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SCREENING FOR CULTURABLE MICROORGANISMS FROM CAVE ENVIRONMENTS (SLOVENIA)

PROUČEVANJE MIKROORGANIZMOV IZ JAMSKIH OKOLIJ (SLOVENIJA) Z GOJITVENIMI TEHNIKAMI

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Janez Mulec & Polona Zalar & Nadja Zupan Hajna & Maja Rupnik: Proučevanje mikroorganizmov iz jamskih okolij (Slovenija) z gojitvenimi tehnikami

V vzorcih iz treh različnih jam smo proučevali mikroorganizme, ki jih lahko gojimo v laboratorijskih razmerah. V jami Pečina v Borštu smo prisotnost mikroorganizmov proučevali v preperelem apnencu, jamskem srebru in v jamski ponvici, kjer se na površini velikokrat pojavljajo kalcitne ploščice; v Martinski jami v preperelem apnencu, v Snežni jami na Raduhi pa kalcitno jamsko mleko. Podatki o številu mikroorganizmov so bili v nekaterih primerih dopolnjeni še z identifikacijo širše skupine ali rodu. Rezultati kažejo, da so poleg še nekaterih drugih bakterijskih in glivnih taksonov fluorescentne pseudomonade prevladujoči mikroorganizmi.
Ključne besede: jamska mikrobiologija, preperel apnenec, jamsko srebro, kalcitne ploščice, kalcitno jamsko mleko, jama Pečina v Borštu, Martinska jama, Snežna jama na Raduhi, kras, Slovenija.

Abstract UDC: 579.2:551.44(497.4)

Janez Mulec & Polona Zalar & Nadja Zupan Hajna & Maja Rupnik: Screening for culturable microorganisms from cave environments (Slovenia)

Various microenvironments in three different caves were screened for the presence of indigenous culturable microorganisms: extremely weathered limestone in Pečina v Borštu and Martinska jama, cave silver and calcite rafts on the surface of subterranean ponds in Pečina v Borštu and calcite moonmilk speleotheme in Snežna jama of Raduha mountain. The counts of viable cells collected are supplemented with laboratory data necessary to establish genus or wider taxonomic group level identity of isolates. Besides other bacterial and fungal groups flourescent pseudomonads are prevailing among isolates.
Key words: cave microbiology, weathered limestone, cave silver, calcite rafts, calcite moonmilk, Pečina v Borštu cave, Martinska jama cave, Snežna jama cave of Raduha montain, karst, Slovenia.

INTRODUCTION

The studied culturable microorganisms are from different cave microenvironments and caves: Pečina v Borštu, Martinska jama and Snežna jama on Raduha (Fig. 1). Caves Pečina v Borštu and Martinska jama are located in the karst area of Matarsko Podolje in SW Slovenia. Weathered limestone is widely present in both caves (Zupan Hajna, 2002). Cave Snežna jama na Raduhi is situated in the Alpine area of N Slovenia. The cave is well known due to the permanent ice presence in entrance part and the large amount of moonmilk speleothems.

Caves

Pečina v Borštu cave (566 m above sea level) is situated about 100 m above the Jezerina blind valley where a small stream sinks on the contact between Eocene flysch rocks and Palaeocene and Cretaceous limestones (Šikić *et al*. 1972). The cave is about 250 m long and elongated in the S-N direction. This cave was formed in Upper Cretaceous limestone (K_2^2) (Šikić *et al*. 1972). The limestone beds dip toward NE at an angle 50-55°. The main tectonic structures in the area are in "Dinaric" NW-SE direction. An active water flow is not present in the cave system any more. The water percolation through cave ceiling is very strong in some parts after atmospheric precipitation. The average annual temperature in the inner parts of the cave is about 10°C. Oscillation of the temperature in the entrance parts corresponds to the outside temperature changes. The condensation of water droplets appears in the entrance part on the cave walls due to temperature gradient. Walls in the cave are covered with flowstone and speleothems of different ages and forms. Almost all the walls are extremely weathered, which could be seen just in some cases where flowstone was not precipitated. The thickness of the weathered zone in limestone varies from a few millimetres up to several centimetres. Clastic deposits in the cave are allochtonous, originated from Eocene flysch Brkini Hills. The main component of the deposit is quartz sand and silt which also contains some clay minerals.

Martinska jama is situated 565 m above sea level on N slope of Veliki Mavrovec hill at Slavnik mountain foothill. The entrance to the cave is open on the SE slope of a small collapse doline. The length of cave passages is 1004 m and depth is 120 m. The cave was formed in

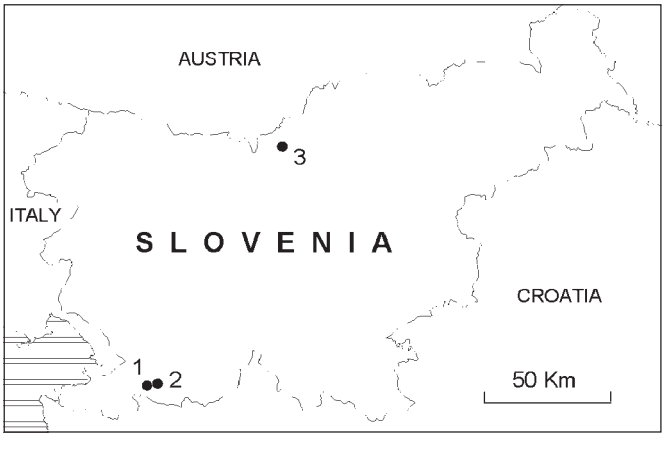

Fig. 1: Geographical locations of the three caves (1, Martinska jama cave; 2, Pečina v Borštu cave; 3, Snežna jama cave).

Cretaceous limestone ($K_{1,2}$) (Šikić *et al*. 1972). Limestone beds dip toward NE at an angle 20-30°. The main tectonic structures in the area are in "Dinaric" NW-SE direction. N-S and in E-W directions are also well expressed. Weathering of the limestone is specially significant for the small side passage named Boeganov rov. The temperature in this passage is virtually constant during the whole year; it varies from 8.7 to 9.0°C. No air current is perceived. There is no permanent water flow in the passage; wall wetness depends only on the atmospheric precipitation and intensity of percolation through the fissures. Sandy clay deposits are found on the passage floor and rocky shelves on the cave walls. Clastic sediments have fluvial origin from the non-carbonate Eocene flysch rocks.

Entrance to Snežna jama is located on the S slope of Raduha Mountain at 1556 m above sea level. It is a horizontal cave with permanent ice in the entrance part. The cave was formed in Upper Triassic ($T_{2,3}$) massive and thick bedded limestone (Mioč *et al*. 1983). Fluvial sediments are present in the form of laminated loams, sand and gravel of noncarbonate origin (Mihevc 2001). In the inner part of the cave there is also a lot of flowstone deposits in the form of precipitated calcite moonmilk (see below).

Cave microenvironments

Karst caves are one of the extreme environments (Pedersen 2000) generally characterized with low nutrient input. In the caves different microenvironments could be inhabited by the microorganisms. The microbial community differences are probably related to environmental cave conditions: humidity, relatively low and stable temperature, nature of the nutrient resources and pH. In this study we selected different caves with specific microenvironments where certain adapted microorganisms could be found. Pečina v Borštu cave was selected due to the presence of microenvironments: silver flashing droplets, a pond with floating calcite rafts and weathered limestone of the cave wall. Martinska jama cave also has some parts of weathered limestone (Boeganov rov). We collected also samples of calcite moonmilk from Snežna jama na Raduhi cave to investigate for the presence of microorganisms.

Silver flashing droplets are known among speleologists as "cave silver" due to weak silver fluorescence when illuminated by a carbide lamp. Such water droplets are formed near the cave entrance where cold and warm air are mixed and their presence is probably connected with condensation. The nature of its fluorescence is not clear yet.

Pond filled with water selected in Pečina v Borštu cave is interesting due to calcite raft formation on its surface. This pond is periodically filled up with water depending on the rain regime. During the rainy period there could be a niche for biofilm foundation and eventual calcite precipitation promoted by the microorganisms present. In some cases mineral precipitation is closely associated with bacterial cells, but this process is generally not controlled by the organism itself. A common mechanism for carbonate mineral deposition by bacteria involves the ability of cells to produce an alkaline microenvironment as a result of their physiological activity (Douglas & Beveridge 1998). Some studies now also indicate that microorganisms play a critical role in processes such as weathering, erosion, sedimentation and cementation (Viles 2000).

The term "moonmilk" is used to describe aggregates of microcrystalline substances of varying composition. Moonmilk could be formed by disintegration of bedrock and speleothems or as mixed deposition of calcite crystals and water (Hill & Forti 1997).

MATERIAL AND METHODS

Culturing techniques

Samples collected aseptically from selected cave microenvironments were transferred to laboratory. To propagate microbial cultures the following media were utilized: (1) Prep medium containing 0.1% yeast extract and 1.5% agar inoculated with 1% weathered bedrock, (2) King B (Difco, USA) a standard medium to support growth of majority of heterotrophic bacteria and detection the presence of fluorescent pigments. (3) 0.1 strength nutrient agar (Merck, Germany), (4) Moonmilk medium: 0.1 % yeast extract, and 1.5% agar and 1% calcite moonmilk sample, and (5) MEA (malt extract agar) for propagation of fungal isolates of nutrient nondemanding species.

Counting of viable cell and characterisation of isolates

Determining of the total cell count was performed as follows: samples were diluted in a physiological solution and 0.1 ml of an appropriate dilution was spread on the agar medium. Plates were incubated at 10°C. After 14 days the colonies of microorganisms were counted and the number of viable cells was calculated as total number of colony forming units (CFU).

After enumeration colonies were randomly sampled and isolated from primary plates by streak plating on King B medium. Pure cultures were tested for: presence of oxidase and catalase, temperature, oxidative vs. fermentative metabolism, production of fluorescent pigments, motility, gelatinase, nitrate reduction, utilization of carbohydrates and amino acids (MacFaddin 1980). Cell morphology and Gram staining were studied using optical microscopy. In the case of violet pigmented bacteria the absorption spectra of crude violacein extract was also performed as cited in Logan & Moss 1992.

Fungus grown on the Prep medium was analysed regarding the morphology, i.e. colony characteristics and micromorphological features like conidiogenesis, structure and size of conidiophores and conidia (Domsch *et al*. 1980, Ellis 1980). Further determination based on DNA sequence comparison of ITS rDNA region after PCR amplification with the specific primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') was also performed. ITS region (550 bp) is a variable noncoding region located between 18S and 28S rDNA genes which also includes a short 5.8S rDNA gene. The comparison of ITS sequences is a suitable method for species determination of certain fungal taxa (Zalar *et al*. 1999).

Biofilm examination

Biofilm foundation was studied using adhesif and colonization technique, which is applicable to environments such as soils, sediments, and nonflowing water systems (Marshall 1997). Microscopic glasses in the cave pond containing calcite rafts were exposed for 5 months, glasses then transferred to laboratory and stained with 0.01% acridine orange. Fluorescence in cells was detected using a microscope equipped with an excitation at 450-490 nm and emission filter at wavelengths 515-565 nm.

RESULTS AND DISCUSSION

Viable cell count

Viable cell count was determined for all microniches studied except for silver flashing droplets (Table 1). Comparison of all microenvironments shows that weathered limestone from Pečina v Borštu cave contained more culturable microorganisms than any other tested niche (1.1×10^6 CFU/ml). Interestingly, these microbes from weathered limestone sample preferred Prep medium (prepared with weathered limestone from Pečina v Borštu cave) for growing, whereas the growth of pond water bacteria was better on King B medium. Obviously, the microorganisms in the weathered limestone from Pečina v Borštu cave sample are well adapted on special nutrients available in such microenvironment.

Viable cell count from two different caves with weathered limestone (Martinska jama cave and Pečina v Borštu cave) differed in the range of 10^3 (Table 1). Microorganisms from Martinska jama cave sample grew on Prep medium prepared from this cave weathered limestone. Interestingly, microbial growth from the Martinska jama cave weathered limestone sample was not detected when inoculated Prep medium was prepared with weathered limestone taken from Pečina v Borštu cave. This indicates that microflora from Martinska jama cave weathered limestone is probably highly adapted to certain conditions or nutrients available in this microniche.

Weathered zones of limestone are a result of incomplete dissolution by the carbonic acid in a cave environment under specific conditions (Zupan Hajna 2002). Despite that the same geological process presumably ruled the weathering process in both cases, we detected a higher number of culturable microorganisms from Pečina v Borštu cave than from Martinska jama cave. This is the first report of estimation of total microbial count from such a microenvironment. Still, as reviewed elsewhere (Amann *et al*. 1995) classical microbiological growth techniques detect only small proportion of all microorganisms present and the direct microscopic cell counts exceed viable cell counts by several orders of magnitude from different natural environments.

Viable cell count of the calcite moonmilk sample from Snežna jama cave (Table 1) revealed a few magnitudes lover microbiale populations ($3.9\text{-}6.4\times10^2$ CFU/ml) on selected cultivation media

Table 1: Counts of the viable microbial cells isolated from different cave microenvironments and caves.

	HABITAT ***(microniche)***				
	Pečina v Borštu cave			***Martinska jama cave***	***Snežna jama cave***
	SILVER FLASHING DROPLETS	POND	WEATHERED LIMESTONE	WEATHERED LIMESTONE	MOONMILK
VIABLE-CELL COUNT	**COLONY FORMING UNITS (CFU) / ml**				
King B	nd	2.5×10^4	4.0×10^5	nd	nd
Moonmilk medium	na	na	na	na	6.4×10^2
Prep medium	nd	1.0×10^4	1.1×10^6	1.3×10^3	na
0.1×NA	nd	1.5×10^4	7.0×10^5	nd	3.9×10^2

na - not applicable, nd - not done

compared to the weathered limestone sample from Pečina v Borštu cave. The role of microbes in a process of calcite moonmilk formation is not clear yet. In some cases their presence is not essential for calcite moonmilk formation (Borsato *et al*. 2000).

Microbial identification and grouping

Bacteria isolated in the pure culture from the Pečina v Borštu cave samples were divided into five groups based on performed tests (see Material and methods) (Table 2). Fluorescent pseudomonads were characterised by the production of fluorescent pigment on King B medium (Fig. 3). All of them were motile Gram negative rods, with an oxidative metabolism, but differed in the utilisation of sugars and amino acids. Oxidative Gram negative bacteria with no production of fluorescent pigments were defined as Gram negative non-fermentatives. The third group represented violet pigmented strains, which were Gram negative rods with a fermentative metabolism. Regarding the absorption spectra the pigment was violacein (Logan & Moss 1992). The strains therefore most probably belonged to the genus *Chromobacter*. The remining two groups of Gram positive cocci and Gram positive irregular rods were characterised by their microscopic morphology.

The fungus grown on Prep medium from Pečina v Borštu cave was further characterized by the molecular methods. Regarding morphological characteristics and sequencing data (see Material and methods) the isolated fungus from weathered limestone belonged to the *Cladosporium herbarum* group (Fig. 2). Species *C. herbarum* appears with relatively high incidence as a saprophyte on decaying organic material, i.e. plant material, soil, sediments, seawater, and also in extreme environments, such as guano caves (Domsch *et al*. 1980). Until now it was not isolated from the oligotrophic karst cave niches. Experiments showed that fungi are able to penetrate actively the marbles and limestones without initiative activity of other organisms and also without previously decayed rock caused by physicochemical parameters (Sterflinger & Krumbein 1997). It would be interesting to investigate if this fungus itself could promote rock weathering,

Table 2: Bacterial diversity in the three cave microenvironments from Pečina v Borštu cave.

	HABITAT ***(microniche)***		
	SILVER FLASHING DROPLETS	POND	WEATHERED LIMESTONE
BACTERIAL GROUPS	**NUMBER OF ISOLATED STAINS**		
FLUORESCENT PSEUDOMONADS	1	6	5
GRAM NEGATIVE NON-FERMENTATIVES		6	2
GRAM NEGATIVE FERMENTATIVES (violet pigmented)		2	
GRAM POSITIVE COCCI		1	
GRAM POSITIVE IRREGULAR RODS (*Actinomyces* like)		4	4

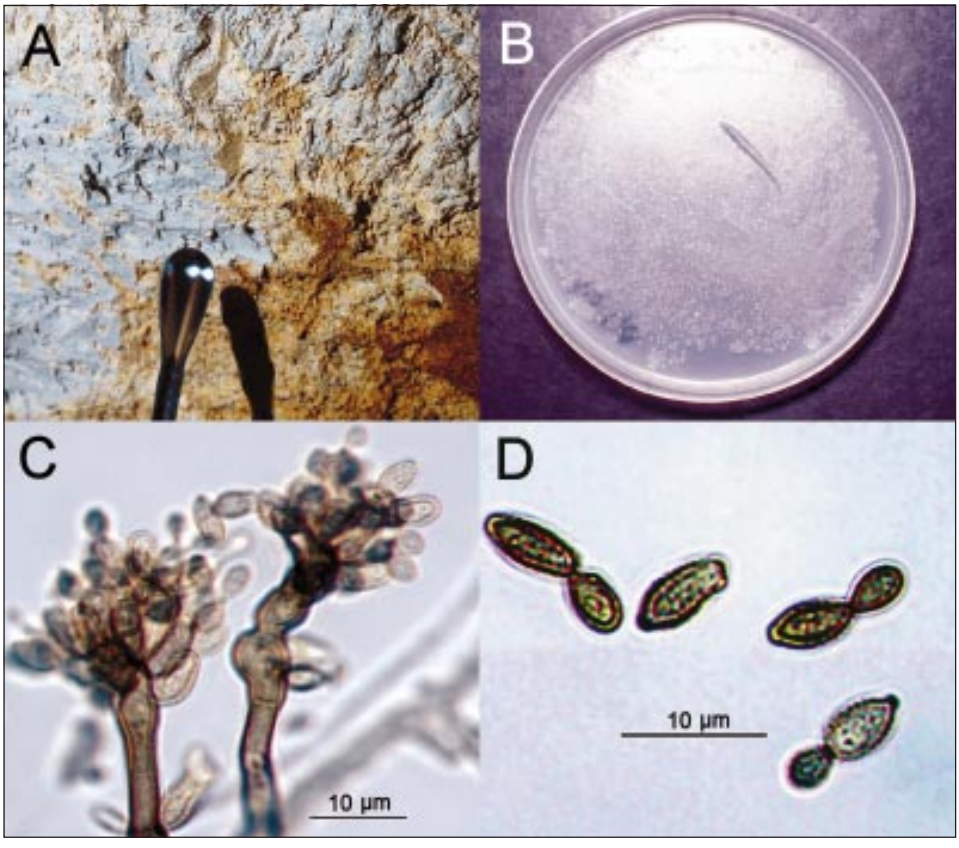

Fig. 2: A: Weathered limestone microenvironment from Pečina v Borštu cave. B: Bacteria isolated from weathered limestone on the Prep agar medium. C: Micromorphology of the fungus Cladosporium herbarum*, conidiophores. D:* C. herbarum *conidia. Bar represents 10 µm. (Photo: A, Jože Žumer; B, Jurij Hajna; C, D; Polona Zalar)*

since there is one species of the same genus (*C. sphaerospermum*), which was found on a Marble statue in Greece (Sterflinger, personal communication).

There is a whole series of effects on weathering related simply to the physical presence and biochemical activity of a microbial community on a rock or mineral surface (Viles 2000). It has been recently shown that streptomycetes are able to destroy stalactites by their metabolic activity (Groth *et al*. 1999). We also proved presence of strains belonging to an *Actinomycetes*-like group in the weathered limestone from Pečina v Borštu cave (Table 1) which could participate in weathering process. On the other hand some bacteria may be involved in a carbonate mineral deposition as experienced by Douglas & Beveridge 1998. Bacteria from the cave pond from Pečina v Borštu cave could have a similar role in the formation of calcite rafts, since the incidence of culturable bacteria is relatively high (Table 1) as well as the rate of biofilm formation (Fig. 3).

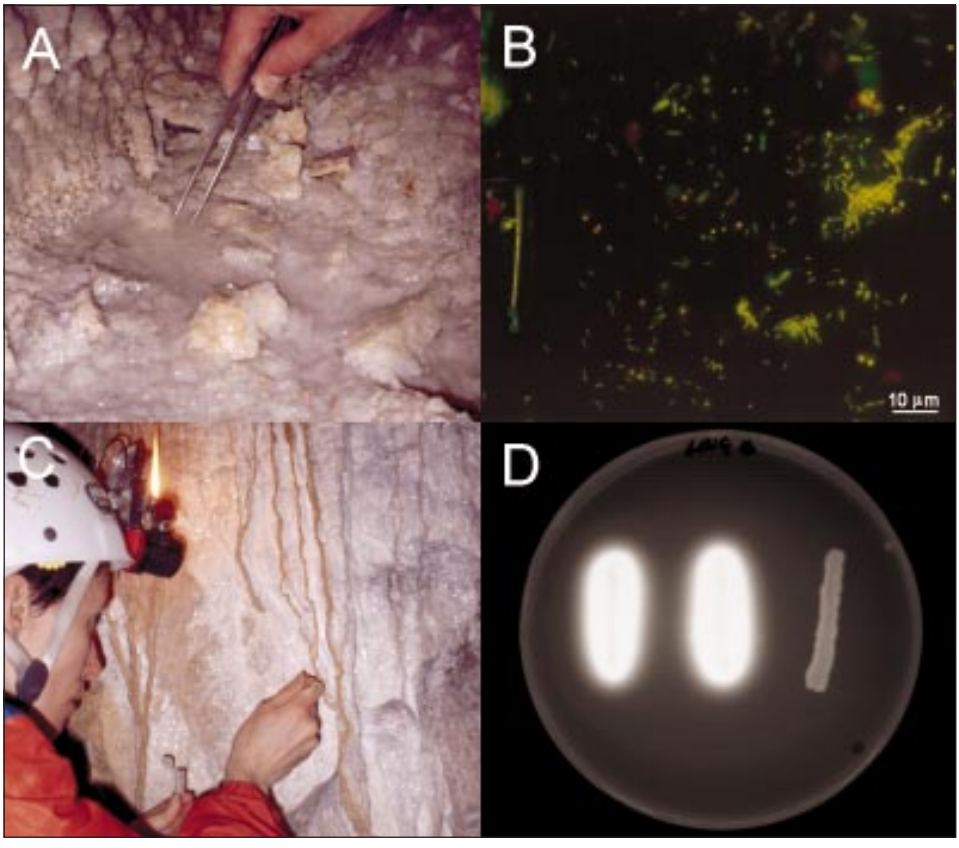

Fig. 3: A: Cave pond with calcite rafts. B: Fluorescence microscopy of the cave pond biofilm after staining with acridine orange. Note different cell morphotypes (rods and cocci). Bar represents 10 µm. C: Sampling procedure of the shiny droplets of condensed water called cave silver. D: Identification of the fluorescent pigment producing strain on KingB medium illuminated with an UV light; left - pioverdin producing positive control strain of Pseudomonas aeruginosa; middle - strain isolated from tha cave pond belonging to the fluorescent pseudomonads group; right - non fluorescent control strain of Pseudomonas stutzeri.
(Photo: A, Jurij Hajna; B, Janez Muhec; C, Nadja Zupan Hajna; D, Barbara Gepič)

Microorganisms in the studied cave microenvironments differed in viable cell count. In the case of Pečina v Borštu cave samples it was shown that microbial flora differs not only in the cell count, but also in the community structure (Table 2). In the silver flashing droplets we isolated only one strain of bacteria belonging to the fluorescent pseudomonads group. However, the presence of these bacteria is not a reason for fluorescence, as droplets show no fluorescence under UV light. In the case of pond sample we isolated quite heterogeneous bacterial flora, belonging to different groups (Table 2). Weathered limestone microorganisms was also heterogeneous. Fluo-

rescent pseudomonads seem to be prevalent microorganisms which can be due to their versatile metabolic pathways. Besides bacteria, fungi also seem to be present in some cave microenvironments. Although they are generally considered as saprophytes, there also may be some genera and species emerging in extreme oligotrophic and other extreme environments.

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PROUČEVANJE MIKROORGANIZMOV IZ RAZLIČNIH JAMSKIH MIKROOKOLIJ (SLOVENIJA) Z GOJITVENIMI TEHNIKAMI

Povzetek

V prispevku je prikazan pregled nekaterih jamskih mikrookolij, ki jih morebiti naseljujejo avtohtoni mikroorganizmi. Proučevani mikroorganizmi so iz različnih jamskih mikrookolij in jam: Pečina v Borštu, Martinska jama ter Snežna jama na Raduhi. V jamah Pečina v Borštu in Martinski jami je v veliki meri prisoten preperel apnenec. Snežna jama na Raduhi je znana po kalcitnem jamskem mleku. Namen raziskave je bil določitev in primerjava mikrobne flore iz različnih jamskih mikrookolij.

Jamo Pečina v Borštu sestavljajo zgornje kredni apnenci. Danes ni več aktivnega vodnega toka. Prenikanje vode, kot posledica padavin, je v nekaterih predelih jame močnejše po izdatnejših padavinah. Temperatura je v jami relativno konstantna. V vhodnih predelih jame se pojavlja takoimenovano jamsko srebro, ki je verjetno posledica kondenzacije vodnih kapljic na steni v vhodnem predelu jame. Pojav je dobil takšno ime, ker kapljice po osvetlitvi s karbidno lučjo šibko srebrno fluorescirajo. V tem mikrookolju smo izolirali fluorescirajoče pseudomonade.

Z vzorčenjem preperelega apnenca (Pečina v Borštu) je bila dokazana relativno visoka mikrobna zastopanost ($1{,}1\times10^6$ CFU/ml), ter vrstna pestrost izolatov. Izolate smo uvrstili v različne skupine na podlagi mikromorfoloških lastnosti, biokemijskih lastnosti ter molekularno-bioloških testov. Tako smo iz preperelega apnenca izolirali in določili glivo, ki spada v skupino *Cladosporium herbarum*. V jami Pečina v Borštu smo vzorčili tudi vodo iz ponvice, na površini katere se odlaga kalcit v obliki kalcitnih ploščic. V tem mikrookolju je bila dokazana največja mikrobna pestrost, z nekoliko nižjim skupnim številom kultivabilnih mikroorganizmov ($2{,}5\times10^4$ CFU/ml) v primerjavi z mikrookoljem preperelega apnenca. Izločanje kalcita v ponvici je morebiti povezano s prisotnostjo mikroorganizmov, saj so tudi pogoji za nastanek biofilma ustrezni.

V Martinski jami, nastali na krednih apnencih, smo vzorčili preperel apnenec in določili $1{,}3\times10^3$ CFU/ml, kar je manj v primerjavi s podobnim vzorcem iz jame Pečina v Borštu. Zanimivo je, da ni bilo mikrobne rasti v primeru, ko smo vzorec preperelega apnenca iz Martinske jame nanesli na gojišče, pripravljeno iz preperelega apnenca iz jame Pečina v Borštu. To kaže, da je mikroflora preperelega apnenca Martinske jame zelo prilagojena na specifično okolje.

V vzorcu kalcitnega jamskega mleka iz Snežne jame na Raduhi, nastale iz zgornjih triasnih apnencev, smo določili $6{,}4\times10^2$ CFU/ml mikroorganizmov.

Rezultati kažejo, da se mikrobna flora v proučevanih mikrookoljih razlikuje tako po številu mikroorganizmov, ki jih lahko gojimo, kot tudi v strukturi združbe. Fluorescirajoče pseudomonade so v večini proučevanih mikrookolij prevladujoči mikroorganizmi.