

Exploring Cave Microbiota: Microbial Diversity, Activity, and Biotechnological Potential in Pristine Parts of Temnata Dupka Cave, Bulgaria

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Abstract

Due to their natural isolation and limited accessibility, sections of caves that are extremely difficult to reach provide an ideal setting for studying unique and diverse microbial communities. This microbial diversity represents a potential reservoir of microorganisms with biosynthetic capabilities including the production of bioactive compounds with potential applications in biotechnology and medicine. Seven swab samples collected from wall surfaces in hard-to-reach galleries in Temnata Dupka Cave, Vratsa Karst Region, Bulgaria were subjected to quantitative and qualitative analysis. Quantitative air sample analysis was conducted at seven different locations within the cave. A total of 49 pure cultures were isolated under aerobic conditions from different galleries in Temnata Dupka Cave. The temperature growth patterns of the isolates were determined. Bacterial isolates from Temnata Dupka Cave were investigated for hydrolytic enzyme production, antimicrobial activity, and antibiotic resistance. A higher prevalence of protease-producing microorganisms (59%) was detected, followed by those exhibiting amylase activity (16%). A total of 53% of the bacterial isolates displayed resistance to at least one of the four antibiotics tested. Around 30% of the tested bacteria demonstrated antimicrobial potential. The results suggest that microorganisms isolated from pristine parts of Temnata Dupka Cave could be a valuable source of different psychrotolerant enzymes and bioactive metabolites. This study represents the first comprehensive report on microbial diversity and biosynthetic potential in hard-to-reach locations within a Temnata Dupka Cave.

Keywords: pristine parts in caves, quantitative analyses, temperature range, antimicrobial activity, antibiotic resistance, enzymatic activity

Резюме

Поради естествената си изолация и липсата на човешка намеса, труднодостъпните части от пещерите създават уникална среда, която подпомага развитието на разнообразни микробни съобщества. Това микробно разнообразие представлява потенциален резервоар от микроорганизми с биосинтетичен потенциал, включително синтез на биоактивни съединения с възможни приложения в биотехнологията и медицината. В това проучване са изследвани седем тампонни проби, събрани от повърхността на стените в труднодостъпни галерии на пещерата Темната дупка, разположена в карстовия район на Враца, България. Пробите бяха подложени на количествен и качествен анализ. Количествен анализ на въздушни проби беше проведен в седем различни точки в пещерата. Общо 49 чисти култури бяха изолирани при аеробни условия, от проби събрани от различни галерии на пещерата Темната дупка. Бяха изследвани температурният диапазон на растеж на изолатите. Бактериалните изолати бяха изследвани за продукция на хидролитични ензими, антимикробна активност и антибиотична резистентност. Установи се преобладаващо присъствие на микроорганизми, продуциращи протеази (59%), следвани от такива с амилалитична активност (16%). Общо 53% от изследваните бактериални изолати проявиха резистентност към поне един от четирите тествани антибиотика. Около 30% от изследваните бактерии демонстрираха антимикробен потенциал. Получените резултати предполагат, че микроорганизмите, изолирани от необлагородената пещера Темната дупка, могат да бъдат ценен източник на различни психротолерантни ензими и

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биоактивни метаболити. Това проучване представлява първото подробно проучване на микробното разнообразие и биосинтетичен потенциал в труднодостъпни участъци на необлагородена българска пещера.

Introduction

Caves are unique ecosystems defined by scarce nutrient availability, consistently low temperatures with minimal seasonal fluctuation, high humidity levels, and the complete absence of light. These extreme conditions foster the development of specialized microbial communities that adapt to survive and thrive in resource-limited environments. In recent years microbiological research related to speleomicroflora has been conducted primarily in urbanized caves, especially those containing valuable historical artifacts such as prehistoric paintings, fossils, and archeological findings. As a result of these studies, significant information has been accumulated regarding the microbial communities within them (Schabereiter-Gurtner *et al.*, 2002; Ikner *et al.*, 2007; Cuezva *et al.*, 2009; Saiz-Jimenez *et al.*, 2011). On the other hand, pristine caves attract scientific interest as they provide habitats essential for studying microbial ecology, biodiversity, and potential applications in various fields. Conducting research in such caves poses considerable technological challenges, which significantly limits these studies. This is largely due to the specific characteristics of each site - these caves lack infrastructure, are often difficult to reach, and require specialized skills and equipment for exploration. However, they tend to remain in their natural state with minimal human impact, enhancing their value for scientific research.

Although caves are perceived as extreme environments with harsh abiotic conditions, they provide an ecological niche for highly specialized microorganisms. The presence and structure of microbial communities depend on factors such as pH, availability of nutrients, light, oxygen content, sulfur, various metals, humidity, and the colonization potential of the substrate (Tomczyk-Żak and Zielenkiewicz, 2015; Martin-Pozas *et al.*, 2020). Caves are oligotrophic habitats, and primary production depends on the presence of chemoautotrophic microorganisms. Recent research indicates that certain caves contain a considerable amount of organic matter, providing favorable conditions for the growth of heterotrophic bacteria (Cañaveras, 2001). However, most caves are oligotrophic systems with less than 2.0 mg of total carbon per liter (TOC). The cave-associated microflora plays a role in the formation of rock deposits (Iquebal *et*

al., 2021a). The microbial activity in caves encompasses not only the formation of biofilms but also processes like calcite precipitation, which significantly contributes to speleogenesis, as well as structural alterations of the surrounding rock (Laiz *et al.*, 1999; Cañaveras *et al.*, 2006). Microbial colonies may also visibly alter rock surfaces, often appearing as spots or color changes caused by ongoing biochemical reactions. The formation of microbial deposits is observed as a result of the microorganisms' energy metabolism. By utilizing environmental resources, they modify the conditions, leading to changes in the chemical properties of minerals. This affects their solubility and results in the formation of layered, banded deposits on the surface. Microorganisms also influence corrosion processes and the subsequent structural changes in the rocks that form caves (Barton, 2006).

Studies of cave microflora have primarily been conducted in karst and basalt caves, as these contain diverse mineral deposits that influence microbial composition. Although cave systems may initially be perceived as habitats with similar conditions, suggesting stability in the composition of microbial communities, various factors such as the physicochemical parameters of the environment, availability of nutrients, air circulation, and complex biological interactions exert a modulatory influence on the structure and composition of the microflora that develops within them (Ghosh *et al.*, 2017; Kosznik-Kwaśnicka *et al.*, 2022). Numerous studies confirm the presence of a permanent autochthonous microflora, which completes its entire life cycle within caves (Simon *et al.*, 2003). At the same time, cave microbial communities are often highly variable and depend on the existing microhabitats within a given cave. Significant differences in microbial diversity, in terms of composition and structure, have been observed in various sections of the same cave (Barton *et al.*, 2007). In terms of microbial diversity, the contribution of allochthonous microflora is also significant. The mechanisms by which allochthonous microorganisms enter the caves are determined both by the type of cave and by the characteristics of the microorganism itself (Bontemps *et al.*, 2022).

Regarding the bacterial diversity observed in caves, recent data indicate that cave bacterial communities are predominantly composed of represent-

atives from phyla *Proteobacteria* and *Actinobacteria*. The widespread presence of *Proteobacteria* species is likely due to their ability to utilize a wide variety of organic compounds, with some species also catalyzing metal transformations. In contrast, *Actinobacteria* species play an active role in biomineralization processes. Representatives of manganese-oxidizing and iron-oxidizing bacteria are frequently found in caves, primarily species from the genera *Leptothrix*, *Gallionella*, and *Clonothrix* (Carmichael *et al.*, 2013). These findings challenge the notion that all caves are extremely energy-poor due to the absence of primary products. Recent studies have identified new methane-oxidizing bacterial species within the genus *Methylobacter*, expanding our understanding of microbial diversity in cave environments (Bontemps *et al.*, 2022). Numerous studies indicate a rich diversity of actinomycetes as part of the cave microflora (Cuezva *et al.*, 2012; Hathaway *et al.*, 2014), with evidence suggesting that many actinomycetes isolated from extreme habitats, like caves, possess significant potential as a source of valuable biomolecules (Bull, 2011). Cave-dwelling actinomycetes have been found to grow in substrate-poor environments and are also capable of utilizing atypical organic matter, such as lignocellulose and humic substances (Laiz Trobajo *et al.*, 2003). In this context, they have developed various biosynthetic pathways that enhance their adaptability, resulting in the synthesis of numerous secondary metabolites. Actinomycetes have proven to be important producers of antibiotics, anthelmintics, immunosuppressive agents, and anticancer compounds (Farda *et al.*, 2022). Other members of the cave microbiota belong to the phyla *Acidobacteria*, *Firmicutes*, and *Nitrospirae*, as well as *Gemmatimonadetes*, *Planctomycetes*, *Verrucomicrobia*, and *Cyanobacteria*. Such bacterial diversity has been discovered in numerous caves across Europe, as well as in the Americas, Asia, and Australia (Cheeptham, 2013; Yasir, 2018; Bontemps *et al.*, 2022). The fungal diversity identified in cave communities is predominantly represented by cosmopolitan saprophytes, associated with infiltrating soil masses, plant material, or insects (Caldeira *et al.*, 2021; Ogórek *et al.*, 2024). Due attention in cave systems should also be given to representatives of the domain *Archaea*. The dominant representatives are found to belong to *Thaumarchaeota* and *Euryarchaeota*. Approximately 70% of the species belong to *Thaumarchaeota*, and according to the literature, they are involved in carbon dioxide fixation and ammonia oxidation processes. The role and activity of *Archaea* representa-

tives in cave habitats remain poorly studied for now (Bontemps *et al.*, 2022). Various studies support the idea that microbial diversification in pristine cave systems is closely related to the presence of diverse microhabitats and specific sources of organic matter (Tomczyk-Żak and Zielenkiewicz, 2016; Bogdan *et al.*, 2023). Another primary focus in many studies in this field worldwide is the hypothesis that caves represent a promising source of novel microbial species capable of producing new antimicrobial compounds. Recent research highlights the significant antimicrobial potential of many cave-dwelling microorganisms, particularly actinomycetes, which are responsible for synthesizing approximately two-thirds of the antibacterial agents widely used today in medicine, industry, and agriculture (Barka *et al.*, 2015; Lima *et al.*, 2023). Another aspect of studying microorganisms isolated from habitats unaffected by human activity, such as pristine caves, is the potential development of antibiotic resistance as an evolutionary response to competition among microorganisms for survival in resource-limited environments (D'Costa *et al.*, 2006; Davies and Davies, 2010). Studies indicate that numerous cave microorganisms have developed a remarkable ability to synthesize bioactive molecules that interact with other microorganisms, forming defensive systems to protect themselves from substances synthesized by other organisms (Souza *et al.*, 2023). In this context, the enzymatic activities of cave microorganisms, influenced by the intense competition within their environment, are of exceptional interest to research (Hamedi *et al.*, 2019). Recently, highly active cave microorganisms have been described with significant amylolytic, proteolytic, and cellulolytic activities (Lima *et al.*, 2023).

In Bulgaria, research on cave microbial diversity remains extremely limited. While most studies have focused on Magura Cave due to its cultural and historical significance, investigations of pristine cave systems are virtually absent. Regarding pristine cave systems in Bulgaria, a microbiological study was published in 2023 and conducted in the Northwestern Stara Planina region. However, this study had a specific taxonomical focus-isolating nontuberculous mycobacteria (Atanasova *et al.*, 2023).

Our study provides novel insights into the microbial diversity and biosynthetic potential of pristine cave environments in Bulgaria. By focusing on the hard-to-reach galleries of Temnata Dupka Cave, where anthropogenic influence is minimal, we aim to address significant knowledge gaps and

contribute to the understanding of cave microbiota as a source of valuable bioactive compounds and enzymes.

Materials and Methods

Sampling site

The Temnata Dupka Cave is a system consisting of two main galleries with adjacent sections, developed across four levels. The northeastern part of the cave system is the longer of the two, spanning over 1000 meters along its transect and serving as the main water channel within the cave. The second section is a gallery extending in a west-northwest direction, with a length of approximately 900 meters. The two main galleries are interconnected by a complex labyrinth of additional galleries, entrances, cave formations, waterfalls, siphons, underground rivers, and lakes. The cave features several lakes of varying depths (ranging from 3 to 12 meters), the most notable being “Zhekov Lake”, which is fed by an underground river. The primary water flow in the cave drains through the karst spring “Zhitolyub”, located beneath the cave. However, during periods of heavy rainfall or snowmelt, water levels rise and overflow through the cave entrance. The temperature inside the cave remains relatively stable throughout the year, depending on the season, ranging between 8°C and 12°C.

Sample collection

The samples were collected during the autumn-winter period, November to December 2023, from Temnata Dupka Cave, located on a rocky ridge 27 meters above the level of the Iskar River, near the Gara Lakatnik in the Western Stara Planina Mountain range (N 43° 05' 19.9" E 23° 23' 10.6" * N 43° 05' 22.9" E 23° 23' 28.3"). This cave is situated within the territory of the Vratsa Mountain Nature Park. Specimens were obtained from seven different locations, including the third level, where the cave entrance is situated, as well as from galleries on the fourth level, which can only be accessed with professional equipment and specialized training. Samples were collected from rock surfaces displaying characteristic formations presumed to have a biological origin, such as shiny or pigmented deposits. Standard sterile cotton swabs were used for sample collection. To investigate microbial presence in the air, Koch's sedimentation method was employed, using Petri dishes containing sterile nutrient media suitable for various physiological groups of microorganisms.

Quantitative analysis

For implementation of Koch's sedimentation

method, the following nutrient media were used: Nutrient agar (NA) for heterotrophic microorganisms; R₂A for oligotrophic microorganisms; Sabouraud agar (SDA) for fungi. The Petri dishes were exposed for 10 min. Microorganisms from air samples were cultured under laboratory conditions at room temperature (22°C) for 24-48 hours. At the end of the incubation period, the number of microbial colonies (N) was recorded and CFU/m³ was calculated using the formula (1):

$$N \text{ (CFU/m}^3\text{)} = \frac{a \times 10^4}{p \times t}, \quad (1)$$

a - number of colonies counted on the Petri dish; *p* - the surface area of the Petri dish in square centimeters; *t* - the exposure time in minutes; 10^4 is a constant used to convert the result into cubic meters.

Quantitative determination of microorganisms from rock surfaces was carried out using standard microbiological methods, including the surface plating method and the serial dilution technique (Most probable number technique, MPN). Each swab was immersed in 5 ml of sterile physiological saline, vortexed thoroughly, and then placed on a shaker for 30 minutes. From the resulting suspensions, tenfold serial dilutions were prepared in a series of six test tubes, each containing 9 ml of sterile physiological saline (10^{-1} to 10^{-6}). From each dilution, a surface plating with 0.1 ml inoculum was performed on solid nutrient media: NA, R₂A, Gauze's agar for actinomycetes, and Ashby's medium for nitrogen-fixing microorganisms. For the MPN method, an inoculum of 1 ml was used, with each dilution being inoculated in duplicate into liquid nutrient media: Saratchandra's medium for nitrifying microorganisms (Basavarajappa *et al.*, 2015), Giltay's medium for denitrifying microorganisms (Qiu *et al.*, 2023), and Nutrient broth (NB) with phenol red was employed to detect ammonification. All inoculated nutrient media were incubated at room temperature for 2 to 5 days. The following formula was applied to determine the MPN of cells per 1 ml using formula (2):

$$N \text{ (MPN/ml)} = \frac{a \times 10^n}{V}, \quad (2)$$

a – the average number of colonies from the appropriate dilution; *10* – the dilution factor; *n* – the dilution number; *V* – a volume of inoculate in ml, and for the MPN method, the McCrady table was used.

Isolation of pure cultures

Various colony types were selected post-incubation and streaked onto the different solid nutrient media. A total of 126 pure cultures were suc-

cessfully isolated, from which 49 isolates were subsequently selected for further experimental work. All isolates were preserved by monthly subculturing on Petri dishes at 4°C. Additionally, the strains were stored at -80°C in physiological saline supplemented with 30% glycerol. The isolates underwent micromorphological analysis to assess cell shape, Gram reaction (Bartholomew and Mittwer, 1952), and spore-forming capability. The micromorphological characteristics of the studied strains were observed under a light microscope (Primo Star, Zeiss) at Plan-Achromat 100x/1,25 oil magnification.

Determination of the temperature range of the bacterial isolates

To investigate the temperature range at which the selected isolates grow, fresh 24-48 hour bacterial cultures were used. Bacterial suspensions were prepared in physiological saline with an optical density of 0.5 McFarland units. The analysis was conducted in sterile 24-well plates. Each well was filled with 2 ml of molten solid nutrient medium (NA, R₂A, Gauze's medium). The plates were incubated at 37°C for 24 hours to check for sterility. Five temperature intervals were selected for culturing the isolates: 4°C, 11°C, 22°C, 28°C, and 37°C. From each bacterial suspension, aliquots of 10 µl were pipetted on the top of the agar medium into each well. The plates were incubated for 1-4 days.

Screening for antimicrobial activity

Bacterial isolates from Temnata Dupka Cave were evaluated for their potential to inhibit the growth of six indicator strains: *Escherichia coli* ATCC 8739, *Bacillus cereus* ATCC 1085, *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* ATCC 8691, *Enterococcus faecalis* ATCC 3915 and *Enterococcus faecium* ATCC 8754. The assessment was performed using the disk diffusion method with cell-free supernatant (Lima *et al.*, 2023). For the preparation of cell-free supernatant from the bacterial isolates, they were cultivated in liquid nutrient media (NB, R₂A). The cultivation was carried out for 3 days. Upon visible growth (turbidity or sediment formation), the bacterial suspensions were transferred into 50 ml centrifuge tubes and centrifuged at 6000 rpm/20 minutes. The resulting supernatants were filtered through membrane filters with a pore size of 0.22 µm (MCE membrane filter, white, gridded, gamma sterile, diameter 47 mm). The aim of this procedure was to obtain a cell-free supernatant containing only extracellular metabolites produced during microbial growth. To investi-

gate their antimicrobial effect, 6 mm diameter disks were cut from filter paper and sterilized using dry heat sterilization (170°C/2 h). The sterilized disks were placed in 24-well plates and loaded with 25 µl of cell-free supernatant. They were left to dry at 37°C for 24-48 hours and then reloaded with an additional 25 µl, bringing the total volume to 50 µl. Indicator cultures (cell suspensions adjusted to 0.5 McFarland units) were spread on Muller-Hinton agar. Five disks soaked with cell-free supernatant were placed in each Petri dish. Tetracycline was used as a positive control, while a disk soaked in sterile broth served as the negative control. After overnight incubation at 37°C, clear zones of inhibition were measured and recorded as zone diameters (in millimeters) around the disks.

Screening for antibiotic resistance

The resistance of bacterial isolates to four antibiotics was determined using the Kirby-Bauer disk diffusion method (1966). Sterile Petri dishes were poured with Mueller-Hinton agar and R₂A. Cell suspensions were prepared from fresh bacterial cultures with an optical density of 0.5 according to the McFarland scale. A lawn inoculation was performed on the solid nutrient medium using 0.1 ml inoculum from each bacterial suspension. The antibiotic disks were placed on the surface, equidistant from one another. The antibiotics used in this study were as follows: Vancomycin - 30 µg, Ampicillin - 10 µg, Gentamicin - 10 µg, and Fosfomycin - 200 µg, based on the standards established by the Clinical and Laboratory Standards Institute (CLSI). The Petri dishes were incubated for 24-48 hours, and the presence/absence of inhibition zones around the antibiotic disks was recorded.

Screening for enzyme activities

The production of hydrolytic enzymes by bacterial isolates from Temnata Dupka Cave was qualitatively assessed using spot plating - 10 µl of inoculum were plated onto the appropriate nutrient medium containing a specific substrate, specific for the corresponding activity, and then incubated. Cultures exhibiting growth, and clearing zones on the respective media were identified as positive. To determine whether the tested microorganisms produce amylase (AmA), inoculation was performed on starch agar. The plates were incubated in the dark at 28°C for five days following the method described by Lima *et al.* (2023). Once growth was observed, the surface of the Petri dish was flooded with Lugol solution. In the case of a positive reaction, the agar medium surrounding the colonies

remains transparent, unlike the rest of the medium, which turns dark blue due to the presence of starch. The production of extracellular protease was assessed using skimmed milk agar, with the presence of a clear zone around the colony serving as an indicator of proteolytic activity (PrA). The cultivation was carried out for the same duration.

Results

Quantitative analysis of microbial isolates from Temnata Dupka Cave

Samples collected from seven locations were analyzed. The examined material consisted of particles from wall surfaces. The origin of the samples is as follows: Samples 1, 2, and 3 – level 3 in Temnata Dupka Cave; Sample 4, 5, 6, and 7 – level 4 in Temnata Dupka Cave (Fig. 1).

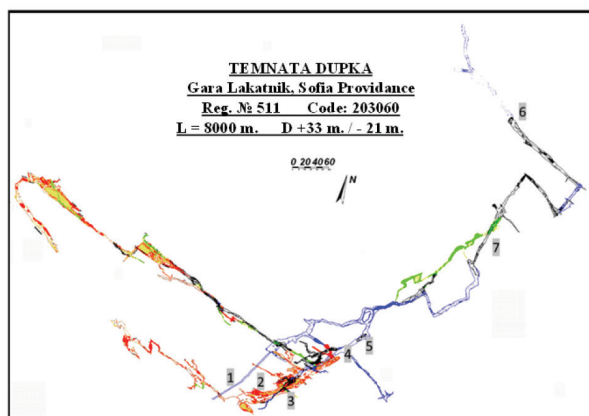


Fig. 1. Sampling locations (Bulgarian Federation of Speleology, 2015)

The experimental work on quantitative assessment focused on determining the abundance of heterotrophic and oligotrophic microorganisms, actinomycetes, and microorganisms involved in the transformation of nitrogen compounds. The quantity of culturable microorganisms is presented in Table 1. On the R₂A medium, Sample (1) exhibited the highest microbial count, reaching up to 10⁹ CFU/ml, which is 5 to 7 orders of magnitude higher

compared to the oligotrophic bacteria in the other samples. For the other groups of microorganisms, the analysis revealed values ranging between 10² and 10⁵ CFU/ml, depending on the sampling locations. The presence of fungi was also recorded, with their quantity not exceeding 2 x 10³ CFU/ml. This group of microorganisms was not included in subsequent studies. The quantitative presence of nitrogen-fixing bacteria, nitrifiers, and denitrifiers was recorded in the analyzed samples. The results are displayed in Table 1.

Nitrifiers were not detected in six of the collected samples, as no color change of Saratchandara's medium from pink to yellow was observed. For the ammonifiers, the concentration was higher in Sample (1) and Sample (3). Regarding the group of denitrifiers, growth was observed in samples from all seven locations, with the highest quantities recorded in Samples (6) and (7). Nitrogen-fixing microorganisms were also detected in all samples, with the highest representation observed in Samples (1) and (4). An analysis of the microbial presence in the air samples collected from the selected locations (which correspond to the sites where the swab samples were collected) within the cave was also conducted (Fig. 2).

In the first three samples, an average quantity of microorganisms was detected as follows: heterotrophs - 3292 cells/1m³; oligotrophs – 4174 cells/1m³; fungi – 588 cells/1m³. In the remaining four locations, the quantity is between 5 and 8.5 times higher, on average: heterotrophs – 19621 cells/1m³; oligotrophs – 22531 cells/1m³; fungi – 5026 cells/1m³.

Isolation and characterization of culturable bacteria from Temnata Dupka Cave

A total of 126 isolates were obtained from which 49 pure cultures were ultimately selected. Thirteen bacterial cultures were isolated on the NA

Table 1. Quantitative analysis of Selected Groups of Microorganisms

Group of microorganisms (Nutrient media)	Sample (1)	Sample (2)	Sample (3)	Sample (4)	Sample (5)	Sample (6)	Sample (7)
Heterotrophs (NA) (CFU/ml)	7×10 ³	2.05×10 ³	2.82×10 ⁴	1.7×10 ⁴	1.8×10 ⁵	1.5×10 ⁴	3.1×10 ³
Oligotrophs (R ₂ A) (CFU/ml)	1.1×10 ⁹	3.05×10 ³	3.96×10 ⁴	7.2×10 ³	6×10 ²	3×10 ²	4×10 ³
Actinomycetes (Gauze's medium) (CFU/ml)	3.9×10 ³	2.75×10 ³	1.6×10 ⁴	6.4×10 ³	4×10 ²	3×10 ²	3.9×10 ³
Nitrogen-fixing (Ashby's medium) (CFU/ml)	3.9×10 ³	2.7×10 ³	3.2×10 ³	6.3×10 ³	8×10 ²	1×10 ²	5×10 ²
Amonifucators (NB with phenol red) (MPN/ml)	6×10 ³	0.6×10 ¹	13×10 ³	13×10 ¹	2.5×10 ¹	0.6×10 ¹	2.5×10 ¹
Nitrificators (Saratchandra's medium) (MPN/ml)	-	-	-	-	-	-	1.2×10 ¹
Denitrificators (Giltay's medium) (MPN/ml)	13×10 ²	1.3×10 ¹	13×10 ²	6×10 ¹	6×10 ¹	1.1×10 ⁶	1.1×10 ⁶

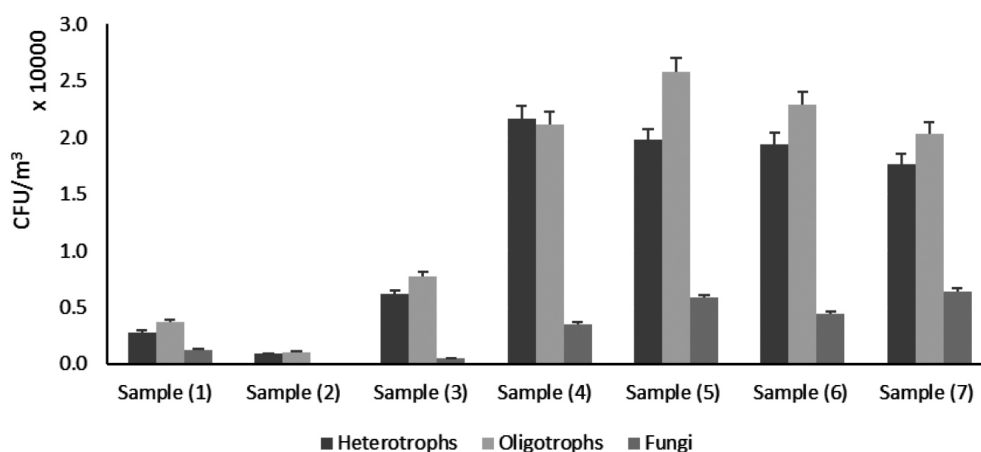


Fig. 2. Quantitative analysis of air samples

medium, fifteen on R₂A, twelve on Gauze's medium, and nine on Ashby's medium. Twenty-eight bacterial isolates were recovered from level 3, and twenty-one from level 4 (Table 2).

The information on the micromorphological characteristics of the bacterial isolates is provided in Table 2. Branching formation was observed in 13 isolates (Td6, Td12, Td22, Td26, Td29, Td36, Td37, Td38, Td39, Td44, Td45, Td48, Td49). For the spherical-shaped cells, formations of the tetrads (Td2, Td3, Td10, Td11, Td14, Td15) and sarcinae (Td5, Td21) types were noted. In the rod-shaped bacteria, the cylindrical form was identified as straight, with two of the isolates displaying visibly more swollen cells compared to the others (Td12, Td28). Forty (including 13 actinomycetes) were identified as Gram-positive. The ability to form endospores was observed in 8 isolates.

Temperature growth patterns

The Temnata Dupka Cave is characterized by a relatively constant temperature throughout the year, ranging between 8°C and 12°C depending on the season. During the sampling period, the recorded temperature was between 8.5°C and 9°C. The growth of Temnata Dupka Cave isolates at different temperatures is shown in Figure 3.

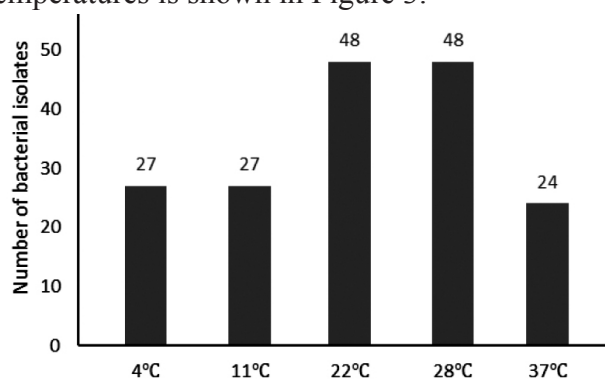


Fig. 3. Growth of Temnata Dupka Cave bacterial isolates at different temperatures

The growth of bacterial isolates from Temnata Dupka was evaluated at temperatures 4°C, 11°C, 22°C, 28°C, 37°C. Twenty-seven bacterial isolates out of the total 49 demonstrated the ability to grow at 4°C and 11°C. Of these, 26 isolates also exhibited growth at 22°C and 28°C. Among the microorganisms capable of growing in the temperature range of 22°C to 28°C, the majority (23 isolates) were additionally able to grow at 37°C. More than half of the isolates (53 %) can be classified as psychrotolerant determined by the ability to grow at both 4°C and above 20°C and most of them have upper limits as 37°C (Tomova *et al.*, 2013).

Investigation of antimicrobial potential of bacterial isolates from Temnata Dupka Cave

All of the isolates from Temnata Dupka Cave obtained as pure cultures were tested for antimicrobial activity. It was established that about 30.6 % of the isolates (five isolates recovered from level 4 of the cave, and then recovered from level 3 of the cave) exhibited inhibitory activity against some of the indicator microorganisms. Seven isolates (Td1, Td17, Td23, Td24, Td41, Td43, and Td48) were able to inhibit the growth of *S. enterica* ATCC 8691 and *E. fecalis* ATCC 3915. Seven isolates (Td3, Td15, Td34, Td36, Td39, Td46, Td49) showed activity against only one of the indicator microorganisms, respectively *S. enterica* ATCC 8691, *E. fecalis* ATCC 3915, *S. aureus* ATCC 6538, *E. coli* ATCC 8739, or *E. faecium* ATCC 8754. No antimicrobial activity against *B. cereus* ATCC 1085 was observed in any of the microorganisms isolated from Temnata Dupka Cave. The diameter of the inhibition zones ranged between 6.5 and 7 mm. The results are shown in Table 3.

Antibiotic resistance of Temnata Dupka Cave bacterial isolates

A total of 49 bacterial isolates were tested against four different antibiotics. In 26 of the iso-

Table 2. Properties of bacteria recovered from Temnata Dupka Cave

Isolate code	Sample origin	Nutrient medium	Gram status	Cell shape	Endospore formation
Td1	Sample 1, level 3	NA	+	Rod	-
Td2	Sample 1, level 3	NA	+	Tetracocci	-
Td3	Sample 1, level 3	NA	+	Tetracocci	-
Td4	Sample 2, level 3	NA	+	Rod	+
Td5	Sample 3, level 3	NA	+	Sarcina	-
Td6	Sample 3, level 3	NA	+	Branching	-
Td7	Sample 3, level 3	NA	-	Rod	-
Td8	Sample 5, level 4	NA	+	Rod	+
Td9	Sample 5, level 4	NA	-	Rod	-
Td10	Sample 4, level 4	NA	+	Tetracocci	-
Td11	Sample 4, level 4	NA	+	Tetracocci	-
Td12	Sample 7, level 4	NA	+	Rod	+
Td13	Sample 7, level 4	NA	+	Branching	-
Td14	Sample 1, level 3	R ₂ A	+	Tetracocci	-
Td15	Sample 1, level 3	R ₂ A	+	Tetracocci	-
Td16	Sample 1, level 3	R ₂ A	+	Spherical	-
Td17	Sample 2, level 3	R ₂ A	-	Rod	-
Td18	Sample 2, level 3	R ₂ A	+	Rod	+
Td19	Sample 3, level 3	R ₂ A	+	Rod	-
Td20	Sample 3, level 3	R ₂ A	-	Rod	-
Td21	Sample 5, level 4	R ₂ A	+	Sarcina	-
Td22	Sample 5, level 4	R ₂ A	+	Branching	-
Td23	Sample 5, level 4	R ₂ A	+	Spherical	-
Td24	Sample 4, level 4	R ₂ A	+	Rod	-
Td25	Sample 4, level 4	R ₂ A	+	Spherical	-
Td26	Sample 7, level 4	R ₂ A	+	Branching	-
Td27	Sample 7, level 4	R ₂ A	+	Rod	+
Td28	Sample 6, level 4	R ₂ A	+	Rod	-
Td29	Sample 2, level 3	Gauze	+	Branching	-
Td30	Sample 2, level 3	Gauze	+	Spherical	-
Td31	Sample 2, level 3	Gauze	+	Spherical	+
Td32	Sample 2, level 3	Gauze	+	Rod	+
Td33	Sample 2, level 3	Gauze	+	Rod	-
Td34	Sample 3, level 3	Gauze	-	Rod	-
Td35	Sample 5, level 4	Gauze	+	Spherical	-
Td36	Sample 4, level 4	Gauze	+	Branching	-
Td37	Sample 4, level 4	Gauze	+	Branching	-
Td38	Sample 7, level 4	Gauze	+	Branching	-
Td39	Sample 6, level 4	Gauze	+	Branching	-
Td40	Sample 3, level 3	Gauze	-	Rod	-
Td41	Sample 1, level 3	Ashby	-	Rod	-
Td42	Sample 1, level 3	Ashby	+	Branching	-
Td43	Sample 2, level 3	Ashby	-	Rod	-
Td44	Sample 2, level 3	Ashby	+	Branching	-
Td45	Sample 3, level 3	Ashby	+	Branching	-
Td46	Sample 3, level 3	Ashby	+	Rod	-
Td47	Sample 3, level 3	Ashby	-	Rod	-
Td48	Sample 5, level 4	Ashby	+	Rod	+
Td49	Sample 4, level 4	Ashby	+	Branching	-

Table 3. Range of inhibition zones (mm)

Isolate code	Pathogen					
	<i>B. cereus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. enterica</i>	<i>E. feacalis</i>	<i>E. faecium</i>
Td1	-	-	-	7	6.5	-
Td3	-	-	-	7	-	-
Td15	-	-	-	-	6.5	-
Td17	-	-	-	7	6.5	-
Td23	-	-	-	7	6.5	-
Td24	-	-	-	7	6.5	-
Td34	-	-	-	7	-	-
Td36	-	7	-	-	-	-
Td39	-	-	7	-	-	-
Td41	-	-	-	7	6.5	-
Td43	-	-	-	7	6.5	-
Td45	-	-	6.5	-	-	7
Td46	-	-	-	-	6.5	-
Td48	-	-	-	7	6.5	-
Td49	-	-	-	-	-	6.5

lates, resistance to at least one of the antibiotics was observed. The remaining 23 strains were sensitive to all antibiotics (Table 4).

The heterotrophic bacteria with laboratory codes from Td1 to Td13 were found to be the most susceptible. The predominant size of the growth inhibition zones was ≥ 30 mm. Only 4 out of the 13 exhibited resistance (R). Td6 and Td13 are resistant to AMP, CN, and FO. The observed resistance was 23.1 % to FO and CN, and 15.4 % to AMP (Fig. 4). Eight isolates from the oligotrophic group exhibited resistance, with isolates Td23 and Td26 showing resistance to all tested antibiotics. Isolates Td18 and Td21 were resistant to two antibiotics, while Td20 and Td28 exhibited resistance only to FO. The percentage of resistance to each of the antibiotics used is shown in Figure 4. Of all the isolates cultivated on Gauze's medium (a total of 12), five were inhibited by all the tested antibiotics. The percentage of resistance to each of the applied antibiotics is shown in Fig. 4. Of the nine isolates initially cultivated on Ashby's medium, two were sensitive to all four antibiotics. The isolates showing resistance to the largest number of antibiotics were Td41, Td42, Td43, Td45, Td46, Td47 and Td48. The distribution of the percentage of resistant isolates is shown in Figure 4.

The total number of Gram-positive microorganisms, including actinomycetes, that exhibit resistance is 22, while the number of Gram-negative microorganisms is 4 (Fig. 5).

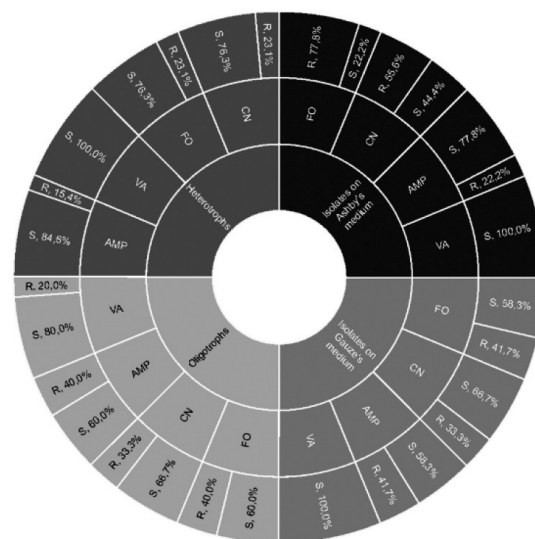
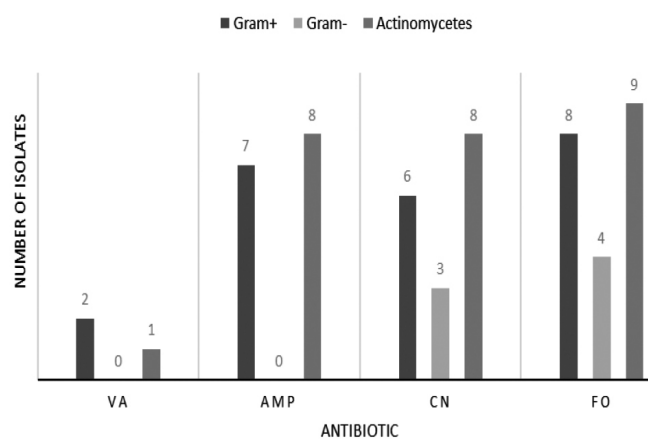
**Fig. 4.** Resistance (R)/Susceptibility (S) of bacterial isolates**Fig. 5.** Distribution of resistance patterns to the antibiotics used

Table 4. Antibiotic resistance of bacterial isolates (R-resistant) (mm)

Isolate code	Antibiotics				Isolate code	Antibiotics			
	VA	AMP	CN	FO		VA	AMP	CN	FO
Td1	25	16	11	R	Td26	R	R	R	R
Td2	>30	>30	>30	>30	Td27	28	>30	>30	22
Td3	>30	>30	>30	>30	Td28	>30	15	17	R
Td4	>30	24	24	11	Td29	21	18	28	R
Td5	26	>30	>30	28	Td30	>30	>30	>30	>30
Td6	>30	R	R	R	Td31	>30	R	24	R
Td7	>30	>30	>30	10	Td32	19	R	R	R
Td8	>30	>30	>30	27	Td33	>30	R	18	13
Td9	>30	12	14	14	Td34	>30	20	12	12
Td10	>30	>30	>30	>30	Td35	>30	>30	>30	>30
Td11	14	14	R	19	Td36	19	R	R	R
Td12	>30	>30	>30	>30	Td37	>30	18	R	R
Td13	>30	R	R	R	Td38	>30	>30	>30	>30
Td14	>30	R	R	R	Td39	>30	R	R	20
Td15	25	>30	>30	22	Td40	24	15	12	8
Td16	28	10	16	11	Td41	23	9	R	R
Td17	24	8	14	12	Td42	15	R	R	R
Td18	20	R	R	9	Td43	>30	16	R	R
Td19	27	>30	>30	>30	Td44	10	20	25	18
Td20	29	27	21	R	Td45	>30	R	14	R
Td21	R	R	14	8	Td46	32	6	R	R
Td22	>30	R	R	R	Td47	24	11	R	R
Td23	R	R	R	R	Td48	>30	13	22	R
Td24	25	18	>30	>30	Td49	8	7	7	8
Td25	>30	>30	>30	>30					

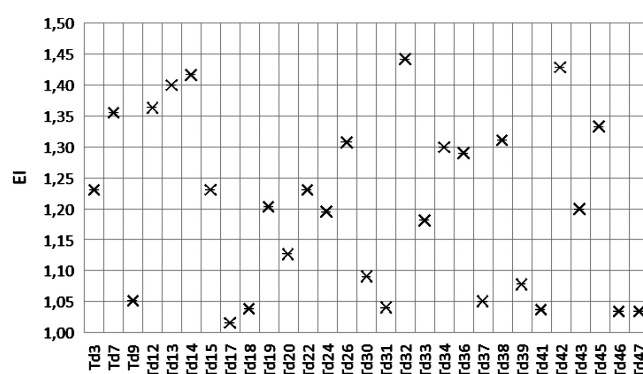
Hydrolytic enzymes activity

Bacteria isolated from Temnata Dupka Cave were tested for hydrolytic enzyme activity on agar media using specific substrates. Isolates were screened for the production of the main hydrolytic enzymes – amylases and proteases. When analyzing the results, we calculated the Enzyme Index (EI) following the method of Oliveira *et al.* (2006).

$$EI = \frac{\text{Total diameter of hydrolysis zone (mm)}}{\text{Diameter of bacterial colony (mm)}}$$

Of the 49 isolates examined, 29 (59 %) exhibited proteolytic activity. Among these 29 isolates, 19 were obtained from Samples (1), (2), and (3), while 10 were from Samples (4), (5), (6), and (7). The analysis of the results showed that the EI ranged between 1.0 and 1.4 (Fig. 6).

Five isolates classified as heterotrophs (Td3, Td7, Td9, Td12, Td13) demonstrated proteolytic activity. Among the microorganisms classified as oligotrophs, 9 (Td14, Td15, Td17, Td18, Td19, Td20,

**Fig. 6.** Comparison of EI in proteolytic activity

Td22, Td24, Td26) exhibited this type of hydrolytic activity. Of the microorganisms initially isolated and maintained on Gauze's medium, 9 (Td30, Td31, Td32, Td33, Td34, Td36, Td37, Td38, Td39) displayed extracellular proteolytic activity, while 6 isolates grown on Ashby's medium (Td41, Td42, Td43, Td45, Td46, Td47) showed such activity. Regarding amylolytic enzyme activity, it was less pronounced, as only 8 (Td3, Td5, Td7, Td17, Td33,

Td34, Td44, Td45) out of the 49 isolates exhibited such activity. For these isolates, the EI ranged between 1.0 and 1.3 (Fig. 7).



Fig. 7. Comparison of EI in hydrolytic activity

Discussion

This study demonstrates that the pristine parts of Temnata Dupka Cave serve as a habitat for a wide diversity of microorganisms. Although caves are considered oligotrophic environments with approximately 2 mg of total organic carbon per liter, very low light levels, low temperatures, and high humidity, cave systems are regarded as specific niches inhabited by various groups of microorganisms exhibiting high adaptability and metabolic plasticity, including endemic species (Schabereiter-Gurtner *et al.*, 2002; Engel *et al.*, 2010; D. Zhu *et al.*, 2020).

Based on this, and the established microbial diversity in other caves (Cheeptham, 2013; Adetutu & Ball, 2014; De Mandal *et al.*, 2017), several groups of microorganisms (in addition to oligotrophs) were selected for quantitative analysis. For this purpose, appropriate nutrient media - NA, R₂A, Gauze, Ashby, Saratchandra, Giltay, and NB with phenol red, and standard microbiological methods were used. The presence of some of these groups of microorganisms has also been studied by other research teams (Cañiveras *et al.*, 2001). The samples were incubated in the dark for a period of 24 to 96 hours to allow sufficient time for the growth of slow-growing bacteria. The number of cultivable microorganisms varied widely, ranging from 1.1×10^9 CFU/ml to 1.1×10^2 CFU/ml across different samples. Our results indicate that the highest number of microorganisms grew on the R₂A medium, which is typically used for cultivating oligotrophs that require limited amounts of organic nutrients, similar to the conditions found in caves. Some researchers suggest that in such cave habitats, the microbial population is approximately 10^6 cells per gram of sediment (Barton *et al.*, 2007). Nevertheless, the oligotrophic nature of the habitat and the

limited accessibility restrict the deposition of large amounts of organic matter, which could explain the higher abundance of oligotrophs.

Various studies highlight the potential of cave microflora and its role in geological processes, which may be closely linked to the ability of cave microbial species to produce various bioactive molecules (Yasir, 2018a). The nitrogen cycle is an essential part of the biogeochemical cycling of elements in nature. In caves, microorganisms play a significant role in this process by employing various metabolic pathways to obtain nutrients and energy under predominantly oligotrophic conditions (Zhu *et al.*, 2022). Our results revealed the highest presence of denitrifiers (3.14×10^5) and ammonifiers (2.7×10^3). Although caves are oligotrophic environments, they can contain localized accumulations of organic matter (bat guano, decomposed plant material), which can support the growth of these microorganisms. Regarding the quantitative analysis of airborne microorganisms, a significant difference was observed between levels 3 and 4 of the cave. Despite Temnata Dupka Cave being designated as a restricted-access site, level 3 remains accessible to tourists, and traces of anthropogenic impact are noticeable. However, microbial sedimentation at the sampling points in level 3 was notably lower. These findings contrast with previous studies reported in the literature. For instance, a study conducted in the Škocjan Cave in Slovenia (Mulec *et al.*, 2017) found that long-unvisited galleries exhibited lower microbial counts compared to regularly visited sections.

Microorganisms inhabiting cave ecosystems often need to adapt to environments with limited nutrient resources or rely on inorganic compounds for growth. These specific requirements for nutrients, energy sources, and environmental conditions make the isolation and maintenance of certain bacterial species in laboratory settings particularly challenging. As expected, we were able to isolate the highest number of pure cultures on nutrient medium R₂A (15 isolates). The determination of key micromorphological characteristics was an essential step in the experimental work. In other studies of cave ecosystems, the identification of fundamental micromorphological traits of isolates has also been conducted to achieve a more comprehensive understanding of microbial communities (Souza *et al.*, 2023).

Although the temperature in Temnata Dupka Cave was below 20°C, it was decided to conduct the initial cultivation at a higher temperature. This

decision was based on studies showing that, under laboratory conditions, cave isolates grow very slowly at the low temperatures typically maintained in caves. Additionally, these studies found that the species diversity of the cultivable microflora is similar when grown at low temperatures and at around 28°C (Laiz *et al.*, 2003; Belyagoubi *et al.*, 2018). Consistent with other research in this field, (Laiz Trobajo *et al.*, 2003a; Tomova *et al.*, 2013) a temperature range was selected accordingly. We found that most of the isolated bacteria from Temnata Dupka Cave (about 46.9 %) are psychrotolerant by the highest growth rate observed between 22 and 37°C. At this stage, it is difficult to specify a definite reason for the lack of growth of 22 bacterial isolates from Temnata Dupka Cave at temperatures of 4 and 11°C, which are similar to those in their natural habitat. The results we obtained for determining the temperature range under laboratory conditions, as well as our observations that the number of bacterial isolates from Temnata Dupka showing growth at higher temperatures compared to their natural habitat is greater, are consistent with findings from other research groups (Laiz *et al.*, 2003; Laiz Trobajo *et al.*, 2003b; Yasir, 2018b).

Cave microorganisms represent a promising reservoir of bioactive compounds. The unique environmental conditions they inhabit, along with the competitive pressure for limited nutrients, drive the evolution of metabolic pathways for the production of diverse secondary metabolites. These metabolites have potential applications as alternative antimicrobial agents for combating various pathogens. In recent years, several studies have substantiated the existence of such metabolite-producing species within cave ecosystems (Tomova *et al.*, 2013; Axenov-Gribanov *et al.*, 2016; Belyagoubi *et al.*, 2018; Lima *et al.*, 2023). Some research teams have reported a high percentage of microorganisms isolated from caves that exhibit antimicrobial activity (Belyagoubi *et al.*, 2018; Ambrožič Avguštin *et al.*, 2019). In contrast, other studies have found a lower proportion of isolates demonstrating antimicrobial properties relative to the total number of isolates (Lima *et al.*, 2023). Studies investigating the antimicrobial activity of cave-derived microorganisms have revealed such potential in both bacterial isolates and actinomycetes (Iquebal *et al.*, 2021b). We tested all 49 Temnata Dupka Cave isolates for antimicrobial activity against a panel of pathogens known to cause various infectious diseases, many of which are resistant to commonly used antibiotics. Of these isolates, only 15 exhibit-

ed antimicrobial activity, primarily against *S. enterica* and *E. faecalis*. Among the active isolates, four were identified as Gram-negative bacteria (Td17, Td34, Td41, Td43), seven as Gram-positive (Td1, Td3, Td15, Td23, Td24, Td46, Td48), and four as actinomycetes (Td36, Td39, Td45, Td49). The relatively low number of actinomycetes displaying antimicrobial activity was unexpected, as previous studies on cave environments have primarily associated antimicrobial properties with actinomycetes (Kim *et al.*, 1998; Yücel & Yamaç, 2010). Antimicrobial compounds hold significant potential for diverse biotechnological applications. Novel antimicrobial agents derived from pristine cave isolates may facilitate the development of new classes of antibiotics for combating infections caused by antibiotic-resistant pathogens. Furthermore, these compounds could serve as biopesticides or feed additives to control agricultural pathogens, thereby reducing dependence on synthetic antibiotics. Additionally, such antimicrobial substances may be employed as natural preservatives in the food and cosmetic industries to prevent the proliferation of harmful microorganisms.

Many microorganisms isolated from caves exhibit intrinsic resistance to various classes of antibiotics (Paun *et al.*, 2021). The presence of resistant bacteria in natural environments such as caves, which have not been subjected to human interference, suggests that antibiotic resistance is an inherent characteristic of microorganisms. The substrate deficiency in isolated cave systems may lead to the formation of very low biomass and surprisingly high microbial diversification, indicating intense competition for resources within the cave environment. In 2020, a strain of *Paenibacillus sp.* LC231 was isolated from Lechuguilla Cave, USA, which exhibited resistance to nearly all classes of clinically important antibiotics while simultaneously producing a novel class of antifungal compounds (Pawlowski *et al.*, 2016). These findings may lead to an important hypothesis: If antibiotic resistance is selected due to nutrient scarcity, then competition in such isolated habitats should also drive the evolution of mechanisms for inhibiting resistance. This hypothesis is grounded in the relationship between the Red Queen Hypothesis and cave microorganisms, emphasizing the role of competition and the evolution of microbial communities in extreme environments such as caves (Pawlowski *et al.*, 2016; Department of Biology, University of Akron, OH, USA, n.d.). This hypothesis, along with the assumption that excessive antibiotic use has not yet sig-

nificantly impacted such extreme habitats (Brown & Balkwill, 2009; Toth *et al.*, 2010), prompted our investigation into whether and to what extent cave isolates exhibit resistance to specific antibiotics. Our research revealed that the most resistant strains were Gram-positive bacteria (12 isolates or 46.1% of all resistant cave isolates) and actinomycetes (10 isolates, or 38.5 % of all resistant isolates). The Gram-negative microorganisms constituted the smallest group (9 isolates), with only 4 of them exhibiting antibiotic resistance (or 15.4 % of all resistant cave isolates). Comparing the effects of the four antibiotics, it was observed that over 93% of the isolates were sensitive to Vancomycin. Sensitivity to Ampicillin and Gentamicin was lower, with 69.4% and 65.3% of the isolates, respectively, showing susceptibility. The lowest sensitivity was recorded for Fosfomycin, with approximately 57.1% of all isolates being susceptible. According to some authors, the increased resistance observed in actinomycetes is attributed to their ability to produce antibiotics themselves. These microorganisms often develop self-protection mechanisms to avoid the effects of their own antibiotics (De Simeis & Serra, 2021; Rütten *et al.*, 2022).

Experimental studies have shown that microorganisms isolated from extreme habitats possess significant potential to produce hydrolytic enzymes, including proteolytic, cellulolytic, and amylolytic enzymes (Lima *et al.*, 2023; Ramos *et al.*, 2018; Oliveira *et al.*, 2006). The presence of extracellular enzymes supports the role of these microorganisms in the cycling of key biogenic elements such as carbon (Lima *et al.*, 2023) and highlights their importance in facilitating biochemical reactions associated with the transformation of organic matter in soil. These processes contribute to the mineralization of organic compounds (Burns *et al.*, 2013), converting them into soluble molecules that can be readily absorbed by cells, in line with their mode of nutrition (Arias *et al.*, 2005). Research conducted by various teams has demonstrated the considerable potential of extreme microorganisms to produce hydrolytic enzymes, particularly proteases and amylases (Lima *et al.*, 2023; Kurdy *et al.*, 2023; Rautela *et al.*, 2017). In our study, we found that the isolates from Temnata Dupka exhibiting enzymatic activity included Gram-positive bacteria, Gram-negative microorganisms, and actinomycetes. A total of 29 strains demonstrated proteolytic activity, of which 12 were Gram-positive, 8 were Gram-negative, and 9 were actinomycetes. Additionally, 8 strains exhibited amylolytic activity, comprising 3

Gram-positive bacterial isolates, 3 Gram-negative bacterial isolates, and 2 actinomycetes. Only six Temnata Dupka isolates (Td3, Td7, Td17, Td33, Td34, and Td45) exhibited both proteolytic and amylolytic activity. Microorganisms are considered the most promising sources of hydrolytic enzymes, primarily due to their rapid growth rates (Gurung *et al.*, 2013). Hydrolytic enzymes, including proteolytic, cellulolytic, and amylolytic enzymes, are among the most widely utilized in various industrial processes, particularly in biotechnology, the food industry, biofuel production, and paper manufacturing (Manisha & Yadav, 2017). Enzymes produced by microorganisms inhabiting extreme environments, such as caves, are regarded as valuable alternatives for the development and enhancement of industrial and biotechnological processes. This is attributed to their remarkable stability under extreme conditions and the significant advantage of being biodegradable (Kumar *et al.*, 2011). The high efficiency of cold-active enzymes at low to moderate temperatures presents significant economic advantages by enabling considerable energy savings in large-scale industrial processes (Russell, 1998; Gerday *et al.*, 2000; Cavicchioli *et al.*, 2011).

Conclusion

The significance of our study lies in the fact that it represents the first detailed investigation into the microbial diversity and potential of pristine cave microorganisms in hard-to-reach sections of cave systems in Bulgaria. Temnata Dupka is a protected site with restricted access to tourists and other visitors. This restricted access allows for the exploration of cave microhabitats that remain unaffected by anthropogenic influences, although some areas can still be reached without professional speleological assistance. The results obtained indicate that psychrotolerant microorganisms may serve as a valuable source of antimicrobial metabolites with promising applications, as well as hydrolytic enzymes, and display antibiotic resistance to various antibiotics. These findings mark the first step toward revealing the unique metabolic activities of microorganisms inhabiting such extreme and isolated environments.

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