

AEROSOLIZED MICROBES FROM ORGANIC RICH MATERIALS: CASE STUDY OF BAT GUANO FROM CAVES IN ROMANIA

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Abstract: Cave air, bat guano, and swabs of bat fur from caves with bat guano in Romania were analyzed by using RIDA[®]COUNT cultivation plates and standard selective media for *Staphylococcus* and *Streptococcus*. The samples of guano varied in concentration of cultivable chemoheterotrophic bacteria (max. 1.9×10^{10} CFU/g), coliforms (max. 2.2×10^8 CFU/g), *Escherichia coli* (max. 1.0×10^8 CFU/g), and yeasts and molds (max. 1.7×10^7 CFU/g). The gravity-settling principle was applied to sample airborne microorganisms, and a new method was developed for evaluation of aerosolization potential. In cave air, the concentration of total bacteria was higher than yeast and molds. In addition to coliforms, enterobacteria, *E. coli*, and unidentified cultivable bacteria in the air samples, we also identified *Chryseomonas luteola*, *Klebsiella pneumoniae*, *Micrococcus*, *Salmonella*, *Staphylococcus*, and *Streptococcus*. In the experiment that provoked microbial aerosolization from guano, 3.35% of total cultivable fungi were aerosolized, up to 0.10% of bacteria, and 0.00% of *E. coli*. The concentration of *Staphylococcus* in the air exceeded counts of *Streptococcus*. The highest concentrations of airborne microorganisms were on the ground level. Using cultivation plates as a robust method we demonstrated that the relative proportion of microbial subgroups in the air remained constant in different seasons, with lower concentrations of airborne microbiota in the autumn. Caves as simplified natural systems demonstrated complex relationships between atmospheric parameters and microorganisms. Bats introduce into caves varying, but not negligible, concentrations of microbes on their fur. Caves with guano had relative high concentration of airborne microbes that may represent a biohazard for animals and humans.

INTRODUCTION

Air is an important habitat for metabolically active and reproducing microbes (Womack et al., 2010) and a vehicle for the transport of different microorganisms. When airborne, microorganisms can travel reasonable distances. For some microbes certain air conditions, such as desiccation, extreme temperature, UV radiation, or chemical and radioactive stressors, can be lethal.

Aerosolization, the production of an aerosol, results in a fine mist or spray containing minute particles that contain biological particles. There are different types of bioaerosol formation caused by wind, animals and humans, or splashing water (Mulec et al., 2012c). It occurs in natural and man-made environments. Human exposure to aerosols of organic-rich materials generates a potential risk and can cause different types of infection. The health hazards of poor air quality can be associated with airborne microbes, and exposure to elevated concentrations of microorganisms can lead to numerous respiratory and dermatological infections, allergies, and other problems (Fabian et al., 2005).

The study of bioaerosols in controlled lab conditions provides valuable information, but trials under natural conditions provide a better insight into the fate of minute biological particles. Due to changeable atmospheric

conditions and interrelated environmental stressors such as wind, UV, and humidity, some natural outdoor environments provide complex study conditions. A natural system that is low in environmental stressors and rich in organic material is karst caves harboring piles of bat guano. Caves are generally natural light-free environments connected with the outside by one or more entries, and with high relative air humidity, constant temperature, and low or negligible air movements (Simon, 2012; White and Culver, 2012). Poulson and Lavoie (2000) considered bat guano one of the most important energy inputs for caves in temperate climate zones. Guano is an important habitat, a source for microbial aerosolization, and a biohazard factor for humans and bats, e.g. *Histoplasma capsulatum* (Alteras, 1966; Jülg et al., 2008).

The objectives of this study were to define the relations between atmospheric parameters and airborne microorganisms that derive from *in situ* organic matter in caves.

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Table 1. Caves studied, with abbreviations used in the figures. The distance from the cave entrance to the most remote bioaerosol sampling site is included. Lithology after Bandrabur and Radu, 1994, Bleahu et al., 1976, and Todoran and Onac 1987; biogeographical regions after ANPM, 2013.

Cave	Biogeographical Region	Entrance a.s.l. (m)	Lithology	Studied Distance / Length of Cave (m)
Adam Cave, AC	Continental	295	Jurassic / Cretaceous limestone	25 / 169
Cheile Turzii Cave, CT	Alpine	552	Jurassic limestone	36 / 120
Fușteica Cave, FC	Continental	200	Jurassic / Cretaceous limestone	47 / 1270
Gura Dobrogei Cave, GD	Steppe	46	Jurassic limestone	125 / 500
Meziad Cave, MC	Alpine	440	Triassic limestone	375 / 4750
Răstoci Cave, RC	Continental	319	Eocene / Oligocene limestone	87 / 507
Topolnița Cave, TC	Continental	434	Jurassic / Cretaceous limestone	320 / 20500

Caves in Romania with bat guano were studied to observe seasonal and air-stratification effects on concentration of bioaerosols, as well as to estimate the level of aerosolized microbes from guano and the role of bats as vectors for microbial transmission. To estimate the percentage of aerosolization, a new method to induce aerosolization was developed and tested in caves. Results from the study are useful for comparison with other organic-rich environments that contain biohazard microbes, such as farms, landfills, and wastewater or compost treatment plants.

MATERIALS AND METHODS

STUDY SITES

Seven caves that are rich in guano deposits and populated by bats in three biogeographic and climate regions in Romania were selected: Avenul lui Adam (Adam Cave), Peștera Cetățeaua Mare din Cheile Turzii (Cetățeaua

țeaua Mare Cave from Gorges of Turda, referred to in the text and tables as Cheile Turzii Cave), Peștera Fușteica (Fușteica Cave), Peștera Liliecilor de la Gura Dobrogei (Bat Cave from Gura Dobrogei, referred to as Gura Dobrogei Cave), Peștera Meziad (Meziad Cave), Peștera de la Răstoci/ Peștera Măgurici (Răstoci Cave), and Peștera Topolnița (Topolnița Cave) (Table 1). Apart from Cheile Turzii Cave, which contains a maternity roost, the other caves host bats all year round, including maternity and hibernation colonies. The most prevalent bat species and number of species in each cave are presented in Table 2.

Adam Cave is located in Băile Herculane area in the Domogled-Cerna Valley National Park, which has 700 to 750 mm of annual precipitation (Munteanu, 2011). The cave entrance is vertical, starting with an 11 m shaft. Temperature is constant year round, with an average of 27 °C that is attributed to intermittent steam vapors; water

Table 2. Bats in studied caves; data from Borda, 2002a, b; Borda et al., 2004; Burghel-Bălăcescu and Avram, 1966; Carbonnel et al., 1996; Coroiu et al., 2007; Pocora and Pocora, 2011.

Cave	Presence	Number of Individuals	Number of Bat Species	Main Guano Contributors
Adam Cave	All year	5000	8	<i>Miniopterus schreibersii</i> <i>Myotis capaccinii</i> <i>Myotis myotis</i> <i>Rhinolophus euryale</i>
Cheile Turzii Cave	Summer	1500	3	<i>Miniopterus schreibersii</i> <i>Myotis myotis</i> / <i>M. oxygnathus</i>
Fușteica Cave	All year	1500	7	<i>Miniopterus schreibersii</i> <i>Myotis capaccinii</i> <i>Myotis myotis</i> / <i>M. oxygnathus</i>
Gura Dobrogei Cave	All year	300	10	<i>Miniopterus schreibersii</i> <i>Myotis daubentonii</i> <i>Myotis myotis</i> / <i>M. oxygnathus</i>
Meziad Cave	All year	5000–7000	10	<i>Miniopterus schreibersii</i> <i>Myotis myotis</i> / <i>M. oxygnathus</i>
Răstoci Cave	All year	200	5	<i>Myotis myotis</i> / <i>M. oxygnathus</i>
Topolnița Cave	All year	1500	10	<i>Miniopterus schreibersii</i> <i>Myotis myotis</i> / <i>M. oxygnathus</i>

temperature is around 42 °C and bursts of vapor at 44.5 to 53 °C (Povară et al., 1972). Because of these particularities, the Adam Cave is unique in Romania, being a tropical-type cave, distinct from all other caves from Romania. Due to the intermittent steam emissions, air mixing is caused by the ascending warm and descending colder air. The air circulation is more obvious in the winter, when temperature differences between cave and external air are more pronounced. The warm air accumulating in the cave becomes a perfect shelter for maternity colonies of bats. The largest guano heap in the Guano Gallery is up to 2.5 m high (Carbonnel et al., 1996).

Cheile Turzii Cave is a small cave located on the right slope of Hașdate Valley in the Trascău Mountains where the average annual precipitation is around 600 mm (Beldean, 2005). In the past, the cave offered shelter for the local people against invasions. The entrance still contains part of a fortification wall that partially obstructs normal air ventilation.

Fușteica Cave is located in the Vilcan Mountains where the annual average precipitation is approximately 800 mm (Costache, 2011). This is an active cave with large guano deposits that are partially flushed away by an intermittent subterranean stream that drains to the Isvarna River (Burghel-Bălăcescu and Avram, 1966). Both maternity and hibernation bat colonies are sheltered in the cave.

Gura Dobrogei Cave is located in Dobrogea in southeastern Romania. Annual precipitation in the region is 350 to 450 mm (Lungu, 2008). In the Fossils Gallery there are large quantities of old and fresh deposits of guano.

Meziad Cave is partly a tourist cave and is located in the Pădurea Craiului Mountains, part of the Apuseni Mountains. The annual average of precipitation in the area is 720 mm (Moza, 2008). The cave is a big subterranean cavity developed on three levels, the main gallery, the first floor, and the second floor. Meziad Cave was included in the study because of big maternity and mating colonies of bats and important fresh guano deposits. During our research activities there has been no electrification and no construction of new trails; this resulted in minimal human visits and preservation of the bat population. The cave has a front gate embedded in an artificial stone wall three meters high.

Răstoci Cave is one of the main caves of the Someșan Plateau of Transylvania, and it is the only cave in that area that shelters a considerable number of bats. The annual average precipitation in the Someșan area for the last hundred years is 635 mm (Sorocovschi and Vodă, 2009).

Topolnița Cave is a large cave located in the Mehedinți Mountains where precipitation is between 900 and 1,000 mm per year (Robu, 2009). The gallery network of the cave is developed on four levels, two dry (one lower and one upper), one semi-active, and the lowest one active. We investigated only the upper inactive part of the cave, where *H. capsulatum* was detected for the first time in Europe (Alteras, 1966). A gate installed in 1960s restricted the

access of bats in this part of the cave and probably induced changes in atmospheric conditions. In 1996 two new openings for bats were cut in the wall, which allowed for re-colonization by bats.

MICROBIOLOGICAL MEDIA AND READING RESULTS

Due to the proven versatility of RIDA[®]COUNT cultivation plates in caves (Mulec et al., 2012a, b) and in organic-rich environments (Oarga et al., 2012), the following varieties of this product were used: for total bacterial counts (RIDA[®]COUNT Total Aerobic Count), for *Escherichia coli* and coliforms (RIDA[®]COUNT *E. coli* Coliform), for enterobacteria (RIDA[®]COUNT Enterobacteriaceae), and for yeast and molds (RIDA[®]COUNT Yeast&Mold Rapid). After 24 and 48 hours of cultivation at 37 °C, readings of bacterial growth were scored. For yeasts and molds, readings were taken after 48 and 72 hours of cultivation at the temperature of 25 °C. The counts of bacteria after 48 and for fungi after 72 hours were considered for statistical analyses. Prolonged incubation for 24 hours gives a more realistic view of the microbial communities, as some cave microbes have demonstrated slow growth on RIDA[®]COUNT media (Mulec et al., 2012a). Isolates that exhibited β-D-glucuronidase and β-D-galactosidase biochemical activities on RIDA[®]COUNT *E. coli*/Coliform plates were considered indicative of *E. coli* (R-Biopharm AG, Germany).

To supplement RIDA[®]COUNT readings with additional indicators of potential pathogenic microbes, we used the standard selective media for *Streptococcus* (Azide bloodagar medium; Holmes and Lermitt, 1955) and *Staphylococcus* (Mannitol salt agar medium; Chapman, 1945). Open Petri plates with these media were placed parallel to RIDA[®]COUNT plates in Meziad Cave and Cheile Turzii Cave. The plates were incubated at 37 °C, and colonies were counted after 24 and 48 hours. This approach is frequently used in Romania to quantify airborne microbiota in organic-rich environments such as domestic farms (Drăghici et al., 2002), zoos (Borda et al., 2012), and caves with bat guano (Borda et al., 2004; 2009; Borda and Borda, 2004).

For subsequent identification of bacterial isolates from Adam Cave and Topolnița Cave, the morphologically distinct colonies developed on Total Aerobic RIDA[®]COUNT were plated on glucose nutrient agar (Oxoid, UK) and MacConkey agar (Sigma-Aldrich). After Gram staining, API[®] strips (Biomérieux, France) were used to identify isolates, API20E for enteric bacteria, API20NE for non-enteric Gram-negative bacteria, and APIStaph for identification of staphylococci and micrococci. The results were interpreted using APIweb software.

SAMPLING PROCEDURE AND ATMOSPHERIC PARAMETERS

Four different types of samples were analyzed: bat guano, cave air, cave air inoculated with guano by our *in situ* aerosolization procedure, and swabs of bats' fur. For

air samples, results are reported as colony-forming units (CFU) per surface in a definite period of sampling, e.g., CFU/20 cm²/20 min, for guano as CFU per gram (w/v), and for swabs as CFU per surface, e.g. CFU/20 cm².

Fresh guano and guano accumulated in caves in previous years were included in the study. For microbiological analysis, guano heaps were aseptically sampled with a spoon in a range from 0 to 5 cm depth. After sampling, characteristics of guano heaps were measured: volume, surface area, and pH. pH was measured at the site using pH indicator strips (EMD Chemicals, Germany) after a sample of guano was homogenized with an equal part of sterile deionised water. Samples for microbiological analyses were transported in a cool box to the lab as soon as possible. After establishing the weight of a guano samples, generally around 2 g, 15 mL of sterile physiological saline was added to the samples and rigorously vortexed. This mixture was serially diluted up to 10⁻⁸. One milliliter of these serial dilutions was applied onto RIDA[®]COUNT test plates. Microbial counts were expressed as colony-forming-units per gram.

To sample airborne microorganisms the gravity-settling method was adopted. Open plates with media were exposed to the cave atmosphere for 20 minutes and, after cultivation in laboratory conditions, microbial counts were expressed as CFU per surface unit (Borda et al., 2004; Mulec, 2008; Mulec et al., 2012a, b). In caves, bioaerosol was sampled on the ground floor at different distances from the cave entrance and guano heaps. Besides the horizontal gradient sampling, a vertical gradient of bioaerosols was also sampled by using a 1.5-m standing rack. To diminish the influence of researchers' presence upon the air quality, sampling was performed consecutively; the first sample was taken at the cave entrance, and the next ones later at previously selected sites, up to the last sampling sites at a guano heap in the cave. If cave morphology at a particular site allowed, sampling was performed in duplicate or triplicate, and the average CFU value was used in subsequent analyses. After sampling was finished, the distances from the cave entrance and guano heap for each individual sampling site was measured. In some caves (Adam Cave, Fușteica Cave, Gura Dobrogei Cave, Răstoci Cave, Topolnița Cave) aerosols were sampled once per year, while in Cheile Turzii Cave and Meziad Cave we also sampled in different periods of the year in order to observe seasonal variability. The summer investigations in these two caves took place once the fresh guano was deposited.

In selected caves (Cheile Turzii Cave, Gura Dobrogei Cave, and Meziad Cave) we performed artificially induced aerosolization to estimate the maximum numbers of cultivable microorganisms from aerosols. A spoon of guano was aseptically transferred to a sterile beaker (diameter 7 cm, height 9 cm, with total volume of 0.346 liter). To provoke *in situ* aerosolization of microbes in guano, the beaker with guano was temporary sealed with

a plastic bag and manually shaken for ten seconds. Big particles of guano were shaken off and removed from the beaker, and the beaker was placed with the opening over the RIDA[®]COUNT media for 20 minutes, so the airborne microbes could settle (Fig. 1). The inoculated RIDA[®]COUNT media was later transferred in the laboratory and incubated as previously described.

During natural aerosol sampling we measured atmospheric parameters, temperature, relative humidity (RH), and air pressure with a Kestrel 4500 Pocket Weather Tracker. The carbon dioxide concentration in the atmosphere was measured with a MI70 Vaisala CO₂ meter.

Swabs of bat coats were sampled to get an idea of how many cultivable microbes bats host on their body surfaces and how much bats contribute to spreading of microbes. A moistened sterile cotton swab was used to swab the body of a bat. The cotton swab was placed into a tube with 4 mL of physiological saline and thoroughly shaken. Finally, one mL of the suspension was spread on RIDA[®]COUNT test plates (Total Aerobic Count, *E. coli*/Coliform, Yeast& Mold Rapid). Cultivation conditions were as described above. Microbial counts from the fur coat were expressed as CFU per surface. Two individuals of *Rhinolophus ferrumequinum* and *R. hipposideros* were swabbed at the beginning of hibernation (October-November 2010).

STATISTICAL EVALUATIONS

Canonical correspondence analysis was used to correlate data of abundance of all cultivable microbial groups, *E. coli* (EC), non-*E. coli* coliforms (NECCO), non-coliform bacteria (NCOBA), and yeasts and molds (Y&M) with environmental variables. The NECCO count was calculated as the number of *E. coli* colonies subtracted from the total coliform counts, and the NCOBA count represented all bacteria excluding coliforms (Oarga et al., 2012). Parametric multivariate analysis was run by the program package CANOCO 4.5 (ter Braak and Šmilauer, 2002). The significance of environmental variables in the analysis was tested by a Monte Carlo permutation test. No transformations of the environmental data were applied.

RESULTS AND DISCUSSION

BAT GUANO

Animal excrement is an important source of nutrients, including bat guano in caves (Deharveng, 2005). Guano contains diverse microbiota (Chroňáková et al., 2009) and is a source for microbial aerosolization, which represents a potential biohazard for humans and bats, (e.g., Alteras, 1966; Jülg et al., 2008). Fresh guano is basic, and older guano becomes acidic (Moulds, 2006). Based on macroscopic observation and pH measurements, guano samples were assigned as fresh if they were up to one season old and neutral to alkaline. No correlations were observed between physical guano parameters (pH, volume, surface) and

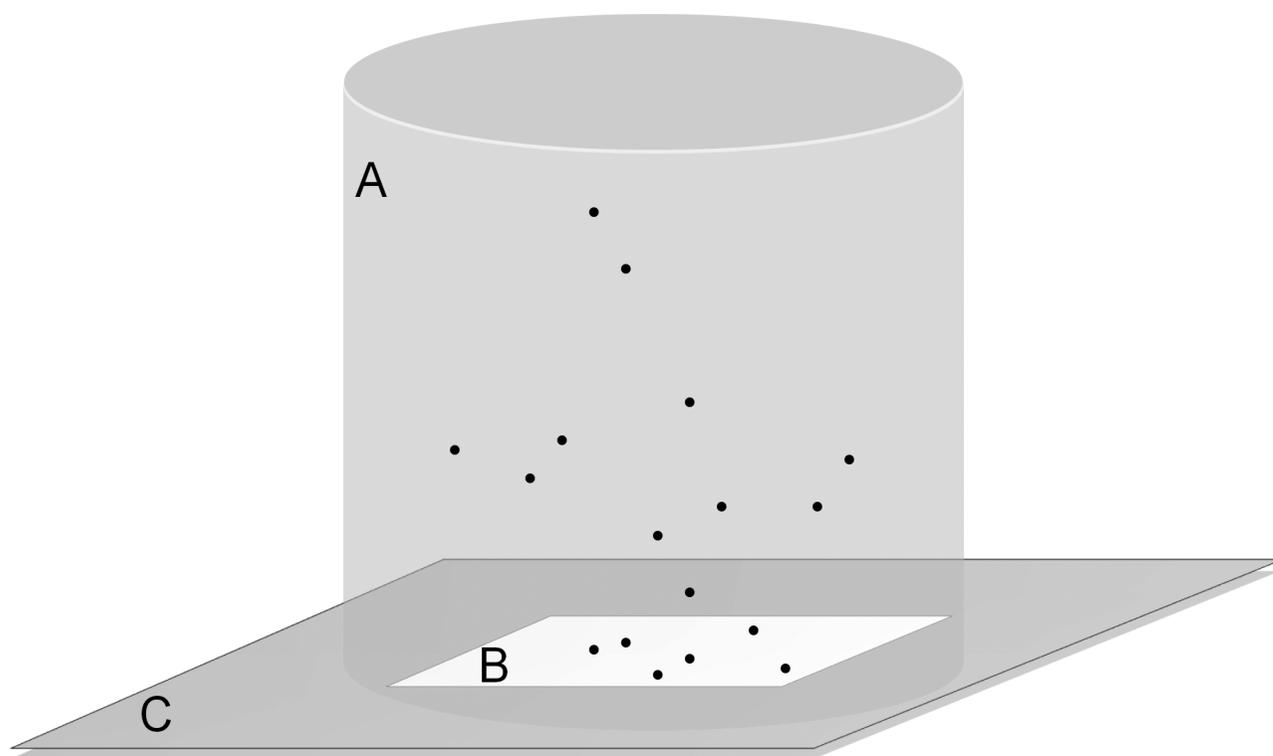


Figure 1. Schematic representation of aerosolization experiment. A, beaker with minute particles represented as dots; B, RIDA®COUNT microbiological medium; C, flexible support with attached microbiological medium.

microbial groups. The biggest guano heap was in Adam Cave (Table 4). The samples of guano varied in concentration of bacteria (up to 1.9×10^{10} CFU/g), coliforms (up to 2.2×10^8 CFU/g), *E. coli* (up to 1.0×10^8 CFU/g), and yeasts and molds (up to 1.7×10^7 CFU/g). The concentrations of fungi varied considerably; interestingly no isolates were retrieved from old guano samples from Gura Dobrogei Cave (Table 5). Coliforms and *E. coli* were not detected in old and dry guano samples from Gura Dobrogei Cave and Topolnița Cave. All guano samples positive on *E. coli*-RIDA®COUNT specific plate were fresh, a few weeks or a few months old. By using the API identification scheme, *Chryseomonas luteola* (99.9% ID) and *Burkholderia cepacia* (99.9% ID) were identified in fresh guano from Adam Cave, and in addition, *B. cepacia* was present in old guano from Topolnița Cave. In a previous study of microbial quantification from guano from Topolnița Cave, Hodoroagea (1972) showed approximately a 150-fold decrease in number of bacteria in the 10–20 cm layer compared with the surface layer (1–5 cm), and fungal concentration was approximately 100-fold higher in the lower layer compared to the guano surface (Hodorogea, 1972).

ATMOSPHERIC CONDITIONS IN CAVES

The highest temperature differences among sampling sites in the same cave were observed in Meziad Cave during sampling in October 2011 (6.6 °C), and in Cheile Turzii

Cave in July 2011 (4.1 °C) (for temperature ranges see Table 3). In both caves, even in colder months, slightly higher temperatures were observed in the sectors with the biggest guano heaps. Temperature differences between sampling sites were high in Meziad Cave also during sampling in November 2010, 2.7 °C, and in Fușteica Cave in October 2010, 2.1 °C. In other caves, temperature differences were smaller. The highest air temperatures were measured in Adam Cave (26.0 to 27.3 °C), with a difference of 1.3 °C between sampling sites. The biggest differences in relative humidity among sites in the same cave were in Meziad Cave in October 2011 at 27.5% and in November 2010 at 14.4%. The next higher differences among sites were in Fușteica Cave (20%), followed by Cheile Turzii Cave (8.4%); this cave had also the lowest measured relative humidity (42.8 to 51.2%). When CO₂ concentration was measured, the biggest differences among sampling sites were in Meziad Cave during November 2010 sampling (175 ppm), followed by Răstoci Cave (133 ppm) and Adam Cave (103 ppm). In Adam Cave, the concentration was the highest among all caves, ranging between 1,307 and 1,410 ppm (Table 3).

AIRBORNE MICROBES

Except in Fușteica Cave and Meziad Cave, the total concentration of airborne bacteria was higher than the total concentration of airborne yeast and molds (Fig. 2). In Adam Cave, the concentration of airborne bacteria was the

Table 3. Ranges of atmospheric parameters during sampling.

Cave	Date (mm/dd/yy)	Temperature (°C)	Relative Humidity (%)	CO ₂ (ppm)
Adam Cave	10/29/10	26.0–27.3	96.4–99.8	1307–1410
Cheile Turzii Cave	07/15/11	14.8–18.9
	10/16/11	8.4–9.3	42.8–51.2	410–470
Fușteica Cave	10/28/10	8.2–10.3	80.0–100.0	421–423
Gura Dobrogei Cave	10/23/11	11.0–12.8
Meziad Cave	11/02/10	11.5–14.2	84.3–98.7	397–572
	07/26/11	13.3–14.6
	10/17/11	7.5–14.1	62.9–90.4	370–416
Rastoci Cave	10/25/10	8.1–8.8	96.0–100.0	418–551
Topolnița Cave	10/30/10	12.7–13.6	96.0–100.0	488–557

highest compared to other caves, probably due to the large volume and contact surface of guano (Table 4). High temperature and high humidity (96.5% to 99.8%) in combination with vaporized compounds from guano that can serve as nutrients for microbial multiplication create an excellent air habitat for microbes. In addition, intermittent thermal emanations in this cave can introduce additional compounds into cave atmosphere and enhance the aerosolization effect. The lowest concentration of airborne microbes was in Fușteica Cave, where guano heaps were located along both sides of the riverbed. The low concentration can be related to the permanent flow of the underground river that constantly washes the central part of the guano deposit and creates continual water saturation of the air, so that aerosol particles settle relatively quickly.

Airborne microorganisms had previously been studied in Romanian caves (Borda and Borda, 2004; Borda et al., 2004) with different ranges of cultivable aerobic bacteria (56 to 1,021 CFU/m³) and fungi (52 to 22,373 CFU/m³). When caves were rich in guano, total aerobic bacteria of up to 11,317 CFU/m³ were detected. A direct comparison with this study is not appropriate due to the use of different sampling methods and cultivation media; in this study RIDA[®]COUNT cultivation plates were used and results are reported as CFU/20 cm²/20 min.

Coliform bacteria and *E. coli*, representative of fecal enterobacteria and common sources of enteric infections (Guentzel, 1996), were screened to observe microbial load that probably originated from feces. Concentrations of

airborne coliforms varied reasonably. Typical *E. coli* colonies on RIDA[®]COUNT *E. coli*/Coliform plates were not detected in the air in Adam Cave, Cheile Turzii Cave, and Fușteica Cave (Fig. 3). Furthermore, despite high concentration of airborne coliforms and total bacteria in Adam Cave and Cheile Turzii Cave, no typical *E. coli* colonies were retrieved on the media, which indicates that *E. coli* is short-lived when airborne and its presence in the cave air is more or less random. Additionally, the results of *in situ* aerosolization showed that *E. coli* is hard to find in aerosols (Fig. 6). *E. coli* does not survive generally more than two or three weeks in low-nutrient environments in a viable and cultivable state (Neidhardt et al., 1996), and indications of its presence in nutrient-poor cave habitats should be carefully examined (Barton and Pace, 2005). Airborne fecal coliforms generally do not survive long outdoors, so the probability of causing infections for wildlife and humans is low (Hughes, 2003). Similar conclusion could be drawn for the underground environment.

Distinct bacterial colonies from Adam Cave and Topolnița Cave that developed on the RIDA[®]COUNT Total Aerobic were identified using API as *Klebsiella pneumoniae* ssp. *ozaenae*, *Salmonella arizonae*, and *Salmonella* spp. The source of these microbes is very likely bats' intestines (e.g., Adesiyun et al., 2009; Di Bella et al., 2003). These microbes are also present in human intestines (Guentzel, 1996). In the air in Topolnița Cave, a non-Enterobacteriaceae isolate, *Chryseomonas luteola* (99.9% ID), was also found.

Table 4. Physical characteristics of studied guano heaps in the investigated caves.

Cave	Studied Guano Heaps	pH	Volume, m ³	Surface, m ²
Adam Cave	1	4.4	32.710	43.90
Cheile Turzii Cave	1	5.8–8.5	0.010–0.400	0.25–4.00
Fușteica Cave	3	4.0–6.5	0.001–0.058	0.30–1.00
Gura Dobrogei Cave	4	4.8–7.2	0.001–0.610	0.09–3.51
Meziad Cave	2	4.7–7.8	0.015–1.360	1.54–6.60
Răstoci Cave	1	4.5	0.049	0.98
Topolnița Cave	2	4.0–6.8	0.375–9.600	3.75–40.00

Table 5. Microbial counts in guano samples expressed as colony-forming units per gram after 48 hours of incubation for bacteria and 72 hours for yeasts and molds.

Cave	Bacteria (CFU/g)	Coliforms (CFU/g)	<i>E.coli</i> (CFU/g)	Yeast and Molds (CFU/g)
Adam Cave	2.1×10^8 – 2.1×10^8	3.37×10^5 – 6.5×10^5	1.4×10^4 – 2.2×10^5	2.5×10^6 – 1.7×10^7
CheileTurzii Cave	6.5×10^7 – 1.9×10^{10}	1.7×10^6 – 2.0×10^8	1.2×10^6 – 1.0×10^8	5.9×10^3 – 1.7×10^7
Fușteica Cave	8.8×10^4 – 1.7×10^6	1.8×10^3 – 3.8×10^4	0.0	1.0×10^5 – 2.3×10^6
Gura Dobrogei Cave	9.3×10^3 – 7.9×10^5	0.0– 9.3×10^4	0.0– 6.1×10^3	0.0– 3.4×10^4
Meziad Cave	3.1×10^5 – 2.2×10^8	3.9×10^3 – 9.8×10^7	0.0– 3.1×10^6	7.2×10^1 – 3.6×10^6
Răstoci Cave	1.2×10^5	2.2×10^3	0.0	6.8×10^4
Topolnița Cave	3.6×10^3 – 1.1×10^9	0.0– 2.2×10^8	0.0– 8.3×10^4	9.2×10^4 – 8.9×10^5

Screening for the presence of *Streptococcus* and *Staphylococcus* was performed; they are an important part of indoor atmospheres (Mandal and Brandl, 2011; Hospodsky et al., 2012). *Staphylococcus* is usually used as an indicator for microbiota from skin and mucous membranes (Aydogdu et al., 2005; Schulz et al., 2004), and *Streptococcus* indicates oral, pharyngeal, and skin bacterial biota and even faecal soil pollution (Kibbey et al., 1978). Except for sampling in Meziad Cave in July 2011, the concentration of *Staphylococcus* in the air exceeded counts of *Streptococcus* (Fig. 4). Concentration of staphylococci and streptococci are frequently elevated in the proximity of guano heaps during the summer when bats are active and fly in their underground roosts (Borda et al., 2004). The biochemical profile of two isolates from Adam Cave atmosphere revealed *Staphylococcus* and *Micrococcus* (99.8% ID). Micrococci are not as common as staphylococci; however, both are frequently present in bat guano (Mohod, 2011; Vandžurová et al., 2013). This group of

microbes can survive in the air for a long time; for example, *Staphylococcus aureus* can survive several months on fabric or dust particles (Mitscherlich and Marth, 1984). Under natural cave conditions airborne *Staphylococcus* and *Streptococcus* are expected to be viable much longer than *E. coli*.

In caves with bat guano, elevated concentrations of airborne bacteria, and to a lesser extent, fungi were detected in our study. We identified *Chryseomonas luteola*, *E. coli*, *Klebsiella pneumoniae* ssp. *ozaenae*, *Micrococcus*, *Salmonella*, *Staphylococcus*, and *Streptococcus*, as well as unidentified cultivable bacteria, coliforms, and enterobacteria. All these microbes can be indicative for bats and guano, and in caves, they might represent a biohazard because they can survive longer as airborne there than in other organic-rich environments with more environmental stressors. High microbial concentrations in guano did not always correspond to high concentration of airborne microbes at the same guano heap (compare Fig. 2 and Table 5).

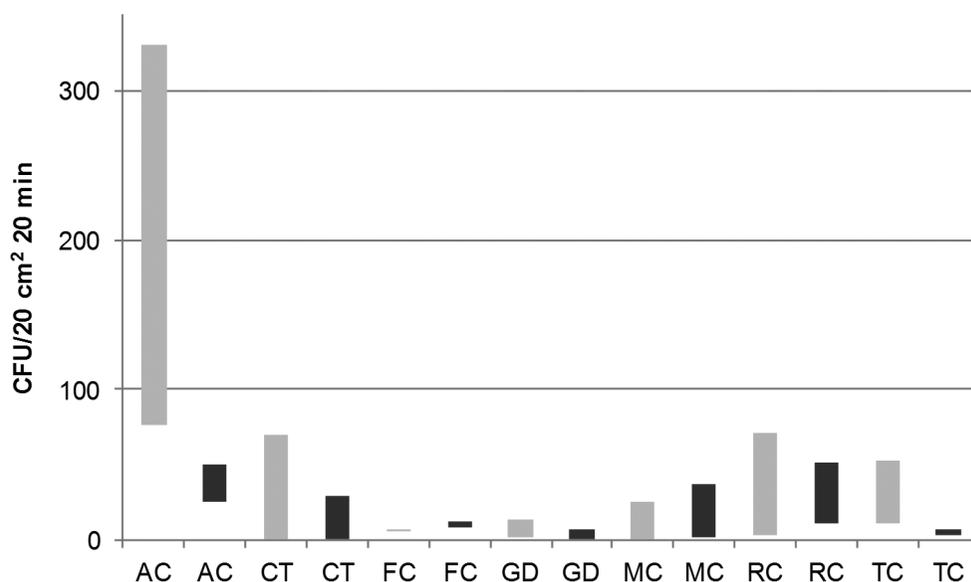


Figure 2. Ranges of concentrations of airborne microbes (grey, total bacteria; black, yeasts and molds) in the air of Adam Cave (AC), Fușteica Cave (FC), Meziad Cave (MC), Răstoci Cave (RC), and Topolnița Cave (TC) in autumn of 2010, and in Cheile Turzii Cave (CT), Gura Dobrogei Cave (GD), and Meziad Cave (MC) in autumn of 2011.

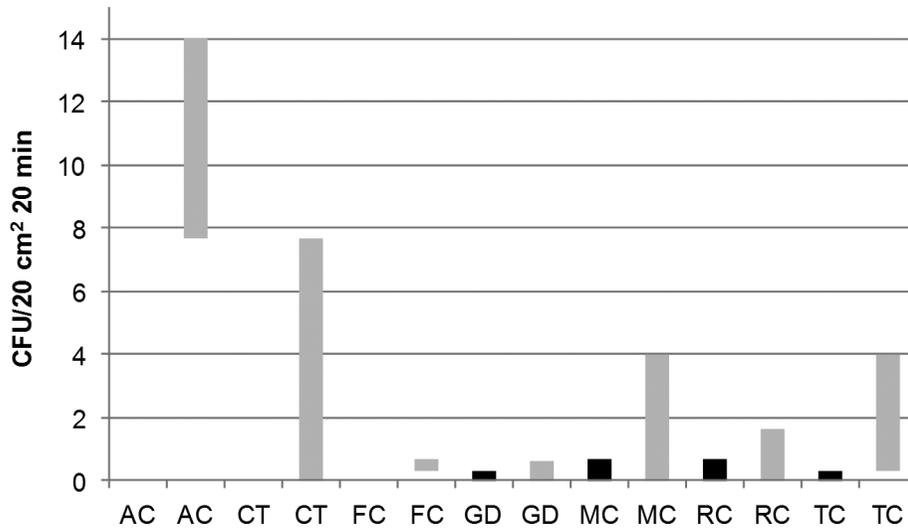


Figure 3. Ranges of concentrations of coliforms (grey) and *E. coli* (black) in the air of Adam Cave (AC), Fușteica Cave (FC), Meziad Cave (MC), Răstoci Cave (RC), and Topolnița Cave (TC) in autumn of 2010, and in Cheile Turzii Cave (CT), Gura Dobrogei cave (GD), and Meziad Cave (MC) in autumn of 2011.

AIRBORNE MICROBES AND ATMOSPHERIC PARAMETERS

Canonical correspondence analysis was performed on the basis of six variables, if available: temperature, relative humidity, air pressure, CO₂, distance to the cave entrance from each individual sampling site, and distance to the guano heap from each individual sampling site. Only a small portion of measured environmental variables explained the variances (Table 6). Measured physical parameters explained the highest variance in the structure of microbial communities in the autumn in Gura Dobrogei Cave (October 2011) by 0.31 and in Meziad Cave (November 2011) by 0.12. In other caves in autumn and also in summer they explained less than 0.09. Temperature had a significant impact ($p < 0.05$) on the bioaerosol

abundance in Meziad Cave in November 2011 and RH in Topolnița Cave in October 2010.

In a study from Postojna Cave, Slovenia, (Mulec et al., 2012c) where the impacting sampling method was used, in the transition periods, i.e., spring and autumn, physical parameters explained variances less (winter 0.62, spring 0.25, summer 0.49, autumn 0.08). The sampling method used in this study is based on gravity settling and is not directly comparable. The method based on settling is very sensitive to any air disturbances that can cause the particles to deviate from their vertical settling route. Other factors that influence settling behavior, in addition to air movements, pressure, temperature, and human and animal movements, are characteristics of an individual particle,

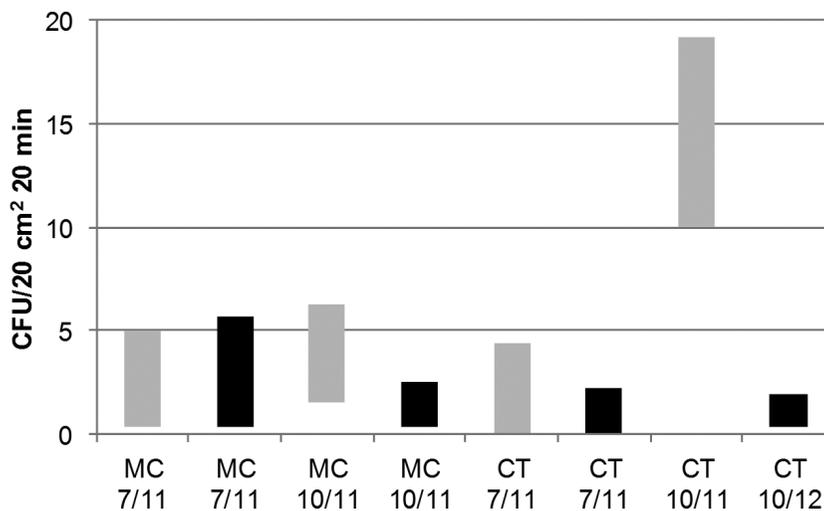


Figure 4. Ranges of concentrations of airborne *Staphylococcus* (grey) and *Streptococcus* (black) in Meziad Cave (MC) and Cheile Turzii Cave (CT) in July (7) and October (10) of 2011.

Table 6. Summary of canonical correspondence analysis analysis using forward selection for explanation of variance by selected variable. Dist_Ent, distance from the closest cave entrance; Dist_Gua, distance to the closest guano heap. AC, Adam Cave; CT, Cheile Turzii Cave; FC, Fușteica Cave; GD, Gura Dobrogei Cave; MC, Meziad Cave; RC, Răstoci Cave; TC, Topolnița Cave. In bold, $p < 0.05$.

Parameter	Cave / Period									
	AC / Oct 10	CT / Jul 11	CT / Oct 11	FC / Oct 10	GD / Oct 11	MC / Nov 10	MC / Oct 11	MC / Oct 11	RC / Oct 10	TC / Oct 10
Temperature										
<i>p</i>	1.00	1.00		1.00	0.24	0.05	1.00	0.14	1.00	1.00
var.	0.08	0.02		0.01	0.02	0.10	0.02	0.05	0.01	0.00
Relative Humidity										
<i>p</i>						1.00		1.00		0.03
var.						0.01		0.00		0.06
Pressure										
<i>p</i>			0.171					0.43	0.37	
var.			0.05					0.02	0.03	
CO ₂										
<i>p</i>									0.38	0.12
var.									0.02	0.00
Dist_Ent										
<i>p</i>							0.22			0.36
var.							0.05			0.03
Dist_Gua										
<i>p</i>		0.35			0.09	0.20	0.43			
var.		0.02			0.29	0.01	0.01			
Variance explained	0.09	0.04	0.05	0.01	0.31	0.12	0.08	0.07	0.06	0.09
Sum of all eigen values	0.08	0.05	0.05	0.01	0.31	0.12	0.08	0.07	0.06	0.09

such as its size and mass, and morphological characteristics of a space that, for example, enables creating of air gaps. Interestingly, distances from guano did not always contribute to the common variance during autumn samplings, but during both summer samplings in Cheile Turzii Cave and in Meziad Cave they did. This can be attributed as a seasonal effect connected with the presence of bats and very fresh guano droppings and its aerosolization. In that period, a pronounced gradient of airborne microbes can be formed radiating from a site of a bat colony and its guano heap, but more data are needed to confirm that. Nevertheless, conditions in cave atmospheres are complex and dynamic and become more complicated when other factors are involved, such as aerosolized microbes/particles from animal excrements. Sampling position in the space has also very important influence on the detection of bioaerosol signals.

VERTICAL MICROBIAL GRADIENT AND EFFECT OF SEASONALITY

Concentration of airborne microorganisms at different heights above the ground varied, indicating that selection of bioaerosols sampling positions is important and should

be clearly reported (Fig. 5). The concentration of airborne microorganisms was generally higher when bioaerosols were sampled at the ground level, in Cheile Turzii Cave on average 3.4 orders of magnitude and in Meziad Cave on average 1.3 orders. In both Cheile Turzii Cave and Meziad Cave, the total microbial counts were higher in summer than in autumn. Total microbial counts were defined as a sum of counts of *E. coli* (EC), non-*E. coli* coliforms (NECCO), non-coliform bacteria (NCOBA), and yeasts and molds (Y&M) (Fig. 5). Coliform bacteria other than *E. coli* were detected in both caves in the summer period. In addition, in Meziad Cave *E. coli* was detected airborne on *E. coli*-specific plates, while in Cheile Turzii Cave these bacteria were not observed in the air at all. Sampling site MC2 in Meziad Cave is located in a big chamber (Pyramid Room, approx. 220,000 m³) with 0.015 m³ of guano, and sampling site MC3 is located in a smaller chamber (Bat Room, approx. 37,500 m³) with a greater quantity of guano (~1.36 m³) that is more scattered in the space. The Bat Room, which had a higher concentration of airborne microbes, shelters from May to August a big nursery colony of about five to seven thousand individuals of *Myotis myotis*/*M. oxygnathus* mixed with *Miniopterus schreibersii*.

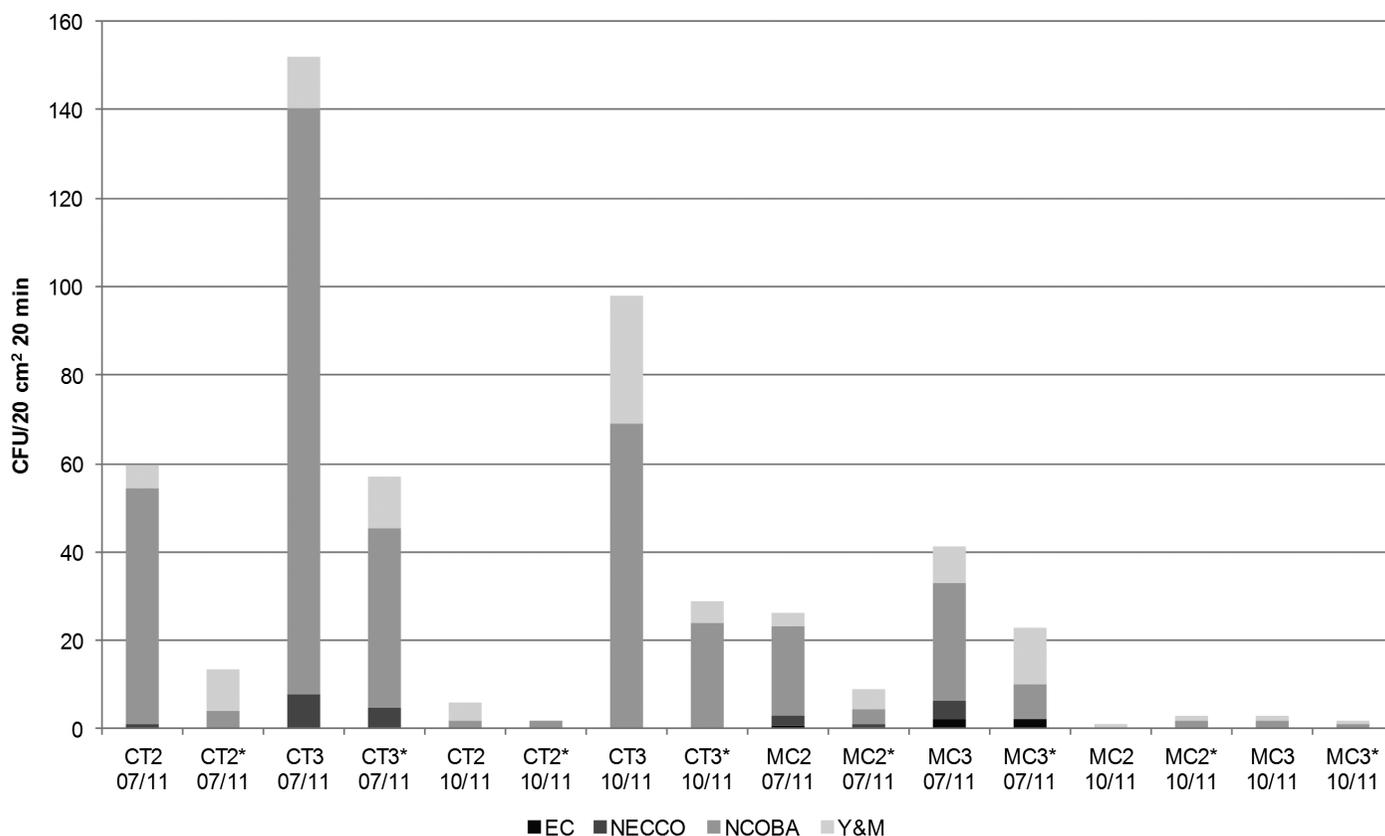


Figure 5. Effect of season and sampling position on concentration of airborne microbes at Cheile Turzii Cave (CT) sampling sites 2 and 3 and at Meziad Cave (MC) sample sites 2 and 3. Sampling was performed in July (7) and October (10) 2011. Samples taken 1.5 meters above the ground are designated by an asterisk; other samples were taken at ground level. EC, *E. coli*; NECCO, other coliforms; NCOBA, non-coliform bacteria; Y&M, yeasts and molds.

The proportion of microbial subgroups seems to be the same at each tested site, with generally smaller concentrations in the autumn when air changes with the subsurface are suppressed and maternity colonies of bats are dispersed. The concentrations of airborne fungi were rather low compared to bacteria (Fig. 5). It is important to underline that sampling at 1.5 m above the ground was chosen as the level of human breathing to point out the possibility of getting in contact with bioaerosols.

AEROSOLIZATION OF MICROBES FROM GUANO

Percentage of aerosolized microbial groups from samples from Cheile Turzii Cave, Gura Dobrogei Cave, and Meziad Cave are summarized in Figure 6. Total bacterial counts ranged from 0.00 to 0.10%, coliforms from 0.00 to 0.16%, *E. coli* 0.00%, enterobacteria from 0.00 to 0.03%, and yeasts and molds from 0.00 to 3.35%. In the three independent sampling campaigns, aerosolization of *E. coli* was unsuccessful, although concentration of this microbe in different tested guanos varied from 0.0 to 1.7×10^7 CFU/g (Table 5). On the other hand, successful aerosolization of coliform bacteria and enterobacteria indicated that aerosolization of *E. coli* may be possible. Transmission of *E. coli* via cave air is probably quite limited.

Artificially induced aerosolization resulted in a large amount of aerosolized fungi from guano. Aerosolization of microbes in caves can be easily enhanced by disturbing guano surfaces while walking. From other microbes-rich materials in different environments, aerosolization can also be caused by wind and animals. Due to their small size, microbes can persist long in the air. For example, droplets more than 5 μm in diameter sediment more quickly on the ground than droplets that are less than 5 μm , which can remain suspended in the air for a long time (D'arcy et al., 2012). As bat guano in Topolnița Cave was already reported to be the source of *H. capsulatum* (Alteras, 1966), the estimation that more than 3% of cultivable fungi may be aerosolized gives an important warning to avoid contact with potential fungal pathogens from guano. High aerosolization potential of fungal spores from guano can be the answer for many cases of guano-associated histoplasmoses reported in landfill, bridge, and wagon-train workers (Gustafson et al., 1981; Huhn et al., 2005), during home renovations (Schoenberger et al., 1988), or among cavers (Ashford et al., 1999).

BATS AS VECTORS FOR MICROORGANISMS

Bats were swabbed to get an estimation on transmission of microbes on their bodies. Swabbing of *Rhinolophus*

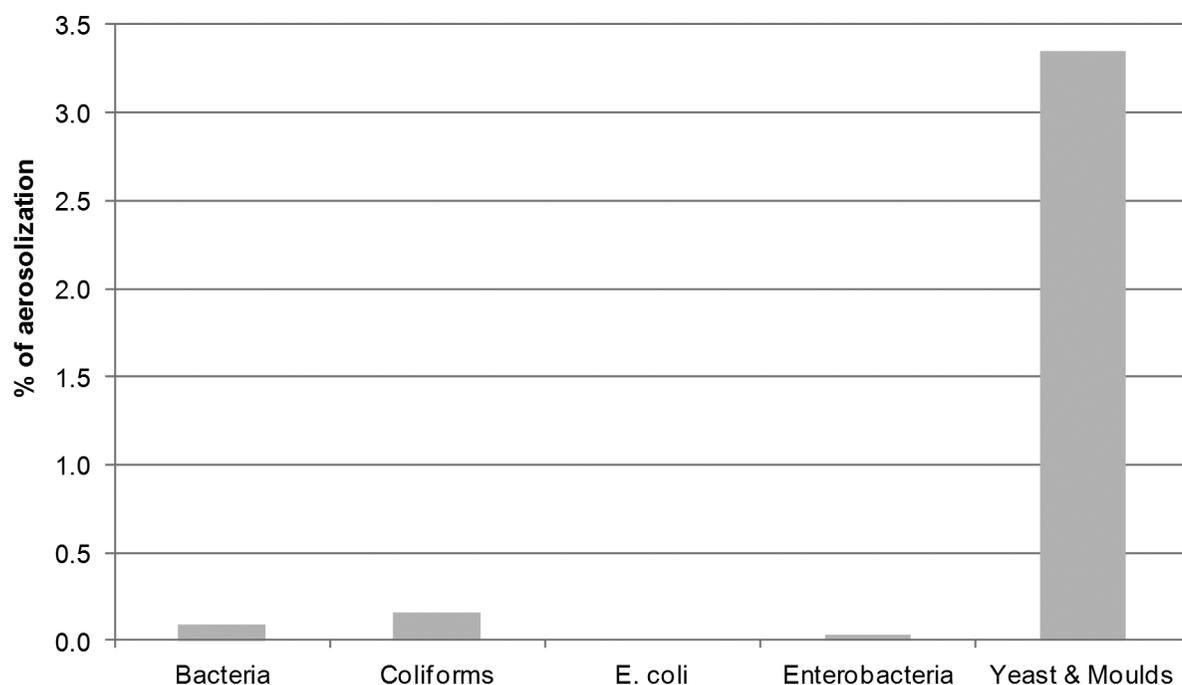


Figure 6. Percentage of aerosolised microbial groups from bat guano.

ferrumequinum coats produced higher numbers of microorganisms than *R. hipposideros* coats (Table 7). Although their body sizes are quite different, *R. ferrumequinum* being almost twice that of *R. hipposideros* (57 to 71 mm versus 37 to 45 mm head and body; 350 to 400 mm versus 192 to 254 mm wingspan), it is possible that behavior is more important for microbial contamination of cave air. In the sampling period at the beginning of November, individuals of *R. hipposideros* were already prepared to hibernate and their daily torpor was already very deep, in contrast to *R. ferrumequinum*, which were still active. Social behavior of bats could also play an important role in spreading of microorganisms; solitary bats rarely get in contact with other bats. *R. hipposideros* usually hang free as isolated individuals and during hibernation wrap themselves completely in their wings, but individuals of *R. ferrumequinum* hibernate in big clusters, where bats coats get in contact because of incomplete wing wrapping. The role of bat species in the propagation and spread of fungi is already known. On one hand their feces serve as a source of

nutrients for microbes, and on the other hand they can be active disseminators of fungi in the environment (Hoff and Bigler, 1981).

CONCLUSIONS

Elevated concentrations of bacteria, including those of enteric origin and to a lesser extent of fungi, were detected in, and around, bat guano. Favorable atmospheric conditions for microbial multiplication, such as high temperature and vaporized compounds from organic matter, produced the highest counts of airborne microorganisms. A cave rich with guano, with high relative humidity, and with a flowing river had the lowest concentrations of airborne microbes. Concentrations of airborne microorganisms were higher at ground level compared to the concentrations at 1.5 m above the ground. Coliform bacteria were frequently detected in air in areas with high organic matter and animal excrements. *E. coli* was rarely found in air, and its successful aerosolization

Table 7. Swab analyses of fur coat of *Rhinolophus* from Meziad Cave expressed as CFU/20 cm² after 48 hours of plate incubation for bacteria and 72 hours for yeast and molds.

Bat Species	Total Bacteria (CFU/20 cm ²)	<i>E.coli</i> (CFU/20 cm ²)	Coliforms (CFU/20 cm ²)	Yeast and Molds (CFU/20 cm ²)
<i>R. ferrumequinum</i> 1	821	0	0	1067
<i>R. ferrumequinum</i> 2	2005	0	135	1030
<i>R. hipposideros</i> 1	0	0	0	21
<i>R. hipposideros</i> 2	5	0	0	7

was not achieved. Microbial aerosolization rate from guano was for bacteria up to 0.10%, and for fungi up to 3.35%. *Staphylococcus* and *Streptococcus* were found in air close to bat guano. In the summer, microbial load in the cave air was elevated, which we attribute to the presence of bats. The proportion of microbial groups was preserved in the summer and autumn periods. Bats and especially their social behavior are crucial for introduction and spread of airborne microbes. Airborne microbes indicative of bats and guano and especially a high potential of fungal aerosolization represent a biohazard for animals and humans in caves. Activities that lead to aerosol formation from animal excrements should be avoided. In comparison to other external environments, caves provide a simplified system of studying bioaerosols, although even in caves complex relationships between atmospheric and microbiological parameters co-exist.

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