatives to traditional products, reducing environmental impact and promoting a more sustainable approach to industry. Moreover, studying pristine environments can provide important insights into the fundamental processes that underpin ecosystem function, such as nutrient cycling, carbon storage, and microbial interactions. This knowledge can be used to inform conservation strategies and policies to protect these environments, which are often fragile and vulnerable to human activity. Overall, the continued research in pristine environments and the discovery of new compounds offer a promising opportunity for both scientific advancement and sustainable development. By harnessing the potential of these environments and developing new green solutions, we can create a more sustainable and environmentally friendly future.

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