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Soil and Sediments in Natural Underground Ecosystems as a Source of Culturable Micromycetes: A Case Study of the Brestovská Cave (Western Tatras, Slovakia)

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Abstract: Soil and sediment host microorganisms are able to survive in extremely resource-limited environments. Therefore, more and more attention is being paid to cave sediments as a reservoir of microbiota. The aim of this study is the speleomycological evaluation of the culturable soil and sediment fungal communities in the Brestovská Cave. To explore the origins of fungi, speleomycological studies were conducted both inside and outside the cave under investigation. Additionally, two incubation temperatures (5 and 24 °C) were used to increase the species spectrum of isolated fungi. To achieve the most accurate species identification, we combined an assessment of morphological characteristics of the isolates with molecular sequencing (ITS, internal transcribed spacer). Twenty different species were found and the most frequent was Penicillium commune, followed by Trichosporiella cerebriformis and Pseudogymnoascus pannorum. To our knowledge, our study has enabled the first identification of fungal species such as Penicillium swiecicki, Cephalotrichum hinnuleum, Cosmpospora berkeleyana, Lecythophora hoffmannii, Ambomucor seriatoinflatus, and Mortierella minutissima in underground sites. Our data showed that the abundance and composition of the fungal community varied between the indoor and outdoor samples and thus from the entrance and less visited sites deeper in the cave.

Keywords: caves mycology; Slovakian geoheritage; cave protection; new species

1. Introduction

Cave ecosystems display unique environmental conditions that are unfavorable for the life and development of most living organisms [1]. They appear as highly zonal environments with characteristics defined by the ground waters, surrounding rocks, and karst morphology [2–4]. The conditions of subterranean facilities significantly differ from the surface and include, i.e., a constant temperature through the year (oscillating between 6 to 10 °C), high humidity (often close to saturation) [5], minimal to no light, restricted or absent air flow with the outside environment, oligotrophy, and sometimes elevated levels of CO₂ [6,7]. All of the above occur especially in the deeper parts of the underground sites [8–11]. As a result, only a few microorganisms possess specific adaptations to cope with such living conditions [9,12]. Thus, the extreme microclimate within the underground makes it a unique source of extremophilic bacterial or fungal isolates [7].

Fungi in the cave environments play important roles—mainly as parasites/pathogens, saprotrophs (decomposers), or mutualistic fungi (e.g., mycorrhizae), but also participants in geological processes [4,13,14]. Some fungal species are known to parasitize insects [15–17], while others may contribute to the inorganic nutrient pool replenishment by dissolving the bedrock [18]. Another unique feature of underground fungi is their ability to take part in the formation of speleothems [19].

The majority of fungal taxa frequently found in caves are cosmopolitan species and might inhabit various environments associated with soils, plant material, or insects [14].
In general, cave environments are nutrient-poor [13,20,21], and hence, they are extreme habitats providing specific ecological niches only for highly specialized microorganisms. However, not every underground site is oligotrophic, as some caves receive a significant input of soil and organic matter [22]. They originate mainly from dripping waters and animal feces [22–24]. Soil and organic substrates can be also brought inside due to external air currents, as well as accidentally by tourists [25,26].

Ascomycota is the dominant fungal phylum in underground ecosystems, representing ~69% of all cultivated fungi [27]. The Basidiomycota phylum makes up 20%, Zygomycota 6.6%, Mycetozoa 2.6%, Oomycota 1%, and other phyla (namely, Amoebozoa, Chytridiomycota, Microsporidiomycota, and Percolozoa) 0.8% [14]. Some studies have suggested that the most commonly found taxa might be a result of the specific study methodology rather than biological patterns in cave mycology. For instance, the use of nutrient-rich media and incubation at room temperature (above 20 °C) support the growth of the commonly reported fungi that belong to Ascomycota [14,25].

In general, fungi have a high plasticity and their capacity to adopt various forms (filaments, yeasts and yeast-like fungi) in response to adverse conditions allows them to inhabit various environments, including soil [28]. In addition, fungi are capable of producing a wide variety of extracellular enzymes which decompose the underground organic residues (such as soil components) [29–31]. Fungi usually dominate in highly or slightly acidic soils [32]. However, as shown by speleomycological research by Naga et al., fungi can also inhabit alkaline calcareous soils [33]. Additionally, many fungal species are able to act as an effective biosorbent of toxic metals such as cadmium, copper, mercury, lead, and zinc [34].

It should be noted that assessing fungal diversity within underground sites is a relatively new discipline, as in the past, only the emergence of selected fungal species was evaluated [14]. Among them, Pseudogymnoascus destructans (Pd), causing a bat disease called white-nose syndrome, brought growing attention to this matter [35]. Pd is able to persist between hibernation cycles of bats within cave soil before infecting bat roosts. Moreover, it can also propagate in cave sediments, especially in keratin-rich areas due to shed bat hair [36].

The main goal of the present research was to conduct the speleomycological evaluation of the culturable soil and sediment fungal communities in the Brestovská Cave (Western Tatras Mts., Slovakia), which is open to tourists. In order to discuss the possible origin of the fungal species, we determined the number and species composition of fungi in the outdoor and indoor soil and sediments samples.

2. Materials and Methods
2.1. Study Area

The Brestovská Cave, situated near Zuberec village in Slovakia’s Tatra National Park (Western Tatras Mts.) [37], stands at an elevation of 867 m asl. Its total length spans 1890 m, of which only 217 m are accessible to the public, making it the largest tourist-accessible cave in Orava and the sole one available for exploration. This cave is part of a vast hydrological system that has formed at the contact of karstic and non-karstic rocks, featuring a river that flows through it. With a temperature ranging between 4 and 6 °C and with a flowing river within, the cave supports mammalian life. Within the Brestovská Cave, nine species of bats have been identified, among which the greater mouse-eared bat (Myotis myotis) is the most prevalent [38].

2.2. Sample Collection

On 24 August 2017, during the absence of tourists, soil and sediment samples were collected from six sites—one outside (near the cave’s entrance) and five within the cave (Figure 1). The collection involved the upper layer of soil/sediment (to a depth of 10 cm) using sterile plastic tools and storing the samples in sterile bags. From each location under study, around 1000 g of material was collected in three replicates. These samples were then
transported to the lab under cooled conditions (10 ± 2.0 °C) and preserved at 5 ± 0.5 °C until the mycological analysis was conducted, which occurred within 7 days of collection.

2.3. Isolation of Fungi from Samples

Before analysis, the samples were aseptically homogenized, and 3 g of the soil/sediment was transferred into sterile, individually sealed conical polypropylene tubes (50 mL) with screw-on lids (FL Medical, Padova, Italy), along with 12 mL of a saline solution (0.85% NaCl). These mixtures were then agitated at an ambient temperature (20 min; 24 ± 1 °C). Subsequently, the samples were diluted at ratios of 50, 500, or 5000 times and vortexed, and 100 µL aliquots were evenly spread onto triple replicate plates containing potato dextrose agar (PDA, Bio-maxima, Lublin, Poland). The plates were incubated at temperatures of 5 ± 0.5 °C and 24 ± 0.5 °C for durations ranging from 5 to 42 days in darkness, from the emergence to the final appearance of colonies. Post-incubation, fungal colonies were enumerated, and the average number of colony-forming units, represented as CFU per g of soil/sediment, were calculated. Fungal colonies were then transferred onto fresh plates with the same agar for subculturing and incubated in darkness at 5 ± 0.5 °C and 24 ± 0.5 °C for periods between 4 and 35 days. Following this incubation period, fungi were isolated using the single-spore technique and subsequently grown on PDA slants for further morphological and molecular identification.

2.4. Fungal Identification

For fungal identification, a dual approach encompassing both phenotypic and genotypic techniques was employed. Pure cultures underwent examination through microscopic and macroscopic analyses. The initial phenotypic classification utilized several mediums including PDA, Czapek yeast autolysate agar (CYA, 30.0 g L⁻¹ sucrose, 15 g L⁻¹ agar, 5.0 g L⁻¹ yeast extract, 3.0 g L⁻¹ NaNO₃, 1.0 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ KCl, 0.5 g L⁻¹ MgSO₄·7H₂O, and 0.01 g L⁻¹ FeSO₄·7H₂O), Czapek–Doxagar (1.2% agar, BioMaxima, Poland), and malt extract agar (MEA, BioMaxima, Lublin, Poland) in the case of *Penicillium* spp., focusing on characteristics like colony hue, expansion, and the presence
of distinctive morphological features such as spores, following established diagnostic keys and monographs [39–61].

Species confirmation involved sequencing the internal transcribed spacer (ITS) regions of fungal rDNA. DNA extraction from PDA-cultivated fungal colonies was performed using the Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Gdańsk, Polska), with ITS regions amplified through PCR using specific primers (ITS1, 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4, 5′-TCTTCCGCTATTGATATGC-3′) [62]. PCR was performed using a T100 Thermal Cycler (Bio-Rad, Berkeley, CA, USA), according to our previous protocol [63]. The PCR products underwent agarose gel electrophoresis on a 1.2% agarose gel, purification (Clean-Up kit, A&A Biotechnology), and sequencing (Macrogen Europe, Amsterdam, The Netherlands).

2.5. Data Analyses

The sequences of PCR products were examined using the BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html, accessed on 10 December 2022). Fungal ITS sequences underwent comparison with those in the NCBI’s GenBank through the BLAST algorithm (http://www.ncbi.nlm.nih.gov/; accessed on 14 December 2022). Criteria established by Zhang et al. [64] guided the interpretation of sequence similarities: a match of ≥97% confirmed the genus and species, a match between 95% and 97% identified the genus only, and a match <95% led to classification at higher taxonomic levels or as ‘unassigned’. These sequences were then uploaded to the GenBank database, accessed on 19 December 2022.

To analyze the count of fungal colonies, the Statistica 12.0 software (StatSoft Polska Sp. z o.o., Kraków, Poland) was utilized, incorporating a one-way analysis of variance (ANOVA) and Tukey’s honest significant difference (HSD) test with a significance level of α ≤0.05. To assess the diversity of fungal species at various sites, the Shannon Diversity Index (H) was employed, calculated with the formula H = −∑ Pi(lnPi), where Pi represents the proportion of each species within the sample [65].

3. Results

The study of micromycetes in sediments/soil of the Brestovská Cave was carried out at one outdoor and five indoor sites using the culturing procedure (PDA medium, incubation at 5 and 24 ± 0.5 °C) (Figure 1). The phenotypic analysis of the obtained fungi allowed for their classification into 20 different species, and molecular studies confirmed those observations. The obtained fungi belong to four phyla (Ascomycota—75% of isolates, Mortierellomycota—15%, Mucoromycota—5%, and Basidiomycota—5% isolates) and thirteen families (Aspergillaceae—represent 20% of isolates, Mortierellaceae—15% of isolates, Coniochaetaceae and Microascaceae—each represent 10% of isolates, and each of the following represent 5% of isolates—Cordycipitaceae, Dermateaceae, Hypocreaceae, Mrakiaceae, Mucoraceae, Myxotrichaceae, Nectriaceae, Oxyrogenaceae, and Pseudonotiaceae). Most of the species belong to filamentous fungi (Ambomucor serialoinflatus, Cephalotrichum himuleum, Chrysosporium merdarium, Cosmopora berkeleyana, Doratomyces stemonitis, Lecanicillium muscarium, Lecythophora hoffmannii, Mortierella elongata, Mortierella hyalina, Mortierella minitissima, Oidiodendron truncatum, Paecilomyces farinosus, Penicillium camemberti, Penicillium chrysogenum, Penicillium commune, Penicillium swiecickii, Pseudogymnoascus pannorum, and Trichoderma atroviride), although two species belong to basidiomycetous and ascomycetous yeast (Tausonia pullulans and Trichosporiella cerebriformis, respectively). The nucleotide sequences of fungal ITS rDNA acquired during this study were deposited in GenBank and assigned accession numbers from OQ073897 to OQ073916. BLAST analysis of these sequences revealed E values of zero, indicating highly significant matches, with identity percentages ranging from 98.98% to 100% and query coverage spanning from 99% to 100%, as detailed in Table 1.
Table 1. Cultured fungi detected in soil and sediment samples of the Brestovská Cave and the results of BLAST analysis (all E values were zero): 1—current name.

<table>
<thead>
<tr>
<th>Isolate Number</th>
<th>Identified Species (Synonyms)</th>
<th>Phylum</th>
<th>Family</th>
<th>GenBank Accession No.</th>
<th>The Sequence Length (bp)</th>
<th>Query Cover %</th>
<th>Identity %</th>
<th>Accession</th>
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<td>Mucoraceae</td>
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<td>Onygenaceae</td>
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The density of mycobiota in the Brestovská Cave at 5 °C ranged from 8.32 to 49.92 CFU × 10^2 per 1 g of sediment/soil samples inside the underground facility and was 91.65 CFU × 10^2 per 1 g of outdoors samples. In the case of the incubation temperature at 24 °C, the overall concentrations of fungal propagules obtained inside the cave were from 9.16 to 156.66 CFU × 10^2 per 1 g, and outside, the concentration was 177.76 CFU × 10^2 per 1 g (Figure 2). In the case of incubation at 5 °C, the highest value of fungal propagule concentration was recorded at site I, although statistically (Tukey HSD test at α ≤ 0.05), this location did not differ significantly from location no. II, III, IV, and VI. In turn, the lowest concentration of fungi was noted at site V, although statistically, this location also did not differ significantly from location no. II, III, IV, and VI. However, significant differences were noted only between locations I and V (p = 0.004211). Meanwhile, the highest statistically significant density of mycobiota in the Brestovská Cave grown at 24 °C was recorded at location no. I and II, and the lowest at location no. V (p = 0.000197). Moreover, in three study sites (I, II, and IV) out of six, statistical differences in the number of obtained fungi between the incubation temperatures of the samples were noted (p = 0.02343, p = 0.04203, and p = 0.034669)—Figure 2.
were cultured only at a temperature of incubation of 5 °C (Figure 5, Table A1).
P. pannorum
also dominated (45.46% of all cultured fungi in the study) in the outside of the cave and 61.18% for the inside samples). In the case of the samples incubated at 5 °C, as at 24 °C (68.76% of all cultured fungi in the study for the outside samples and 42.31% of all cultured fungi in the study, respectively) of the Brestovská Cave at 5 °C as well, the inside samples). In the case of the samples incubated at 5 °C, T. cerebriformis dominated (28.89% of all cultured fungi in the study) in the outside of the cave and P. commune (28.89% of all cultured fungi in the study) in the inside of the cave (Figure 3).

In total, all 20 fungal species identified in this study were discovered within the Brestovská Cave, with only 8 of them (M. elongata, M. hyalina, M. minutissima, O. truncatum, P. commune, P. pannorum, T. atroviride, and T. cerebriformis) isolated from the indoor soil/sediment samples considering both incubation temperatures. In turn, separating the results according to the incubation temperature, in both cases, outside the cave, 4 fungal species were isolated from the sediment and soil samples, and in the case of samples taken inside the cave, 12 species were obtained at 5 °C, and only 9 species at 24 °C (Figure 3, Table A1). Cephalotrichum hinnuleum, Ch. merdarium, C. berkeleyana, D. stemonitis, L. muscarium, M. elongata, M. minutissima, O. truncatum, P. camemberti, T. pullulans, and T. cerebriformis were cultured only at a temperature of incubation of 5 °C (Figure 4, Table A1). In turn, A. seriatoinflatus, L. muscarium, M. hyalina, P. farinosus, P. chrysogenum, P. commune, P. swiecickii, and T. atroviride were isolated only by using an incubation temperature of 24 °C (Figure 5, Table A1).

Penicillium commune dominated overall in the outside and inside samples (45.37% and 42.31% of all cultured fungi in the study, respectively) of the Brestovská Cave at 5 °C as well as at 24 °C (68.76% of all cultured fungi in the study for the outside samples and 61.18% for the inside samples). In the case of the samples incubated at 5 °C, T. cerebriformis dominated (45.46% of all cultured fungi in the study) in the outside of the cave and P. pannorum (28.89% of all cultured fungi in the study) in the inside of the cave (Figure 3).

Similar tendencies regarding the most frequently isolated fungal species were noted in individual locations inside the underground facility, as in the case without division into individual locations of the study, with the exception of study site no. II at 5 °C. Namely, P. pannorum also dominated at 5 °C in study sites no. III, IV, V, and VI (from 33.31% to 58.76%, which corresponded to 2.78 to 20.55 CFU × 10² per 1 g of sediment/soil samples), but O. truncatum was most abundant in location no II at 5 °C and accounted for 33.33% of all fungi obtained in this location (16.66 CFU × 10² per 1 g)—Figure 4, Table A1. In turn, the most frequently isolated species at 24 °C from all five surveyed locations inside the Brestovská Cave was also P. commune, which accounted for 31.92% to 91.70% of all fungi cultured at individual study sites, which corresponded to 6.66 to 91.67 CFU × 10² per 1 g (Figure 5, Table A1).
Figure 3. The percentage of each fungal species contributing to the total fungi cultured from all study sites of soil and sediment samples of the Brestovská Cave.

Figure 4. The percentage contribution of each fungal species to the overall fungi cultured at 5 °C from specific study sites within the soil and sediment samples of the Brestovská Cave.
A similar situation was also noted for the general Shannon index calculated for both incubation temperatures (0.724 for outside samples and 0.837 for no. II and 0.804 for no. III). Moreover, there was positive correlation between the Shannon Diversity Index and the concentrations of fungal propagules for samples incubated at 24 °C and overall for samples incubated at both temperatures (p < 0.05; r = 0.87 and r = 0.41 for 24 °C, and r = 0.20, respectively). However, in the case of incubation at 5 °C, the above correlation was negative (p < 0.05; r = 0.67) (Figure 6).
4. Discussion

Numerous speleomycological studies conducted in recent years have indicated differences in fungal communities depending on the geographic location of the tested object, the location of the study within a given object, the season of the year, the external environment, and the presence of visitors and animals living in them. The presence of animals and the different seasons are among the most important determinants of the occurrence of fungi in underground ecosystems [14,26,66–69]. This was partly confirmed by our previous study in the Brestovská Cave, which investigated the occurrence of psychrophilic and psychrotolerant airborne fungi in air samples [1] as well as the presence of keratinophilic and keratinolytic fungi [7]. This underground ecosystem is distinguished by its unique aeromyco-cota composition, as highlighted by our research findings. For the first time, we identified the presence of fungal species such as Coniothyrium pyrimum, Cystobasidium laryngis, Filobasidium wieringae, Leucosporidium drummii, Mortierella parvispora, Mrakia blollopis, Nakazawaea holstii, and Vishniacozyma victoriae in the air of underground locations. Notably, C. pyrimum, C. laryngis, L. drummii, M. blollopis, and N. holstii were previously undetected in any part of underground ecosystems [1]. During the study of keratinophilic and keratinolytic species in the Brestovská Cave, we discovered for the first time the presence of two species in underground sites: Chrysosporium europae and Penicillium charlesii [7]. Additionally, in that case, we discovered that study sites located inside the cave displayed much more fungal species than on the outside [7]. This conclusion was in contrary to the belief that cave microorganisms are usually carried in the dust from the outside. Taking into account that soil microorganisms are responsible for critical ecosystem processes (biogeochemical cycle of carbon and other nutrients, soil aggregation etc., Acosta-Martinez et al. [70]), investigation of their biodiversity in deeper cave parts seems crucial. Considering the above, we decided to revisit the previously investigated Brestovská Cave and study the fungi inhabiting the soil/sediments in this cave.

Additionally, in our opinion, such analyses are important not only because of the possibility of discovering new fungal species [7], but above all, because the underground object is a popular touristic attraction and contact with potentially pathogenic fungal matter (e.g., spores) [24] might cause serious health issues, especially in individuals with impaired immune systems [71].

Identification revealed the presence of microscopic fungi belonging to various species of distinct environmental interactions, with the highest prevalence of Ascomycota phylum, which is consistent with previous studies [14]. Four Penicillium spp. were found in tested samples: P. chrysogenum, P. camemberti, P. commune, and P. swiecicki. The genus is commonly found in soil, especially in the rhizosphere [72] where it is usually beneficial for plants. Underground ecosystems are also enriched with other Penicillium spp., such as P. chrysogenum, P. camemberti, and P. commune, which are common in European caves [7,24,68,73], but they are also isolated in other parts of the world, e.g., P. chrysogenum was identified in cave soil samples in Russia, Korea, and Thailand, P. camemberti in Australia, and P. commune in northeastern USA [14]. Penicillium chrysogenum was isolated in our previous aeromycological studies of the Brestovská Cave from the air inside and outside of the cave [1]. Moreover, to the best of our knowledge, P. swiecicki was never previously isolated from caves or other underground sites. Overall, Penicillium spp. are known for their adaptation to cold environments due to the cold-active enzymes like endo-1,4-β-glucanasesor phosphatases [74,75]. They have been frequently isolated not only from caves but also from glaciers or Arctic soil [76]. Among Penicillium spp., P. commune was the most abundant taxa isolated, which is commonly associated with cheese spoilage [77]. However, this species is also a known producer of mycotoxins, such as cyclopiazonic acid, penitrem A and roquefortine [78,79]. Despite the fact that roquefortine and penitrem A are not volatile [80] and should not pose a threat to tourists, both display highly neurotoxic effects if accidently ingested [78]. They might be harmful to animals and critters feeding on cave sediments.

Another fungus with high prevalence in extreme environments and also present in the soil of the Brestovská Cave is Pseudogymnoascus pannorum, closely related to P. destructans
which causes white-nose syndrome in bats. Although *P. pannorum* (basionym: *Sporotrichum pannorum* Link 1824; obligate synonyms: *Chrysosporium pannorum* Hughes 1958 and *Géomyces pannorum* Sigler and Carmichael 1976) [53] is not known for its pathogenicity toward bats, it can on rare occasions cause disease in humans and dogs [81,82]. This fungus was also detected in our previous study in the Brestovská Cave—it was isolated from external and internal air samples [1] and internal soil/sediment samples during a study on keratinophilic and keratinolytic fungi [7]. Most likely, its presence in underground locations is attributed to the utilization of various substrates like keratin or complex carbohydrates but also to its tolerance to high salinity [82,83]. Another keratinophilic fungus isolated in the present study is *Chrysosporium merdarium* (basionym: *Sporotrichum merdarium* Link 1818) [46] which was also isolated from internal air samples in our previous study in the Brestovská Cave [1]. However, it had also been isolated from caves in other countries, like Hungary, Russia, and the Czech Republic [14]. Like other keratinophilic fungi, *Chrysosporium* spp. can cause the breakdown of keratinous substrates and potentially infect skin or nails. *Chrysosporium* spp. have been occasionally associated with human disease; however, there is no evidence of *C. merdarium* infections [84–86].

Other new records of species found in cave soil and sediment are *Cephalotrichum himuleum*, which is usually isolated from the rhizosphere, decaying plants and dung [87], and *Cosmospora berkeleyana* (basionym: *Verticillium berkeleyanum* P. Karst. 1891; obligate synonyms: *Acremonium berkeleyanum* (P. Karst.) Gams 1982) [52], which is usually found on fruiting bodies of fungi, but also from soil and rabbit dung [88]. *Lecytophora* (now transferred to *Cioniochaeta*) *hoffmannii* (basionym: *Margarinomyces hoffmannii* J.F.H. Beyma 1938; obligate synonyms: *Phialophora hoffmannii* (J.F.H. Beyma) Schol-Schwarz. 1970) [50], also never before isolated from caves, is an intriguing discovery in our study, since it is a potential pathogen of humans and animals. Although a saprophyte in the environment, *L. hoffmannii* has been associated with human infections resulting in keratitis or subcutaneous abscesses, particularly in immunocompromised patients [89,90].

Among isolated Ascomycota, *Doratomyces stemonitis* (basionym: *Isaria stemonitis* Pers. 1797; obligate synonyms: *Stysanus stemonitis* (Pers.) Corda 1837 and *Periconia stemonitis* (Pers.) Pers. 1801) [47,49] was previously found in caves in the USA, Russia, Slovakia, Japan, and Korea [14]. It is not surprising since this species belongs to coprophilous fungi, inhabiting feces mainly of herbivores. A set of secreted enzymes, like those engaged in the degradation of cellulose, facilitates the degradation of indigestible parts of plants and enables *D. stemonitis* to inhabit this specific environment [91].

*Leccamillium muscarium* (basionym: *Cephalosporium muscarium* Petch 1931; taxon synonyms: *Cephalosporium aphidicola* Petch 1931 and *Verticillium hemileiae* Bouriquet [51]) is an entomopathogenic fungus, frequently isolated from whitefly (*Trialeurodes vaporariorum*) [51] and having a high psychrotolerance, with the ability of some strains isolated from Antarctica to propagate even at 0 °C. Facilitation of the development in harsh conditions may be also due to the activity of lytic enzymes, degrading various substrates like chitin [93]. Another cold-adapted species isolated in the present study is *Oidiodendron truncatum*. We also found its presence during our earlier aeromycological study in the Brestovská Cave [1]. According to some reports, this species was also found in cave soil in Russia and in air samples from Czech Republic caves [14,94].

An important entomopathogen that can inhabit the cave environment is *Paecilomyces farinosus* (current name: *Cordyceps farinosa* (Holmsk.) Kepler, B. Shrestha and Spatafora 2017; basionym: *Ramaria farinosa* Holmsk. 1781; obligate synonyms: *Isaria farinosa* (Holmsk.) Fr., 1832, *Spicaria farinosa* (Holmsk.) Vuill., 1911, *Clavaria farinosa* (Holmsk.) Dicks., 1790, *Corynoides farinosa* (Holmsk.) Gray 1821 and *Penicillium farinosum* (Holmsk.) Biourge 1923) [44,59]. Although it is distributed globally, the cave isolates were found mainly in Europe, and cave soil samples that contained this species were obtained from Slovakia and Italy [14,95].
Among soilborne fungi, *Trichoderma atroviridae* was isolated from the soil and sediment of the Brestovská Cave, which is consistent with previous information about fungi inhabiting other Slovak caves [14]. *Trichosporella cerebriformis* (basionym: *Sporotrichum cerebriforme* de Vries and Kleine-Natrop 1957) [48] is also a species that could be found in soil, with some adaptations to cold environments since it had been isolated from Arctic tundra [96]. Cave soil isolates of this species have been found in the Brestovská Cave but also in caves of the Czech Republic and Russia [14].

Four isolates of Mucormycota phylum were obtained from the soil and sediment of the Brestovská Cave. *Ambomucor seriatoinflatus* was previously isolated from soil samples from Mongolia, China, and Alaska (with low prevalence); however, this is its first occurrence in the cave ecosystems [97,98]. Moreover, three species of the *Mortierella* genus were found in our study. *Mortielella elongate* (current name: *Linnemannia elongata* (Linnem.) Vandepol and Bonito 2020) [43,61] is one of the most prevalent fungi found in soil, with various adaptations to harsh environmental conditions, like low temperatures [99,100]. On the other hand, cave ecosystems are rarely inhabited by this species. Besides the Brestovská Cave, it was isolated only from caves in Belgium and from insect samples [14]. *M. hyalina* (current name: *Linnemannia hyalina* (Harz) Vandepol and Bonito 2020; basionym: *Hydrophora hyalina* Harz 1871) [61] exhibits higher prevalence in cave ecosystems, having been previously isolated from caves in the Czech Republic and Great Britain [14]. On the other hand, the third species—*M. minutissima* (current name: *Podila minutissima* (Tiegh.) Vandepol and Bonito 2020) [39]—had never been reported in caves until now. This species was frequently isolated from soil, decaying plant and animal tissues, or fecal matter; however, due to its psychrotolerant nature, it could be adapted to the cold cave environment [101,102].

The sole species of the Basidiomycota phylum isolated from Brestovská Cave soil and sediment samples is *Tausonia pullulans* (basionym: *Oidium pullulans* Lindner 1901; obligate synonyms: *Basidiotrichosporon pullulans* (Lindner) 1977, *Monilia pullulans* (Lindner) Kloecker 1924, *Oospora pullulans* (Lindner) Sacc. 1906, *Trichosporon pullulans* (Lindner) 1942 and *Guehomyces pullulans* (Lindner) Fell and Scorzetti 2004) [57]. This yeast is commonly found in plant material and soil samples and is considered a psychrotolerant, with a set of produced cold-active enzymes, such as lignin-degrading enzymes or lipases. It also harbors a collection of hydrolytic enzymes, whose activities provide nutrients for fungal growth from various substrates [103,104]. Basidiomycota fungi are generally found in association with substrates rich in nutrients, such as wood and manure, within underground settings. However, these types of substrates are not common components of cave environments, although wooden beams can often be found in old mines, and some caves may house substantial guano deposits [14]. The scarcity of large, nutrient-dense substrates within caves could account for the comparative rarity of Basidiomycota, especially when contrasted with Ascomycota.

A huge role in the effectiveness of the isolation of individual fungal species from samples using culture methods is played by the types of substrates used and the temperature during incubation [83,105]. On the one hand, the Basidiomycota phylum is difficult to culture and identify [69,106], so the methods used in most studies are directed toward the detection of Ascomycota. Zygomycota fungi, characterized by their prolific spore production and swift growth rates, are relatively easy to identify, which could lead to an overestimation of their abundance within cave environments [14]. On the other hand, using specific culture media and temperatures, it is possible to target the isolations toward given functional groups of fungi [24]. This is evidenced, among others, by our previous study in the Brestovská Cave. From the same samples, we carried out isolation targeted at keratinophilic and keratinolytic fungi using Vanbreuseghem hair bait [7]. This resulted in acquiring fungal species that were not detected during the study using PDA medium and the two incubation temperatures. Nevertheless, culture-based analysis is still the most popular method for speleomycological research [1,6,10,14], which is effective for detecting even the spores of slow-growing fungi such as Basidiomycota when the proper medium, incubation temperature, and low sample concentration are used [69].
5. Conclusions

This study sheds new light on the diversity of fungi residing in the soil and sediments of the Brestovská Cave in Slovakia, enriching our understanding of these ecosystems. We successfully isolated 20 fungal species, with 12 unique to the cave’s interior and 8 found both inside and outside. Among the most frequently isolated was *P. commune*, a species known for mycotoxin production. Remarkably, our study marks the first identification of six fungal species in underground ecosystems: *P. swiecickii*, *C. hinnuleum*, *C. berkeleyana*, *L. hoffmannii*, *A. seriatoinflatus*, and *M. minutissima*. This not only enhances our biological knowledge of these species but also highlights the potential risks they pose to immuno-compromised visitors and cave-dwelling fauna. The density of mycobiota decreased with distance from the cave entrance and was primarily influenced by the external environment. In turn, the observation of greater species diversity inside the cave compared to outside it can most likely be attributed to its unique ecosystem and suggests a need for further investigation into spore dispersion mechanisms, such as insect vectors, to develop protective measures for both cave preservation and visitor safety.

**Author Contributions:** Conceptualization, R.O.; methodology, R.O.; software, R.O.; validation, R.O. and J.S.; formal analysis, R.O.; investigation, R.O. and J.B.; resources, R.O.; data curation, R.O. and J.S.; writing—original draft preparation, R.O., J.B., K.S., A.P. and J.S.; writing—review and editing, R.O., J.B., K.S., A.P. and J.S.; visualization, R.O.; supervision, R.O.; project administration, R.O.; funding acquisition, J.S. All authors have read and agreed to the published version of the manuscript.

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**Appendix A**

**Table A1:** The average number (CFU \( \times 10^2 \) per 1 g) of fungi cultured from soil and sediment samples of the Brestovská Cave: 1— not detected.

<table>
<thead>
<tr>
<th>Fungal Species</th>
<th>Fungi Cultured at 5 °C</th>
<th>Fungi Cultured at 24 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td><strong>Ambomucor seriatoinflatus</strong></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Cephalotrichum hinnuleum</strong></td>
<td></td>
<td>1.25</td>
</tr>
<tr>
<td><strong>Chrysosporium merdarium</strong></td>
<td></td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Cosmospora berkeleyana</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Doratomyces stemonitis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lecanicillium muscarium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lecythophora hoffmannii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mortierella elongata</strong></td>
<td>16.66</td>
<td>2.91</td>
</tr>
<tr>
<td><strong>Mortierella hyalina</strong></td>
<td>25.00</td>
<td>15.83</td>
</tr>
<tr>
<td><strong>Mortierella minutissima</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oidiadoron truncatum</strong></td>
<td>8.33</td>
<td>16.66</td>
</tr>
<tr>
<td><strong>Paeilomyces farinosus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Penicillium camemberti</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Penicillium chrysogenum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Penicillium commune</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Penicillium swiecickii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pseudogymnoascus pannorum</strong></td>
<td></td>
<td>2.50</td>
</tr>
<tr>
<td><strong>Tausonia pullulans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trichoderma atroviride</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trichosporiella cerebriformis</strong></td>
<td>41.66</td>
<td></td>
</tr>
</tbody>
</table>


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