BABEŞ-BOLYAI UNIVERSITY
FACULTY OF BIOLOGY AND GEOLOGY
DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

Summary

Scientific Supervisor

Prof. Dr. Horia Leonard BANCIU

PhD student

Diana Felicia PANAIT

CLUJ-NAPOCA

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Diversity and potential roles of prokaryotic communities in Romanian cave ecosystems

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Preface

The current doctoral thesis represents the product of five years (2018-2023) of research performed at the Department of Molecular Biology and Biotechnology, from the Faculty of Biology and Geology, Babeș-Bolyai University (Cluj-Napoca, Romania), rigorously supervised by Prof. Dr. *Habil*. Horia Leonard Banciu.

This research takes a snapshot of the diversity and function of bacterial communities associated to dry and wet sediments in a selected number of caves spanning pristine or with little human impact and show caves from Romania. It is focused on addressing key questions about microbial, predominantly bacterial, communities – such as their taxonomy, (functional) diversity, adaptations to specific niches, and connectivity – to better understand their roles within cave food webs, using a range of approaches to achieve these insights.

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The funding of this doctoral research also included the doctoral grant, and a scholarship grant awarded through the project POCU/380/6/13/123886 "*Antreprenoriat pentru inovare prin cercetare doctorală și postdoctorală"*.

Outline of the thesis

Caves are habitats characterized by limiting conditions such as low nutrient availability, low temperature, and lack of light. Terrestrial cave ecosystems are inhabited by diverse microorganisms that play important ecological roles. The major inhabitants of these ecosystems are bacterial communities, and investigation of these communities sheds light on the adaptation mechanism (e.g., adaptive strategies to low temperature and nutrient availability), metabolic capacities, and ecological functions of bacteria thriving in these harsh environments (Tomczyk-Żak and Zielenkiewicz, 2016).

The **general aim** of this thesis is to explore and elucidate the diversity, functionality and involvement of prokaryotic communities in trophic chains in caves from Romania. This doctoral thesis aims:

i. to investigate bacterial diversity in pristine/non-touristic areas of six Romanian caves by employing both culture-dependent and culture-independent techniques;
ii. to evaluate the cold adaptability/viability of isolated bacteria associated to cave sediments;
iii. to address the capabilities of cave microbial communities to metabolize diverse organic nutrients by using community-level physiological fingerprinting;

Chapter I: Diversity and freeze tolerance of culturable bacteria associated with sediments from Romanian caves

The oligotrophic cave habitats are populated by microbial communities that play key roles in supporting the typically short-length food-chains of cave ecosystems (Epure et al., 2012), being involved in sulfur oxidation (Yun et al., 2018), manganese oxidation (Rossi, 2010), methane oxidation processes (Cheng et al., 2022) or formation of the so called moonmilk deposits (Sanchez-Moral, 2012). Despite the many studies which focused on isolating bacteria from cold environments, research on bacteria specifically isolated from caves and their adaptations to extreme temperatures, whether cold or hot, remains limited. Consequently, there is a significant opportunity to search for novel microorganisms in cave (Engel, 2010) and explore their adaptations to cold environments (Ruiz-Blas et al., 2023).

This chapter focuses on assessing the culturable bacterial diversity associated to the sediments sampled from the Cloşani, Ferice, Leşu, Muierilor, Movile, and Urşilor caves. Furthermore, the viability of bacteria isolated following a freeze cycle of almost three years was assessed to evaluate their tolerance to cold (freezing) conditions. The high occurrence of specific species (such as those belonging to *Pseudomonas* and *Bacillus*) indicates that they may actively contribute to the nutrient cycling and ecological interactions within the microbial communities of investigated cave ecosystems. *Bacillales* species such as *Bacillus thuringiensis* and *Bacillus mycoides* appear as highly resilient to freezing most probably due to their spore-forming physiology. On the other hand, species related to *Sphingomonadales*, *Pseudomonadales*, and *Burkholderiales* are more abundant in fresh samples, suggesting they are more sensitive to freezing or naturally more prevalent in fresh sediments. Freezing significantly impacted bacterial recovery, with a decrease in the number of culturable bacterial isolates observed in samples collected from caves like Cloşani, Ferice, and Movile. By comparing fresh and post-freezing conditions culturable bacterial diversity, to the best of our knowledge, this study provides novel insights into bacterial survival strategies. The relevance of this study could offer potential implications for environmental microbiology, climate change research, and biopreservation.

Chapter II: Community-level physiological profiling of carbon substrate metabolization by microbial communities associated with cave sediments and water

Chapter II was published as **Panait, D.F.**, Panait, A.M., Cristea, A., Levei, E.A., Moldovan, O.M., and Banciu, H.L., (2025), Community-level physiological profiling of carbon substrate metabolization by microbial communities associated to sediments and water in karstic caves from Romania, preprint bioRxiv 2025.02.17.638648; https://doi.org/10.1101/2025.02.17.638648

In **Chapter II** we address the functional diversity patterns of microbial communities from Cloșani, Ferice, Muierilor, Leșu, and Topolnița caves capable of utilizing carbon substrates by employing Biolog® EcoPlate™-based assay. The findings highlighted in this chapter reveal that the microbial communities within water samples exhibited the highest metabolic diversity, showing the capacity to utilize a broad range of C-sources such as D-galacturonic acid , L-asparagine, and Tween 80. On the other hand, the sediment samples showed lower activity than that water-asociated counterpart with substrate-specific preferences for L-serine and L-arginine, while the C-substrate utilization pattern by the communities present in moonmilk samples showed low activity, limited to utilization at higher rates for Tween 80 and D-galacturonic acid. These results suggest that environmental conditions drive temporal patterns in C-sources utilization, providing a foundation for future studies to explore the mechanisms underlying these dynamics and their broader implications for cave ecosystems' functioning.

Chapter III: Diversity, distribution and organic substrates preferences of microbial communities of a low anthropic activity cave in North-Western Romania

Chapter III was published as **D. F. Bogdan**, A. I. Baricz, I. Chiciudean, P. A. Bulzu, A. Cristea, R. Năstase-Bucur, E. A. Levei, O. Cadar, C. Sitar, H. L. Banciu and O. T. Moldovan, Diversity, distribution and organic substrates preferences of microbial communities of a low anthropic activity cave in North-Western Romania, in *Frontiers in Microbiology* 14:962452., https://doi.org/10.3389/fmicb.2023.962452

Chapter III focuses on investigating the microbial, particularly the bacterial, diversity, abundance, distribution and organic substrate preferences in *Peştera cu Apă din Valea Leşului* (Leşu Cave) located in Apuseni Mountains, North-Western Romania. For this, 16S rRNA gene amplicon sequencing and community-level physiological profiling alongside the assessment of environmental parameters in cave sediments and water were employed. The findings of this study revealed that variations in abundance at phylum level suggest *Pseudomonadota* (synonym *Proteobacteria*) as the most prevalent phylum across samples, while at order level these abundances depend on sample types. For the most dominant orders the distribution pattern showed a positive correlation with carbon sources such as putrescine, γ-amino butyric acid, and D-malic acid, while particular cases were positively correlated with polymers (Tween 40, Tween 80 and α-cyclodextrin), carbohydrates (α-D-lactose, i-erythritol, D-mannitol) and most of the carboxylic and ketonic acids. The results of this chapter are first indications of microbial diversity from a low anthropic cave located in North-Western Romania, Leşu Cave, and the metabolic potential of the microbial communities inhabiting this ecosystem. This study is original in its comprehensive approach, combining 16S rRNA gene amplicon sequencing with community-level physiological profiling (CLPP) providing novel insigths into both the distribution of bacterial taxa and their capabilities on degrading organic substrates. Overall, this chapter serves as a starting point for further studies in elucidating the links between the taxonomic and functional diversity of subterranean bacterial communities.

Chapter IV: Taxonomic diversity and abundance of microbial communities in sulfide-rich sediments of Movile Cave

Part of the Chapter IV was published as Chiciudean, I., Russo, G., **Bogdan, D.F.**, Levei, E.A., Faur, L., Hillebrand-Voiculescu, A., Moldovan, O.T., and Banciu, H.L. Competition-cooperation in the chemoautotrophic ecosystem of Movile Cave: first metagenomic approach on sediments. *Environmental Microbiome* 17, 44 (2022). https://doi.org/10.1186/s40793-022-00438-w

Chapter IV focuses on the exploration of microbial diversity associated to dry sediments in the chemoautotrophic ecosystem of Movile Cave (South-Eastern Romania) by using shotgun metagenomics. The results highlight significant taxonomic diversity within Movile Cave sediments, with microbial communities consisting of representatives affiliated to 22 phyla. Dominant groups include *Acidobacteriota*, *Chloroflexota*, *Proteobacteria*, and *Planctomycetota*, alongside candidate phyla such as "*Ca*. Patescibacteria", "*Ca*. Zixibacteria", "*Ca*. KSB1", and "*Ca*. Krumholzibacteriota". Notably, over 60% of metagenome-assembled genomes (MAGs) remain unaffiliated with known genera, while 30% are identified as new species. This is the first report on microbial diversity in the dry sediments of the chemoautotrophic Movile Cave ecosystem, as previous studies have focused exclusively on the ecology of its sulfide-rich, oxygen-depleted pools.

Chapter V: Taxonomic diversity and abundance of microbial communities in sulfide-rich sediments of Movile Cave

Chapter V summarizes the main achievements of this thesis along with general concluding remarks, originality, and perspectives.

V.1. General conclusions

This thesis gives a comprehensive examination of the diversity, metabolic capabilities, and ecological functions of prokaryotic communities residing in various cave habitats from Romania, such as sediments, pool water and substrate (limon), and moonmilk. The integration of culture-based, physiological, and metagenomic methodologies revealed insights into microbial diversity and the largely uncharted taxonomic and functional microbial landscape present in these nutrient-limited environments.

V.2. Original contributions of the thesis

The main contributions of the thesis to cave microbiology, such as the (1) comparison of fresh and post-freezing culturable bacterial diversity, (2) the use of Generalized Additive Models (GAMs) for analyzing Community-Level Physiological Profile (CLPP) data in cave settings, (3) integration of 16S rRNA gene amplicon sequencing with CLPP for Leşu Cave and lastly, (4) it provides metagenome-assembled genome (MAG) datasets for Movile Cave, identifying novel candidate taxa and chemolithoautotrophy as a strategy to survive in this type of ecosystem.

V.3. Perspectives

Overall, these findings reported in the present PhD thesis highlight the critical role of bacterial communities in (organic) nutrient cycling and ecosystem stability within these unique cave habitats, emphasizing the importance of microbial contributions in sustaining subterranean food webs. Future research should focus on enhancing the comprehension of microbial adaptations to extreme subterranean environments by employing integrated omics approaches which could validate the presence of functional genes associated with cold/hot tolerance, chemolithoautotrophy and nutrient cycling. Expanding statistical and computational models (e.g., GAMs) could yield more profound insights into microbial interactions and metabolic networks.

List of publications included in the thesis as chapters

Panait, D.F., Panait, A.M., Cristea, A., Levei, E.A., Moldovan, O.M., and Banciu, H.L., (2025), Community-level physiological profiling of carbon substrate metabolization by microbial communities associated to sediments and water in karstic caves from Romania, preprint bioRxiv 2025.02.17.638648; https://doi.org/10.1101/2025.02.17.638648, (**Chapter II**)

Bogdan D.F., Baricz A.I., Chiciudean I., Bulzu P.A., Cristea A., Năstase-Bucur R., Levei E.A., Cadar O., Sitar C., Banciu H.L. and Moldovan O.T., (2023), Diversity, distribution and organic substrates preferences of microbial communities of a low anthropic activity cave in North-Western Romania. *Front. Microbiol*. 14:962452, https://doi.org/10.3389/fmicb.2023.962452 (**Chapter III**)

Chiciudean, I., Russo, G., **Bogdan, D.F.**, Levei, E.A., Faur, L., Hillebrand-Voiculescu, A., Moldovan, O.T., and Banciu, H.L. Competition-cooperation in the chemoautotrophic ecosystem of Movile Cave: first metagenomic approach on sediments. *Environmental Microbiome* **17**, 44 (2022). https://doi.org/10.1186/s40793-022-00438-w (**Chapter IV**)

List of publications not included in the thesis

Porav, A. S., Bocăneală, M., Fălămaş, A., **Bogdan, D. F.**, Barbu-Tudoran, L., Hegeduş, A., & Dragoş, N. (2020). Sequential aqueous two-phase system for simultaneous purification of cyanobacterial phycobiliproteins. *Bioresource technology*, *315*, 123794, https://doi.org/10.1016/j.biortech.2020.123794

Attendances at International and National Conferences

1. **Bogdan, D.F.**, Cristea, A., Buda, D. M., Baricz A. I., Ionescu, M., Crăciun, A., Moldovan, O. T., and Banciu, H. L., (2019), *Diversity and antibiotic resistance of heterotrophic bacteria isolated from Muierilor Cave (Romania)*
   - **Poster** at Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology (SRBBM), Iaşi, Romania (abstract available in Journal of Experimental & Molecular Biology, ISSN: 2601-6974).
2. **Bogdan, D.F.**, Cristea, A., Buda, D. M., Baricz A. I., Moldovan, O. T., and Banciu, H. L., (2019), *A snapshot on the culturable aerobic heterotrophic bacteria isolated from Muierilor Cave (Romania)*.

- **Poster** at Annual Conference of the Faculty of Biology and Geology *BIO.T.A. – Biodiversitate, Tradiţii şi Actualitate*, Cluj-Napoca, Romania.

3. Kovacs, E., Levei, E.-A., Török, A. I., Cadar, O., Sitar, C., Faur, L., Năstase-Bucur, R., Kenesz, M., Petculescu, A., **Bogdan, D. F.**, Chiciudean, I., Banciu, H. L., Atkinson, I., Fruth, V., and Moldovan, O. T, (2020) *Chemical characterization of sediments in the Topolniţa karst cave (SW Romania)*.
    - **Oral presentation**, 47th International Conference of Slovak Society of Chemical Engineering, Slovakia (online).
4. **Bogdan, D. F.**, Chiciudean, I., Baricz, A.-I., Szekeres, E., Ionescu, M., Muşat, N., Remizovschi, A., Levei, E. A., Năstase-Bucur, R. M., Sitar, C., Kenesz, M., Mirea, I. C., Moldovan, O. T., & Banciu, H. L., (2020), *Metabolic fingerprinting of microbial communities from cave ecosystems: ecological and socio-economical relevance*
    - **Oral presentation** – contest at National Conference for PhD Students "Impact of my research project in society", 3rd edition, University of Bucharest (online).
5. Vaniga, N. P., **Bogdan, D. F.**, Moldovan, O. T., and Banciu, H. L., (2021), *Diversity of cultivable heterotrophic bacteria from three Romanian karst caves*
    - **Oral presentation** at 6th edition of the *Young Researchers in BioSciences* International Symposium, Studia Universitatis Babeş-Bolyai, Biologia, 2021, Vol 66, Issue 1, p32, ISSN 1221-8103, Cluj-Napoca, Romania.
6. **Bogdan, D. F.**, Chiciudean, I., Bulzu, P. A., Baricz, A. I., Năstase-Bucur, R., Levei, E. A., Sitar, C., Mirea, I. C., Moldovan, O. T., and Banciu, H. L., (2021), *Assessment of bacterial diversity and community-level physiological profiles associated to sediments and water from Peştera cu Apă din Valea Leşului (Romania)*
    - **Oral presentation** at Annual Conference of the Faculty of Biology and Geology *BIO.T.A. – Biodiversitate, Tradiţii şi Actualitate*, Cluj-Napoca, Romania.
7. Chiciudean, I., Russo, G., **Bogdan, D. F.**, Levei, E. A., Faur, L., Moldovan, O. T., and Banciu, H. L., (2021), *Ecological interactions inferred from metagenomic analysis of Movile Cave sediments*
    - **Poster** at Annual Conference of the Faculty of Biology and Geology *BIO.T.A. – Biodiversitate, Tradiţii şi Actualitate*, Cluj-Napoca, Romania.
8. **Bogdan, D. F.**, Baricz, A. I., Chiciudean, I., Bulzu, P. A., Năstase-Bucur, R., Levei, E. A., Sitar, C., Mirea, I. C., Banciu, H. L., and Moldovan, O. T., (2022), *Evaluation of bacterial*

diversity and community-level physiological profiles associated to sediments and water from a karst cave

- **Poster** at Annual Conference of Federation of European Microbiological Societies, Belgrade, Serbia (abstract book available online: https://www.femsbelgrade2022.org/abstract-book).

9. Chiciudean, I., **Bogdan, D. F.**, Moldovan, O. T., and Banciu, H. L., (2022), *A glimpse into the biosynthetic potential and resistome of microbial communities inhabiting sulfidic, chemoautotrophic Movile Cave*
   - **Oral presentation** at 25th International Conference on Subterranean Biology – ARPHA Conference abstracts, Cluj-Napoca, Romania (abstract available online: https://doi.org/10.3897/aca.5.e86928)
10. **Panait, D.F.**, Porav, A. S., and Barbu, L., (2023), *Scanning transmission electron microscopy (STEM) use in determination of 3D structures of metalloproteins. Case study: cyanobacterial photosystem I*
    - **Oral presentation** at Annual Conference of Romanian Electron Microscopy Society (CREMS), Cluj-Napoca, Romania.

Involvement in various projects

1. **2018**, **member** – Developing the entrepreneurial spirit among UBB students with the target group, **CNSIS-FDI-2018-0096**.
2. **2018–2022**, **member** (Research assistant) - National grant **PN-III-P4-ID-PCCF-2016-0016**, contract no. 15/10.10.2018; title: „Food chains in the dark: diversity and evolutionary processes in caves (DARKFOOD)"; responsible partner P1-UBB: Prof. Dr. Habil. Horia Leonard Banciu.
3. **2023-2024, education expert – ROSE AG 299/SGU/NC/II/23.12.2019**

Work mobilities

1. **Guest Research Assistant**, **2021** (4 months), Helmholtz Centre for Environmental Research (UFZ), Leipzig, Germany.
2. **Erasmus teaching mobility** grant, **June 2023**, Mutah University, Jordan.
3. **Erasmus teaching mobility** grant, **March 2024**, University of DaNang, Vietnam
4. **Erasmus teaching mobility** grant, **April-May 2024**, Baku Engineering University, Azerbaijan

5. **Erasmus staff training mobility** grant, **September 2024**, Clarkson University, United States of America.

Teaching activity related to thesis topic

Includes a list of Bachelor and Master thesis supervised as second coordinator:

1. Bachelor thesis of Luminița Ciucă, *Amprentarea metabolică a comunităților bacteriene din peșteri prin metoda Biolog™ ECOPLATE*, **2020**.
2. Bachelor thesis of Nicoleta Vaniga, *Identificarea moleculară a unor izolate bacteriene din probe de peșteră*, **2020**.
3. Master thesis of Roxana Marin, *Analiza tiparelor de diversitate metabolică a comunitpților bacteriene din peșterile Izvorul Tăușoarelor și Topolnița prin metoda Biolog™ ECOPLATE*, **2021**.
4. Master thesis of Anamaria Petruț, *Izolarea și identificarea diversității bacteriene în sedimente de la Peștera Movile și determinarea profilului de rezistență la antibiotice a acestora*, **2021**.
5. Master thesis of Nicoleta Vaniga, *Izolarea și identificarea moleculară a unor izolate bacteriene din sedimente de peșteră supuse unui ciclu de îngheț-dezgheț artificial*, **2022**.