

Article

Effects of Speleotherapy on Aerobiota: A Case Study from the Sežana Hospital Cave, Slovenia

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Abstract: Speleotherapy is one of the non-pharmacological methods for the treatment and rehabilitation of patients with chronic respiratory diseases, especially those with chronic obstructive pulmonary disease (COPD) and asthma. On the one hand, one of the alleged main advantages of speleotherapeutic caves is the low microbial load in the air and the absence of other aeroallergens, but on the other hand, due to the lack of comprehensive air monitoring, there is little information on the pristine and human-influenced aerobiota in such environments. The aim of this study was to assess the anthropogenic effects of speleotherapy on the air microbiota and to investigate its potential impact on human health in Sežana Hospital Cave (Slovenia). From May 2020 to January 2023, air samples were collected in the cave before and after speleotherapeutic activities using two different volumetric air sampling methods—impaction and impingement—to isolate airborne microbiota. Along with sampling, environmental data were measured (CO₂, humidity, wind, and temperature) to explore the anthropogenic effects on the aerobiota. While the presence of patients increased microbial concentrations by at least 83.3%, other parameters exhibited a lower impact or were attributed to seasonal changes. The structure and dynamics of the airborne microbiota are similar to those in show caves, indicating anthropization of the cave. Locally, concentrations of culturable microorganisms above 1000 CFU/m³ were detected, which could have negative or unpredictable effects on the autochthonous microbiota and possibly on human health. A mixture of bacteria and fungi typically associated with human microbiota was found in the air and identified by MALDI-TOF MS with a 90.9% identification success rate. *Micrococcus luteus*, *Kocuria rosea*, *Staphylococcus hominis*, and *Staphylococcus capitis* were identified as reliable indicators of cave anthropization.

Keywords: bioaerosol; air sampling; cave anthropization; speleotherapy; COPD; asthma



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1. Introduction

Chronic respiratory diseases are among the most common non-communicable diseases worldwide, largely due to widespread exposure to harmful environmental stimuli as well as genetic and lifestyle factors [1–3]. Among them, chronic obstructive pulmonary disease (COPD) and asthma are the most prevalent diseases affecting both children and adults, with a global prevalence of 3.9% and 3.6%, respectively. COPD and asthma are also the leading causes of death associated with chronic respiratory diseases worldwide. In the European Union, for example, there were 2,348,184 deaths attributable to COPD between 1994 and 2010 [1,4,5]. The treatment of COPD and asthma is quite complex as there is no cure. Therefore, the treatment of these diseases aims to control symptoms, prevent disease progression, restore patients' daily activities, and minimize the impact of the disease on

quality of life [2,4,6]. Traditionally, treatment is pharmacologic, but patients may also benefit from non-pharmacologic treatment approaches [2,6]. As COPD and asthma are strongly influenced by environmental factors such as tobacco smoke, pollen, molds, and other aeroallergens, it is logical that environmental modification could be beneficial [2,6–8]. The aggravation of COPD and asthma can be influenced by environmental factors, including bioaerosols. Bioaerosols, i.e., airborne particles of biological origin, include a wide range of organisms and substances, such as bacteria, fungi, viruses, pollen, and spores. These particles can cause respiratory diseases, inflammation, allergic reactions, and infections when inhaled [9–11]. Therefore, environmental measures that include reducing allergens in the home (e.g., house cleaning or dust mite control) and/or eliminating irritants (e.g., avoidance of secondhand smoke) are often recommended in the management of asthma [2,6,7]. More advanced environmental modification strategies have also been proposed, such as medical rehabilitation in spas, high-altitude climatotherapy, and speleotherapy [2,7,8,12]. The latter uses the climatic conditions of caves and salt mines for the rehabilitation treatment of chronic respiratory diseases, in particular COPD and asthma, as well as for some dermatological diseases, such as atopic dermatitis in children [12–14].

Speleotherapy began to gain attention after the Second World War, particularly in Eastern and Central Europe, and is associated with beneficial effects on patients with chronic and allergic respiratory diseases [8,12,15,16]. The reason for the clinical improvement of these diseases lies in the microclimatic characteristics of the caves that mitigate the inflammatory response—high relative humidity, constant air temperature, aerosolized minerals, little or no wind, short exposure to occasional natural elevated levels of radiation, and the absence of external pollutants, mites, pollen, and other particles that exacerbate chronic respiratory diseases [8,12,17,18]. Together, these factors facilitate expectoration, relax the respiratory muscles, stimulate the self-cleaning function of the lungs, and have an anesthetic effect [8,12]. Speleotherapy, therefore, helps asthmatics treat the disease themselves and minimize the use of medications [19–21]. The positive effect of speleotherapy is usually seen after 3–4 weeks of treatment with a daily stay in a cave for 3–6 h, depending on the temperature in the subterranean environment [19,21]. Low levels of air biocontamination are, therefore, considered an important factor in speleotherapy, but the data about microbiological air monitoring in speleotherapeutic caves are still scarce [20,22–25]. Monitoring of bioaerosols and climatic conditions in speleotherapeutic caves is important from the point of view of both the therapy and environmental protection [22,23,26]. Therefore, the question arises whether patients and medical staff can significantly alter the composition and concentration of microbes in the air to such an extent that the environment in the cave becomes less favorable for both patients with respiratory diseases and the indigenous microbiota of the cave itself.

Anthropogenic changes in aerobiota have already been described in show caves, which are accessible to the public for guided tours and equipped for tourist visits, with varying degrees of anthropization [25–28]. To complement these data, we studied the natural and human-influenced aerobiota in a karst cave that serves as a speleotherapeutic center in Slovenia. This underground facility has been an integral part of the Sežana Hospital since 1993 and is used for the treatment and rehabilitation of patients with chronic respiratory diseases [29]. The aim of this study was to assess the anthropogenic effect of speleotherapy on the air microbiota and consider its potential impact on human health in the Sežana Hospital Cave.

2. Materials and Methods

2.1. Sampling Sites

The air samples were taken in an artificial cave used for speleotherapy by Sežana Hospital in Sežana, Slovenia (pronounced [sɛːˈʒaːna], 45°42′33.6672″ N, 13°52′25.7448″ E, 364 m a.s.l.). Sežana Hospital is a specialized hospital for the treatment and rehabilitation of patients with chronic lung diseases. Since 1993, a significant part of respiratory rehabilitation (speleotherapy) has taken place in a partly artificial karst cave directly behind

the hospital. Karst caves are natural underground passages formed by the dissolution of soluble rocks such as limestone, dolomite, and gypsum and are characterized by features such as stalactites, stalagmites, and underground rivers. The cave was dug during the Second World War and is an approximately 207-meter-long tunnel-like passage with two entrances on each side and a smaller, elongated chamber in the middle, which was originally used for storage purposes (Figure 1). The central part of the cave, where most speleotherapeutic activities take place, has a volume of approximately 407 m³ (Telovadnica, Jedilnica), while the total volume of the cave is approximately 1321 m³. The sampling sites in Sežana Hospital Cave were selected on the basis of the speleotherapeutic activities taking place in the cave. The first sampling site was a small corridor that runs perpendicular to the main tunnel and is used for lunch breaks during therapy sessions (Jedilnica, dining room in Slovenian) (Figure 1). The second sampling site was located in the middle of the small chamber used for meditation and educational purposes (Spalnica, bedroom in Slovenian), and the third sampling site was located at the other end of the same chamber used for physical activities (Telovadnica, gym in Slovenian) (Figure 1). The rehabilitation program lasts five days a week for three weeks. On average, the cave is in operation for 40 weeks a year. The group consists of 15–18 patients who are referred to Sežana Hospital by their pulmonologists or general practitioners. Patients bring their medical records, which show that they meet the requirements for admission to the rehabilitation program. During the first few days, patients are examined in a doctor's office and undergo physical fitness tests, pulmonary function tests, and basic laboratory tests. Over the next three weeks, patients learn how to manage their medication and learn breathing exercises, expectoration, and exercises to increase general physical fitness and muscle performance. Most of this rehabilitation therapy, around four hours a day, takes place in the cave.

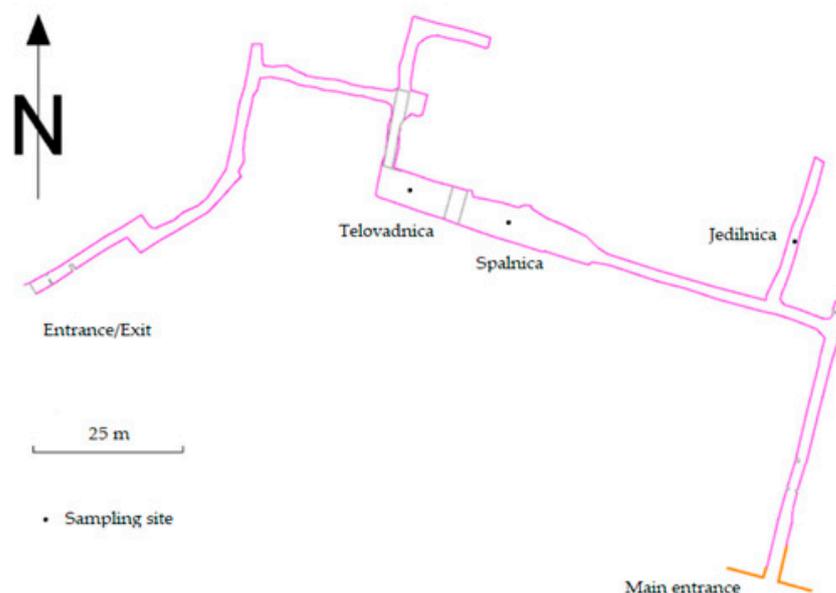


Figure 1. Sampling sites are in Sežana Hospital Cave. The floor plan is adapted from the register of the Karst Research Institute, the Research Center of the Slovenian Academy of Sciences and Arts.

2.2. Air Sampling and Measurement of Environmental Parameters

The air sampling began in the morning, starting with the sampling of the outdoor air 5 m in front of the main entrance of the cave. Then, a few hours before the cave was opened to patients, air samples were collected inside the cave to obtain a natural background. Atmospheric parameters were simultaneously measured. Immediately after the exit of the patient group, the second round of sampling began to observe a direct influence of the patients on the microclimate in the cave and the air microbiota. The measurements and aerosols were collected 1.0 m above the ground on a portable platform. Air sampling

and measurements in the Sežana Hospital Cave were carried out in all sections: 29 May 2020, 7 July 2020, 27 November 2021, 2 June 2022, and 10 January 2023. Two different air samplers were used simultaneously to collect bioaerosols for comparison. Both air samplers were selected because they are ISO 14698 certified and were used in our previous study [27] and in our routine microbiological air monitoring of operating theaters. A VWR® SAS Super DUO 360 Air Sampler (VWR International, Radnor, PA, USA) with an airflow of 180 L/min was used to collect bioaerosols directly onto contact agar plates, while a Coriolis® μ Cyclonic Air Sampler (Bertin Technologies, Saint Quentin en Yvelines, France) with an airflow of 150 L/min was used to collect bioaerosols in saline solution. In the case of the impactor, 0.5 m³ of air was sampled simultaneously on two contact plates (Replicate Organism Detection and Counting, RODAC) with two different growth media (see Section 2.3). Airborne particles were collected from a total air volume of 4.5 m³ in 10 mL sterile saline solution (0.9% NaCl) using the Coriolis® μ Air Sampler. Prior to sampling, the flow tube and the surfaces of both air samplers were disinfected with 96% ethanol. After sampling in the cave, the RODAC plates were sealed with parafilm. The liquid was aseptically divided in a laboratory to perform individual microbiological analyses, culture-dependent biomass estimations, and microbial identifications.

Parallel to the microbiological sampling, environmental parameters were continuously measured at 30 s intervals before and after the speleotherapeutic activities in the cave. Temperature and relative humidity were measured with a portable Kestrel 4500 PocketWeather Tracker (Kestrel Instruments, Boothwyn, PA, USA). Atmospheric CO₂ was measured with a Vaisala MI70 portable carbon dioxide meter (Vaisala, Helsinki, Finland). Wind speed and flow were measured with the VelociCalc Multi-Function Ventilation Meter 9565 (TSI Incorporated, Shoreview, MN, USA).

2.3. Microbial Cultivation

2.3.1. Microbial Cultivation after Sampling with the Impactor

For air sampling with the impactor, two different RODAC plates were used simultaneously on each of the two heads of the SAS Super DUO 360 Air Sampler: BD™ Columbia Agar with 5.0% sheep blood (BA) (Becton-Dickinson, Franklin Lakes, NJ, USA) and Sabouraud Dextrose Agar supplemented with chloramphenicol (SDA) (Oxoid Limited, Basingstoke, UK). BA and SDA were used to estimate and identify the culturable fraction of airborne bacteria and fungi, respectively. Both media were prepared according to the manufacturer's instructions and poured into the RODAC Petri dishes. After solidification, the control strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as controls for BA, as suggested by the manufacturer. *Aspergillus brasiliensis* ATCC 16404 and *Candida albicans* ATCC 10231 were used as controls for SDA accordingly.

After air sampling, the RODAC plates were sealed with parafilm and transported to the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Slovenia, where they were incubated at 37 °C for 48 h (BA) and at 20 °C for 7 days (SDA). The plates were checked daily for growth. The colony-forming units (CFU) counted were expressed per volume of air (CFU/m³) according to the equation provided by the manufacturer (VWR International, Radnor, PA, USA). In order to better compare the microbial concentration before and after the speleotherapeutic activities, the A/B index was calculated (after/before, A/B), which indicates how much higher or lower the concentration is after the therapy. Different morphotypes of bacterial colonies from all primary selection agar media were inoculated onto BA and incubated at 37 °C for 24 to 48 h to be subsequently identified by MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry). Different fungal morphotypes from all primary selection agar media were inoculated onto Sabouraud dextrose agar supplemented with gentamicin and chloramphenicol (SGC2) (bioMérieux, Marcy-l'Étoile, France) and incubated at 37 °C or 30 °C (depending on their preliminary morphological identification) for 24 to 72 h to be subsequently identified by their morphological characteristics and MALDI-TOF MS.

2.3.2. Microbial Cultivation after Sampling with the Impinger

Cultivation of airborne microorganisms from samples obtained with the Coriolis[®] μ Cyclonic Air Sampler was performed as previously described [27], but with the addition of mycological media. Briefly, BA (Becton Dickinson, Franklin Lakes, NJ, USA) and SGC2 (bioMérieux, Marcy-l'Étoile, France) agar plates were used to estimate and identify the culturable fraction of airborne bacteria and fungi, respectively. A portion (200 μ L) of the liquid samples was evenly distributed on the BA and SGC2 plates, and then the plates were incubated at 37 °C for 48 h and at 20 °C for 7 days, respectively. The colony-forming units (CFU) counted were expressed per volume of air (CFU/m³) according to the equation provided by the manufacturer (Bertin Technologies, Saint Quentin en Yvelines, France). In order to better compare the microbial concentration before and after the speleotherapeutic activities, the A/B index was calculated (after/before, A/B), which indicates how much higher or lower the concentration is after the therapy. Different morphotypes of bacterial colonies from all primary selection agar media were inoculated onto BA and incubated for 24 to 48 h at 37 °C for subsequent identification by MALDI-TOF MS. Different fungal morphotypes from all primary selection agar media were inoculated onto SGC2 and incubated at 37 °C or 30 °C (depending on preliminary morphological identification) for 24 to 72 h to be subsequently identified by their morphological characteristics and MALDI-TOF MS.

2.4. Microbial Identification

The pure microbial isolates on the BA and SGC2 plates were identified using MALDI-TOF MS and a formic acid on-spot extraction method, as previously described [27,30]. A 24- to 48-h-old colony was spread on the MALDI steel plate and overlaid with 1 μ L of 98% formic acid. After drying, the sample was covered with 1 μ L of photoabsorbent saturated α -cyano-4-hydroxycinnamic acid (HCCA) matrix solution in 50% acetonitrile–2.5% trifluoroacetic acid (Bruker Daltonik, Bremen, Germany) and allowed to dry before subsequent analysis with a linear-mode microflex LT/SH MALDI-TOF MS system (Bruker Daltonik). The spectra obtained were analyzed using the MALDI-TOF Biotyper[®] (MBT) Compact HT software with the Main Spectra Library BDAL v. 2023 for bacteria and yeasts and Filamentous Fungi v. 2023 for molds (Bruker Daltonik). The Bruker bacterial test standard was used for calibration according to the manufacturer's instructions. The quality of the identification was evaluated using the score from 0 to 3 given by the manufacturer. A score of ≥ 2.00 indicates reliable identification at the species level; a score of 1.70 to 1.99 indicates reliable identification at the genus level; and a score of < 1.70 was interpreted as no identification. In addition to MALDI-TOF MS identification, the filamentous fungi were identified on the basis of their growth and morphological characteristics [31,32].

2.5. Statistical Analysis

Descriptive statistics were used to present the distribution of absolute values of environmental parameters (temperature, relative humidity, wind, and CO₂ concentration) and the quantification of airborne microorganisms in the observed cave before and after the speleotherapeutic session. Average values and standard deviations are given for temperature, relative humidity, wind, CO₂, and microbial concentration. The influence of patients on the measured variables (microbial concentration and CO₂) was determined using the Wilcoxon signed rank test adjusted by the Bonferroni correction. *p*-values of less than 0.05 were considered statistically significant. Statistical analyses were performed using IBM[®] SPSS[®] for Windows version 26 (SPSS Inc., IBM Company, Chicago, IL, USA) and Excel[®] for Windows[®] (Microsoft[™], Redmond, WA, USA).

3. Results

3.1. Environmental Parameters

Table 1 shows the results for CO₂ air concentrations, air temperature, airflow, and relative humidity (RH). The data for these environmental parameters are presented as

minimum, maximum, and median values for each of the three sampling sites in the Sežana Hospital Cave. Wind speed (m/s) is presented in more detail in Table S1 along with other environmental parameters: a range between the minimum and maximum values and an arithmetic mean.

Table 1. Environmental parameters: Air CO₂ concentration, temperature, airflow, and relative humidity from May 2020 to January 2023 at the Jedilnica, Spalnica, and Telovadnica sampling sites in the Sežana Hospital Cave before and after speleotherapeutic activities.

Location		Before Therapeutic Activities				After Therapeutic Activities			
		T (°C)	CO ₂ (ppm)	RH (%)	Airflow (L/s)	T (°C)	CO ₂ (ppm)	RH (%)	Airflow (L/s)
Jedilnica	Mean	15.0	1694.6	82.4	0.14	14.1	1528.3	93.7	0.20
	Minimum	14.2	489.0	77.4	0.07	13.2	846.0	78.8	0.09
	Maximum	16.5	4757.9	84.6	0.21	16.2	2687.8	100.0	0.34
	Standard deviation	1.0	2048.4	3.4	0.06	1.4	810.1	10.1	0.10
Spalnica	Mean	13.4	2112.8	93.3	0.17	13.8	1480.1	95.1	0.22
	Minimum	13.0	457.3	90.6	0.09	13.1	939.0	88.7	0.00
	Maximum	14.1	6092.6	95.6	0.24	14.9	2121.6	100.0	0.52
	Standard deviation	0.5	2680.9	2.1	0.06	0.8	486.7	5.0	0.22
Telovadnica	Mean	13.2	2219.8	96.9	0.16	13.4	1886.8	99.4	0.37
	Minimum	12.8	508.0	93.4	0.01	13.0	1003.8	97.8	0.22
	Maximum	13.7	6160.7	99.4	0.41	13.9	2860.4	100.0	0.64
	Standard deviation	0.4	2673.7	2.5	0.18	0.4	792.6	1.1	0.19

The average air temperature was stable during all measurements and ranged between 12.8 °C and 16.5 °C, with an average of 13.9 °C before and 13.8 °C after the speleotherapeutic activities (Tables 1 and S1). The average CO₂ concentrations ranged from 457.3 ppm to 6160.7 ppm and from 846.0 ppm to 2860.4 ppm before and after the speleotherapeutic activities (Table 1). There was always a certain level of airflow in the cave. The airflow can be partially controlled because one door (labeled “entrance/exit” in Figure 1) has an integrated hatch. The airflow in the Sežana Hospital Cave was the strongest on 7 July 2020 at the Telovadnica sampling site after the speleotherapeutic activities, with 0.64 L/s (Tables 1 and S1). The hatch was partially open during the entire sampling period. In general, the airflow was stronger after speleotherapeutic activities in 66.7% (8/12) of samplings. The RH ranged from 77.4% to 100%, with an average of 90.9% before and 96.1% after the speleotherapeutic activities.

3.2. Quantification and Identification of Culturable Airborne Microorganisms

This study focuses on the cultivation of aerobiota and compares two aerobiological sampling methods, the impinger and the impactor, followed by identification using MALDI-TOF MS. Tables 2 and 3 show the total concentrations of culturable bacteria and fungi in CFU/m³ and the number of species identified for both air sampling methods before and after the speleotherapeutic activities. In order to better compare the microbial concentration before and after the speleotherapeutic activities, the A/B index was calculated (after/before, A/B), which indicates how much higher or lower the concentration is after the therapy (Tables 2 and 3).

Table 2. Microbial concentrations (CFU/m³) and number of identified species before and after speleotherapeutic activities in the air samples collected by impactors from the Jedilnica, Spalnica, and Telovadnica sampling sites in the Sežana Hospital Cave from May 2020 to January 2023. The A/B index (after/before speleotherapeutic activities, A/B) shows the changes in concentration and number of species between samples taken after and before speleotherapeutic activities.

SAS Duo Air Sampler								
Location	Date	Microbes— Before Patients (CFU/m ³)	Microbes— After Patients (CFU/m ³)	A/B Index— Microbial Load	No. of Microbial Species— Before Patients	No. of Microbial Species— After Patients	A/B Index— Species	No. of Patients and Staff
Jedilnica	7 July 2020	102	354	3.5	10	10	1.1	15
	27 November 2021	112	232	2.1	5	5	1.0	10
	2 June 2022	106	214	2.0	9	9	1.1	19
	10 January 2023	88	610	6.9	10	10	1.1	15
		10.2 *	182.6 *					
Spalnica	7 July 2020	144	163	1.1	7	7	0.8	15
	27 November 2021	143	185	1.3	4	4	1.3	10
	2 June 2022	64	218	3.4	7	7	0.6	19
	10 January 2023	54	138	2.6	8	8	1.1	15
		48.9 *	33.9 *					
Telovadnica	7 July 2020	169	198	1.2	8	8	0.8	15
	27 November 2021	202	148	0.7	3	3	0.6	10
	2 June 2022	152	190	1.3	11	11	1.4	19
	10 January 2023	96	650	6.8	7	7	0.9	15
		44.3 *	236.7 *					

* Standard deviation.

Table 3. Microbial concentrations (CFU/m³) and number of identified species before and after speleotherapeutic activities in the air samples collected by impinger from the Jedilnica, Spalnica and Telovadnica sampling sites in the Sežana Hospital Cave from May 2020 to January 2023. The A/B index (after/before speleotherapeutic activities, A/B) shows the changes in concentration and number of species between samples taken after and before speleotherapeutic activities.

Coriolis Air Sampler								
		Microbes— Before Patients (CFU/m ³)	Microbes— After Patients (CFU/m ³)	A/B Index— Microbial Load	No. of Microbial Species— Before Patients	No. of Microbial Species— After Patients	A/B Index— Species	No. of Patients and Staff
Jedilnica	7 July 2020	134	757	5.6	4	7	1.8	15
	27 November 2021	50	3873	77.5	1	4	4.0	10
	2 June 2022	653	886	1.4	5	9	1.8	19
	10 January 2023	151	784	5.2	4	10	2.5	15
		274.2 *	1533.0 *					
Spalnica	7 July 2020	217	518	2.4	5	3	0.6	15
	27 November 2021	185	1820	9.8	3	4	1.3	10
	2 June 2022	818	435	0.5	8	7	0.9	19
	10 January 2023	304	951	3.1	8	5	0.6	15
		295.6 *	634.4 *					
Telovadnica	7 July 2020	117	469	4.0	2	7	3.5	15
	27 November 2021	2370	4558	1.9	2	4	2.0	10
	2 June 2022	501	301	0.6	5	5	1.0	19
	10 January 2023	317	934	2.9	5	6	1.2	15
		1041.0 *	2012.9 *					

* Standard deviation.

The highest concentrations of culturable microorganisms were measured at the Telovadnica sampling site with both sampling methods before and after speleotherapeutic activities (Table 2). We detected higher microbial concentrations after the speleotherapeutic

activities in 91.7% (11/12) of the cases with the SAS sampler and in 83.3% (10/12) of the cases with the Coriolis sampler (Tables 2 and 3).

The influence of patients and medical staff on the measured variables (median concentration of microorganisms, CO₂, and RH) is shown in Table 4. The differences in microbial concentrations measured with the SAS air sampler in Jedilnica and Spalnica show borderline results in terms of statistical significance ($p = 0.068$). The same applies to the microbial concentrations in Jedilnica, which were determined with the Coriolis air sampler (Table 4). In the other cases, the influence of cave visitors was not statistically significant ($p > 0.05$). For the environmental parameters, only the RH in Jedilnica showed borderline results in terms of statistical significance ($p = 0.068$). A comparison of the results obtained with the SAS and the Coriolis air sampler was not carried out due to technical differences between the two methods.

Table 4. The influence of patients and hospital staff on measured variables (median concentration of microorganisms, CO₂, and percentage of relative humidity, RH) in air samples from 2020 to 2023 in Jedilnica, Spalnica, and Telovadnica sampling sites in the Sežana Hospital Cave. Air was sampled simultaneously by the impactor and impinge methods.

Location		SAS Impactor (CFU/m ³)	<i>p</i>	Coriolis Impinger (CFU/m ³)	<i>p</i>	CO ₂ (ppm)	<i>p</i>	RH (%)	<i>p</i>
Jedilnica	Before therapeutic activities	104.0	0.068	142.5	0.068	765.8	0.715	83.8	0.068
	After therapeutic activities	293.0		835.0		1289.7		97.9	
Spalnica	Before therapeutic activities	103.5	0.068	260.5	0.273	950.60	0.715	93.5	0.465
	After therapeutic activities	174.0		734.5		1429.9		95.9	
Telovadnica	Before therapeutic activities	160.5	0.465	409.0	0.144	1105.3	0.715	97.3	0.066

3.3. Identification of Culturable Airborne Microorganisms

A total of 74 bacterial species from 29 genera and 15 fungal species from seven genera were identified from air samples collected from May 2020 to January 2023 in three sampling sites in the Sežana Hospital Cave (Tables S2–S4). A relatively large number of isolates, 52.3% (1274/2672) from the “before patients” samples and 21.8% (2580/3300) from the “after patients” samples using the SAS air sampler, could not be identified due to low MALDI scores (<1.70) (Table 5). Using the Coriolis air sampler, we were unable to identify 26.2% (1923/7335) of the isolates from the “before patients” samples and 22.6% (3688/16350) from the “after patient” samples (Table 5).

The most common genera from the *Firmicutes* group (also known as *Bacillota*) were *Bacillus* and *Staphylococcus*, which accounted for up to 24.8% of the isolates per sampling site (Table S5). Genus *Staphylococcus* was present at all sampling sites (Table S5). In samples taken before speleotherapeutic activities, staphylococci represented 0.9–21.9% of isolates, and after speleotherapeutic activities, they represented 0.7–11.6% of isolates. Using MALDI-TOF MS, we were able to identify 12 different *Staphylococcus* sp. (Table S5). The highest percentage of *Staphylococcus* isolates, 21.9%, was detected in the Jedilnica sampling site on 10 January 2023 before the speleotherapeutic activities with the Coriolis air sampler (Table S2). Among the *Actinobacteria* (also known as *Actinomycetota*), *Micrococcus*, *Dietzia*, and *Kocuria* were the three most frequently isolated genera, accounting for up to 55.6% of isolates per sample (Table S5). *Micrococcus luteus* was one of the most abundant bacterial species in all samples, accounting for 31.3–55.6% in the “before patients” samples and 18.1–52.2% in the “after patients” samples (Table S5). Among the *Proteobacteria* (also known as *Pseudomonadota*), *Acinetobacter*, *Serratia*, and *Pseudomonas* were the three most frequently

isolated genera, accounting for up to 16.3% of isolates per sample (Table S5). The kingdom *Fungi* was represented by 15 species from the Ascomycota and *Basidiomycota* (Table S5). We were able to identify 11 mold species and five yeast species. The most frequently encountered ascomycetous species were *Penicillium* spp., which accounted for up to 42.6% of all isolates per sample (Table S6). Among the opportunistic and allergenic filamentous fungi, *Alternaria alternata* reached the highest proportion of isolates—30.4%—in the Spalnica sampling site on 7 July 2020 with the Coriolis air sampler before speleotherapeutic activities (Table S3). Among the yeasts, *Debaryomyces hansenii* (formerly *Candida famata*) was detected most frequently, with 28.8% of isolates on 7 July 2020 in Telovadnica before the speleotherapeutic activities (Table S4).

Table 5. Number and percentage of microbial isolates identified (MALDI score ≥ 1.70 , genus level identification) per sampling site (Jedilnica, Spalnica, and Telovadnica) and comparison between different sampling methods.

		Jedilnica		Spalnica		Telovadnica		Locations Combined	
		SAS	Coriolis	SAS	Coriolis	SAS	Coriolis	SAS	Coriolis
Non-identified microorganisms (CFU/m³)	Before patients	412	550	514	622	472	751	1398	1923
	After patients	288	2621	217	500	215	567	720	3688
All isolated microorganisms (CFU/m³)	Before patients	850	1672	869	2041	953	3622	2672	7335
	After patients	1410	6300	704	3743	1186	6262	3300	16,305
% of identified microorganisms	Before patients	51.5	67.1	40.9	69.5	50.5	79.3	47.7	73.8
	After patients	79.6	58.4	69.2	86.6	81.9	90.9	78.2	77.4

SAS—VWR® SAS Super DUO 360 Air Sampler (VWR International, Radnor, PA, USA); Coriolis—Coriolis® μ Cyclonic Air Sampler (Bertin Technologies, Saint Quentin en Yvelines, France).

3.4. Microbial Indicators of Cave Anthropization

Among the 89 identified bacterial and fungal species (Tables S5 and S6), we identified up to 22 species that are associated with the human microbiota and could serve as indicators of cave anthropization (Table 6). Half of these microorganisms (11/22) belong to the genus *Staphylococcus*, a known colonizer of human skin [33]. *K. rosea*, *M. luteus*, *S. capitis*, and *S. hominis* were isolated from all three sampling sites and are considered strong anthropogenic indicators. According to the American Biological Safety Association (<https://my.absa.org/Riskgroups> (accessed on 8 March 2024)), less than half (8/20) of the bacteria mentioned belong to risk group 2 as they have a limited pathogenic potential. Others (12/20) cause infections so rarely that they were not assigned to a risk group (Table 6). Among the fungi, the ascomycetous yeast *Meyerozyma guilliermondii* (formerly *Candida guilliermondii*) was also isolated at all three sampling sites. These yeasts are part of the human skin and gut microbiota but are also found in other environments such as soil, plants, insects, seawater, and processed foods [34]. A similar problem exists with some other bacteria, such as *Acinetobacter* spp., *Enterococcus moraviensis*, and *Bacillus licheniformis* (Table S5), as these bacteria are often associated with environments other than humans, e.g., soil, water, food, and various animals [33,35].

Table 6. Potential microbial indicators of cave anthropization (MALDI-TOF MS score value > 2.00, species level identification).

Microorganisms	Sampling Sites *	Risk Group (Country)	Human Microbiota
Bacteria			
<i>Aerococcus viridans</i>	1, 2	2 (BE, CH, DE)	skin, urinary tract
<i>Corynebacterium amycolatum</i>	1	2 (CH, DE)	skin
<i>Corynebacterium aurimucosum</i>	1, 3	-	skin, vaginal mucosa
<i>Kocuria marina</i>	1, 2	-	skin
<i>Kocuria palustris</i>	1, 2	-	skin
<i>Kocuria rhizophila</i>	2	-	skin
<i>Kocuria rosea</i>	1, 2, 3	-	skin
<i>Micrococcus luteus</i>	1, 2, 3	2 (BE, CH, DE, NIH)	skin
<i>Rothia dentocariosa</i>	1	-	oral mucosa
<i>Staphylococcus capitis</i>	1, 2, 3	-	skin, scalp
<i>Staphylococcus cohnii</i>	1, 3	-	skin
<i>Staphylococcus epidermidis</i>	1, 2	2 (BE, CH, DE)	skin, nasopharynx
<i>Staphylococcus haemolyticus</i>	2, 3	2 (CH, DE)	skin
<i>Staphylococcus hominis</i>	1, 2, 3	-	skin
<i>Staphylococcus lugdunensis</i>	1, 2	2 (BE, CH, DE)	skin
<i>Staphylococcus pettenkoferi</i>	1	-	skin, urinary tract
<i>Staphylococcus petrasii</i>	1	-	skin
<i>Staphylococcus saprophyticus</i>	2	2 (BE, CA, CH, DE)	skin
<i>Staphylococcus schleiferi</i>	1	2 (BE, CH, DE)	skin
<i>Staphylococcus warneri</i>	1, 2	-	skin
Fungi			
<i>Cutaneotrichosporon dermatis</i>	2, 3	-	skin
<i>Meyerozyma guilliermondii</i>	1, 2, 3	2 (EU, UK)	skin, intestinal tract

BE—Belgium; CA—Canada; CH—Switzerland; DE—Germany; EU—European Union; UK—United Kingdom.
 * 1: Jedilnica sampling site; 2: Spalnica sampling site; 3: Telovadnica sampling site.

3.5. Outdoor Air

The results for the outdoor air, which were determined with the SAS air sampler, are listed in Table 7. We were able to detect four bacterial and seven fungal species. The most common isolates were *Penicillium* spp. and *Cladosporium* spp., with concentrations of up to 28 CFU/m³ per species. The highest microbial concentration was measured on 27 November 2021 with 122 CFU/m³. The identification success rate of isolates varied between 62.2% in May 2020 and 88.2% in January 2023.

Table 7. Bacterial and fungal isolates (MALDI Score ≥ 2) were obtained from the outdoor air samples from May 2020 to January 2023.

Bacteria	Microbial Concentration (CFU/m ³)				
	29 May 2020	7 July 2020	27 November 2021	2 June 2022	10 January 2023
<i>Arthrobacter parietis</i>	NP	NP	4	NP	2
<i>Bacillus pumilus</i>	NP	NP	4	NP	6

Table 7. Cont.

Bacteria	Microbial Concentration (CFU/m ³)				
	29 May 2020	7 July 2020	27 November 2021	2 June 2022	10 January 2023
<i>Pantoea eucrina</i>	NP	2	NP	NP	4
<i>Paenarthrobacter ilicis</i>	NP	NP	4	NP	NP
Fungi	NP	NP	NP	NP	NP
<i>Aspergillus fumigatus</i> *	4	2	4	2	4
<i>Aspergillus flavus</i> *	NP	2	NP	2	4
<i>Aspergillus glaucus</i> *	NP	NP	8	NP	12
<i>Cladosporium</i> * sp.	20	20	28	26	24
<i>Penicillium brevicompactum</i> *	2	4	6	4	8
<i>Penicillium italicum</i> *	4	2	8	4	8
<i>Penicillium</i> * sp.	16	20	22	20	18
Total microorganisms	74	72	122	88	102
Non-identified microorganisms	28	20	26	30	12
% of identified microorganisms	62.2	72.2	78.7	65.9	88.2

NP—Not present; * Identification is confirmed by the morphological characteristics of the isolates [31,32].

4. Discussion

4.1. Environmental Parameters

In the Sežana Hospital Cave, the air temperature ranged between 12.8 °C and 16.5 °C and did not change in the presence of patients and staff. It has already been proven that a stable air temperature is beneficial for patients with chronic respiratory diseases. Cold and hot temperatures have a negative effect and can lead to exacerbation of the disease, especially in COPD patients and children with asthma [36–38]. One of the therapeutic advantages of speleotherapeutic caves is precisely the stable low air temperature, which, in combination with high relative humidity, improves mucociliary clearance [15,39]. RH varied between 77.4% and 100%, with the lowest value measured at the Jedilnica sampling site. This is consistent with other studies in which lower RH near the entrance is typical, while RH inside the cave increases [40,41]. As already mentioned, high RH is also one of the positive climate factors in speleotherapy, as low RH is associated with an increase in emergency admissions in asthmatics, according to some studies [15,42]. Therefore, the deeper parts of the Sežana Hospital Cave, the Spalnica and Telovadnica sampling sites, are more suitable for speleotherapeutic activities, as the average RH is 93.3% and 96.9%, respectively [15,39]. At 82.4%, the Jedilnica sampling site has a lower RH than most low-temperature caves, which makes this part of the cave less favorable for speleotherapeutic activities [39,43]. Interestingly, the RH was influenced by the patients and the medical staff, as the RH was higher after the therapies, and the results were even in the statistical borderline range ($p = 0.068$). CO₂ concentrations were also generally higher after speleotherapeutic activities, suggesting an anthropogenic influence on the cave microclimate, although these changes in concentrations were not as obvious as in some studies of show caves [44–46] and were also not statistically significant ($p = 0.715$). The largest increase in CO₂ concentration after therapy was found on 27 November 2021 in the Jedilnica sampling site, when it increased threefold, but on the other hand, on 7 July 2020, we even found a decrease in CO₂ after therapy at all sampling sites. One of the possible explanations for these differences is the increased artificial ventilation of the cave with a hatch at the alternative entrance. This hatch may serve to increase or decrease the ventilation of the cave. Nevertheless, we did not measure a higher wind speed or flow on 7 July 2020 than on the other sampling dates, so the behavior of the CO₂ remains partially unexplained and can possibly be clarified by further investigations of the cave. The use of sensors to continuously monitor air param-

eters, including gas concentrations, could reduce uncertainty at this point. However, in agreement with other studies [44,45,47], CO₂ concentrations indicate a seasonal distribution that is highest in the warmer months of the year.

4.2. Culturable Airborne Microorganisms and Speleotherapeutic Activities

The air in the Sežana Hospital Cave was not yet characterized microbiologically in detail. Similar to the show caves, higher concentrations of culturable airborne microorganisms were usually detected in the human presence. Just as tourist visits increase microbial concentrations in the show caves, speleotherapeutic activities in this therapeutic cave do the same in at least 83.3% (10/12) of the samples taken. Regardless of the method of air sampling, microbial concentrations increased 1.1- to 77.5-fold after speleotherapeutic activities, suggesting an anthropogenic influence, although there was no strong statistical association ($p \geq 0.068$). The highest increase in concentration, namely 77.5 times, was measured in Jedilnica on 27 November 2021. On the same day, the highest microbial concentrations of over 3800 CFU/m³ were generally measured. This situation was due to the high concentrations of *Acidovorax* sp. In Spalnica and Telovadnica, we were able to identify it to genus level using MALDI-TOF MS (MALDI Score < 2.00), and in Telovadnica, it even accounted for 59.3% of isolates after speleotherapeutic activities. *Acidovorax* sp. is also found in soil and plants [48] and could be part of the cave microbiota and aerosolized during physical activities. The other explanation lies in the fact that *Acidovorax* spp. is considered to be the core microbiota of human skin in some studies [49] and is, therefore, a strong anthropogenic indicator, although it has only been detected once with the Coriolis air sampler. As previously described [27], higher microbial concentrations were associated with speleotherapeutic treatments (10–19 people present) but not with the number of people present; e.g., 232 CFU/m³ were detected in Jedilnica on 27 November 2021, when only 10 people were present, and only 214 CFU/m³ in Jedilnica on 2 June 2022, when 19 people were present. A similar trend was previously observed in show caves in Slovenia and Romania, also with similar airborne microbial concentrations [26,27,50]. As we can see, Sežana Hospital Cave follows the pattern of show caves.

4.3. Comparison of Air Sampling Methods

Various methods of air sampling have been described so far, which are used in different environments [51,52]. We have opted for two active air sampling methods that are used both in the microbiological control of the hospital environment and in cave microbiology [26,27,53–55]. Speleotherapeutic caves are unique in this respect because they are caves and hospital environments at the same time. In hospitals and in the pharmaceutical industry, similar to cave microbiology, both active and passive air sampling methods are used, but unfortunately, they are not yet standardized [26,50,55,56]. They are roughly described in various national guidelines and in the EU GMP and formalized in ISO 14698-1:2003 [53,54,57]. The choice of method depends primarily on the type of room or the expected level of microbiological cleanliness. Since a low microbial load is expected in speleotherapeutic caves [21,25,58], we did not opt for the settle plate method, as it has a relatively low sensitivity compared to active methods [59,60]. Impingement and impaction air sampling methods are technically very different: they differ in the size and shape of the surface through which the air is pumped (circular for the impactor and square for the impinger), the impactor samples the air directly onto the solid culture medium, and the impinger samples into a physiological solution (or another type of liquid) with which the solid culture medium must be inoculated in the subsequent steps. The differences also lie in the sample volume and the airflow rate. A direct comparison is therefore not possible, although we were able to identify some differences. On average, we obtained higher microbial concentrations with the impinger air sampler than with the impactor sampler, which can possibly be explained by the nine times larger volume of air sampled (4.5 m³ compared to 0.5 m³). At the same time, we were able to detect more microbial species with the SAS air sampler than with the Coriolis sampler. It is possible that a larger

air volume used with the SAS air sampler would lead to more comparable results. At this point, it must be emphasized that the two methods are difficult to compare and that air sampling is, to a certain extent, unpredictable and non-reproducible [59,61]. Further research will clarify the suitability of each sampling method for speleotherapeutic caves, but for now, we can only recommend the use of a single air sampling method in a cave for longitudinal studies to facilitate the comparison of results and to establish guidelines for microbiological monitoring of these “natural” parts of hospitals.

4.4. Cave Anthropization

It has been known for some time that human visitation can have a negative impact on the cave environment, for example, by increasing CO₂ levels and introducing nutrients and allochthonous microorganisms [26,28,46,62–64]. Most research focuses on show caves, especially those exposed to mass tourism, such as Lascaux Cave (France), Nerja Cave (Spain), and Postojna Cave (Slovenia) [27,28,30,62,65]. There are relatively few datasets on the anthropogenic impact on speleotherapeutic caves [23,66]. Almost 13 years ago, Porca et al. made an important contribution to the standardization of the assessment of the anthropogenic impact on the cave ecosystem [67]. In 2018, Bercea et al. expanded and updated the criteria of Porca et al. and defined five categories of anthropogenic impact on caves according to the concentration of bacteria and fungi in the air [26,67]: Class 1: ≤ 50 CFU/m³ (no human impact); Class 2: 50–150 CFU/m³ (alarm signal—regular checks are recommended); Class 3: 150–500 CFU/m³ (regular checks and changes in cave management (e.g., visit schedules, ventilation, etc.) are recommended); Class 4: 500–1000 CFU/m³ (humans already have a strong influence on the cave ecosystem); and Class 5: ≥ 1000 CFU/m³ (irreversible ecological disturbance if high concentrations persist). If we apply this interpretation scheme to our data, we see that we never exceed the Class 5 limit when using the SAS air sampler before speleotherapeutic activities. A strong anthropogenic influence (Class 4) was only observed in Telovadnica on 10 January 2023, while the other results fall into Class 2, where regular monitoring is recommended, with the exception of Telovadnica on 2 June 2022, which falls into Class 3 with 152 CFU/m³. The presence of patients and medical staff leads to an increase in microbial concentrations and thus also to an increase in the class of anthropogenic disturbance: in all cases, after speleotherapeutic activities, we now have Class 3, which recommends regular microbiological monitoring and measures in terms of visiting times, arrangement of ventilation, etc. However, if we look at the results obtained with the Coriolis air sampler, the picture is somewhat different, as we generally found higher microbial concentrations than with the SAS air sampler. In this case, more than 1000 CFU/m³ were detected in Telovadnica on 27 November 2021 (2370 CFU/m³), even before speleotherapeutic activities. On 27 November 2021, after the speleotherapeutic activities, we found an increased microbial load (>1000 CFU/m³) at all sampling sites and, thus, the negative influence of humans on the cave ecosystem. Regardless of the air sampling method, increased microbial concentrations (Class 3) were found after speleotherapeutic activities, which may have a long-term negative impact on the cave ecosystem. However, Bercea et al. and Porca et al. [26,67] recommend regular microbiological monitoring and optimization of visits to the speleotherapeutic cave in terms of frequency, number of people in the group, etc. As the Sežana Hospital Cave is a relatively small cave with low ventilation, the microbial concentration could be reduced by increasing the artificial ventilation through the hatch at the side entrance shortly before and immediately after the speleotherapeutic activities.

4.5. MALDI-TOF MS Identification Success Rate

MALDI-TOF MS has found its place in medical microbiology laboratories, especially in bacteriology, while it is still underestimated in microbial ecology laboratories [68,69]. In recent years, MALDI-TOF MS has also gained visibility in cave microbiology as more and more authors use it for the characterization, especially the identification, of isolated cave microorganisms [27,30,70–72]. In general, we were able to identify more isolates

in the samples after speleotherapeutic activities than in the samples before speleotherapeutic activities. We were able to identify a maximum of 79.3% of the isolates before the speleotherapeutic activities and 90.9% of the isolates after the speleotherapeutic activities, which is in line with some other studies [50,70]. As we have similarly found in Postojna Cave and Škocjan Caves, Slovenia [27,50], the samples collected after speleotherapeutic activities contain a greater proportion of typical human microbiota, particularly bacteria of the genus *Micrococcus* and *Staphylococcus*, which are well represented in the commercially available MALDI-Bruker library. Compared to the results from Postojna and Škocjan caves, where the air was also sampled with the Coriolis air sampler, we were able to identify a higher proportion of isolates: 29.8 percentage points more in the case of the natural background of the cave (73.8% vs. 44.0%) and 10.5 percentage points more after human activities (77.4% vs. 66.9%) [27]. On the one hand, this can be explained by the use of the newer MALDI-Bruker library, which has a broader spectrum of microbial species than in 2018, when we conducted the study in natural show caves [27]. The difference is also reflected in the number of species identified, as we identified 23 more bacterial species in the Sežana Hospital Cave than in the previous study (74 vs. 51) [27]. On the other hand, the better coverage of isolates in the MALDI-Bruker library could indicate a higher degree of anthropization of the Sežana Hospital Cave.

4.6. Culturable Microorganisms and Potential Indicators of Human Impact

Similar to other cave studies, bacteria from the phyla *Actinomycetota* (formerly Actinobacteria), *Bacillota* (formerly Firmicutes), and *Pseudomonadota* (formerly Proteobacteria) were frequently isolated from air samples [50,63,73–76]. We identified 74 different bacterial species from 29 genera, which is comparable to other culture-based studies [27,77,78]. On the one hand, we have succeeded in identifying more bacterial species than in other caves. Compared to Cueva de Ardales (Spain) and Lascaux Cave (France), we identified 33 and 40 more species in Sežana Hospital Cave, respectively (41 vs. 74 and 34 vs. 74), and in the aforementioned Postojna and Škocjan Caves (Slovenia), we identified 23 more species (74 vs. 51) [27,76,78]. On the other hand, we identified 1.8 times fewer bacterial species than in the Cueva del Tesoro (Spain) (74 vs. 130) [78]. As the different studies use different identification methods, a direct comparison may be difficult. If we lower the Bruker cut-off value for reliable species identification from 2.00 to 1.70, as we did in our earlier study for Postojna and Škocjan Caves [27], we obtain 97 different species, which compares favorably with the show caves Gruta de las Meravillas and Cueva del Tesoro (Spain) with 94 and 130 molecularly identified species, respectively, and with the Postojna and Škocjan Caves if we apply the same MALDI Score cut-off value—84 different species [27,78]. In terms of bacterial diversity, Sežana Hospital Cave is similar to other caves with varying degrees of anthropization [27,76,78].

Anthropogenic indicators have already been described in various studies [30,63,73,78,79]. Anthropogenic indicators are mostly bacterial and fungal species that are closely associated with humans as the typical microbiota of the skin, gastrointestinal tract, and upper respiratory tract [27,79]. In Sežana Hospital Cave, 22 potential indicators of anthropization of the cave were identified. Among the most reliable—part of the human core microbiota [30,33,80] and present at all sampling sites—are *M. luteus*, *S. capitis*, *S. hominis*, and *K. rosea* among the bacteria and *M. guilliermondii* among the fungi. According to other studies, *S. epidermidis* could also be among them [27,30,33]. In addition to the specific species mentioned, most coagulase-negative staphylococci are also important anthropogenic indicators [27,30,33]. Potential indicator microorganisms could also include *Acinetobacter* spp., *Acidovorax* spp., *Enterococcus moraviensis*, and *Bacillus licheniformis*, but these can also occur in other environments and are therefore not exclusively associated with the presence of humans [81–83]. Some of the anthropogenic indicators could also be found among the isolated molds. For example, *Penicillium* species are frequently found in human environments and nature, but it is hard to pick a particular species as a certain indicator due to their general ubiquity [84–86]. To obtain a more comprehensive view of the cave microbiota,

we would also need data on microorganisms from cave surfaces that frequently come into contact with humans and those with which humans do not interact.

4.7. Pathogenic Microorganisms in Cave Aerobiota

In most speleotherapeutic caves, aerosolized particles, especially minerals and ions, are studied, while bioaerosols are not given much attention [66,87–90]. There are limited data on air biocontamination and its impact on the health of patients with chronic and allergic respiratory diseases associated with speleotherapy [23,91]. Exposure to certain types of bacteria and fungi has been shown to be significantly associated with COPB and asthma exacerbations [9,92–95]. In addition to the infection, the dysbiosis of the lung microbiota is also important, affecting the local immune system, which determines the balance of inflammation and exacerbation of the disease [96,97]. Since exposure to pathogenic or even saprophytic microorganisms can aggravate the health status of patients with chronic respiratory diseases, the microbiological air quality is an important factor, especially in rooms where patients receive inhalation therapies [20,23,98]. The speleotherapeutic cave is part of the hospital and must, therefore, be maintained to ensure patient safety and protect them from healthcare-associated infections. Caves are particularly interesting in this case because their maintenance and cleaning/disinfection are particularly demanding. In this case, microbiological monitoring is extremely important to detect and control infection risks. In our study, commensals and opportunistic pathogens were identified among the microorganisms, but no primary pathogens were isolated from the air samples. Medically interesting isolates all belong either to risk group 2, as they have a limited pathogenic potential, or they do not belong to any risk group, as they do not normally cause infections in the immunocompetent host. Representatives of the genera *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* were isolated in the Sežana Hospital Cave, the last two even at all three sampling locations. The above-mentioned molds are one of the most common causes of the development and exacerbation of asthma and rhinitis [99,100]. *Aspergillus fumigatus*, which we were only able to detect in low concentrations in Jedilnica, is the clinically most important fungal isolate as it can cause a broad spectrum of pathogenic interactions in patients with COPD and asthma, from allergic sensitization and allergic bronchopulmonary aspergillosis to invasive pulmonary aspergillosis [101–103]. Fortunately, high humidity near saturation is not favorable for the growth of *Aspergillus* spp. [86,104]. The absence of *Aspergillus* spp. in the deeper parts of the Sežana Hospital Cave is beneficial for patients, especially those who are sensitized to *Aspergillus* spp. In addition to *Aspergillus* spp., molds of the genera *Penicillium* and *Cladosporium* also have a considerable influence on the course of asthma and COPD but cannot cause infections, only hypersensitivity reactions [94,99]. In view of the fact that we isolated relatively high concentrations of *Penicillium* spp. (≤ 334 CFU/m³) in the Sežana Hospital Cave, which at times accounted for up to 42.6% of all isolates, we can conclude that a prolonged stay in the cave could have negative effects on the patients [73,105]. A similar species structure and concentration were also found in other speleotherapeutic caves and a spa center [25,91,106]. Here, increased ventilation of the cave and therapies with smaller groups could be recommended, as larger groups are partly responsible for the introduction of microorganisms into the cave [23,50]. *Penicillium* spp. are frequently found indoors, but for a healthy living environment, their concentration should be lower indoors than outdoors [107,108]. In the Sežana Hospital cave, the exact opposite was the case—we found lower concentrations and fewer species in the outdoor air than in the cave. Harmful mold concentrations in caves have not yet been defined [59]. The cut-off values for indoor areas proposed by the World Health Organization (WHO) and the European Commission's report on biological particles in indoor environments (EC) may be helpful for interpretation. The WHO recommends <1000 CFU/m³ for indoor areas [109], while the EC recommends <500 CFU/m³ [110]. If we use the WHO recommendation, we see that the microbial concentrations exceed the cut-off value only on 27 November 2021 when sampling with a Coriolis air sampler. If we use the EC cut-off values, we obtain an excessively high microbial load only before the

speleotherapeutic activities on 10 January 2023 in Telovadnica. If we take into account the isolated mold species and total concentrations, we find that the aerobiota in the Sežana Hospital Cave is similar to that in show caves and does not pose a risk to immunocompetent individuals [26,27]. One of the main characteristics of speleotherapeutic caves is the non-allergic atmosphere, which is somewhat compromised in the Sežana Hospital Cave by the mold species that can exacerbate COPD and asthma [95,110]. The microclimatic parameters (RH, T, and wind) are suitable for speleotherapy and probably compensate for the slightly poorer microbiological results [15,58]. Adequate artificial ventilation of the cave before and after speleotherapeutic activities, optimization of the number of patients, and time spent in the cave would probably also improve this parameter. Further research is needed to better understand the dynamics of aerobiota and its importance for the health of patients undergoing speleotherapy.

4.8. Limitations

This study originally had an ambitious plan to explore the aerobiota in more detail, but due to the COVID-19 pandemic (2020–2022) and the closure of the cave for speleotherapy during this period, air samples were taken less frequently. This limited our insight into the structure and dynamics of the aerobiota. Therefore, microbiological time-series analyses need to be performed to assess anthropization and ecological disturbance more accurately. Although reproducibility was not the aim of this study, the collection of only one sample per sampling site and air sampler limits the information on microbial concentrations. Three replicates for each sampling period and location and the use of sensors to continuously monitor air parameters, including gas concentrations, could provide us with more accurate results.

Another limitation of this study was the use of culture-based methods to analyze the cave aerobiota. The use of metagenomics-based approaches, such as advanced molecular genetic techniques and high-throughput sequencing technologies, would greatly increase our knowledge of the abundance and diversity of airborne microbes.

As far as the identification of microorganisms is concerned, MALDI-TOF MS alone is not an optimal identification method, especially in environmental microbiology [50,70,71]. A number of methods have been proposed for the optimal identification of microorganisms, each with its own advantages and limitations. In addition to MALDI-TOF MS and genome-based methods, other successful methods range from morphology and gas-liquid chromatography of cellular fatty acids to the use of optical methods for label-free detection of bacteria [68,111–113]. Overall, however, these studies emphasize the need for a multi-method approach to microbial identification that combines the strengths of individual methods to achieve optimal results. In addition to species identification, resistance profiles and phylogenetic comparisons could provide more precise information on the anthropogenic origin of the isolated microorganisms.

5. Conclusions

Speleotherapy is one of the non-pharmacological methods for the treatment and rehabilitation of patients with chronic respiratory diseases, especially COPD and asthma. The specific cave environment with constant temperature, high humidity, low ventilation, aerosolized minerals, and low concentrations of microorganisms and radioactivity benefits the rehabilitation of these patients. For successful and safe speleotherapeutic rehabilitation, it is necessary to control the microclimatic parameters, especially the concentration of aerosolized microorganisms, which can have a negative impact on patients. There are little data on microbiological air monitoring in speleotherapeutic caves. The optimal methods for air sampling have not yet been established internationally; due to the expected low microbial load, we recommend the use of volumetric methods. The methods are difficult to compare due to the technical diversity; therefore, we recommend that laboratories decide on one method and apply it consistently. There are no internationally recognized criteria for assessing the air quality in caves. We recommend using the WHO and EC criteria for

indoor microbial concentration and the qualitative criteria from the published literature: absence or lowest possible concentrations of opportunistic and allergenic molds, mainly from the genera *Aspergillus*, *Alternaria*, *Penicillium*, and *Cladosporium*. Our data show that speleotherapeutic caves, similar to show caves, are also exposed to anthropization. By regulating the ventilation of the speleotherapeutic cave and optimizing the number of patients in the group, potentially excessive microbial concentrations are most likely to be successfully reduced. We recommend regular microbiological monitoring, optimization of ventilation, and reducing the number of patients in the groups.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/atmos15050518/s1>, Table S1: Environmental parameters: CO₂, temperature, wind velocity and flow, relative humidity, and radiation from May 2020 to January 2023 in Jedilnica, Spalnica, and Telovadnica sampling sites in Sežana Hospital Cave before and after speleotherapeutic activities; Table S2: Bacterial and fungal isolates (MALDI Score \geq 2.00) from the Jedilnica sampling site in Sežana Hospital Cave; Table S3: Bacterial and fungal isolates (MALDI Score \geq 2.00) from the Spalnica sampling site in Sežana Hospital Cave; Table S4: Bacterial and fungal isolates (MALDI Score \geq 2.00) from the Telovadnica sampling site in Sežana Hospital Cave; Table S5: Bacterial isolates (MALDI Score \geq 2.00) from the Jedilnica, Spalnica, and Telovadnica sampling sites in the Sežana Hospital Cave, their risk group assignment, and typical habitat; Table S6: Fungal isolates (MALDI Score \geq 2.00) from the Jedilnica, Spalnica, and Telovadnica sampling sites in the Sežana Hospital Cave, their risk group assignment, and typical habitat.

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