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Culture-dependent and independent approach for investigating bat guano microbiome

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Abstract. Human-bat interactions, with both positive (understanding the microbial ecology of extreme environments and discovering novel sources of bioactive compounds) and negative (zoonotic infections reservoir) consequences, highlight the necessity to investigate the microbiome of guano deposits, especially under climate change scenarios. This study investigated the microbiome of bat guano deposits from Isverna Cave, Romania, by bridging culture-based, DNA-based and flow cytometry. Our culture-dependent approaches revealed a predominance of Gram-positive bacteria, but also the presence of Gram-negative bacteria of clinical interest (*Serratia fonticola, Hafnia alvei, Moellerella wisconsensis,* and *Escherichia coli*). A modified DNA extraction method, based on the DNeasy PowerSoil Pro protocol, exhibited superior performance, yielding high-quality DNA suitable for downstream applications. Flow cytometry analysis revealed a greater microbial load as compared to cultivation, higher in the hibernation colony versus the maternity colony. This multi-methodological approach provides a framework for further research into the complexity of the bat guano microbiome.

Keywords: Bat guano microbiota, MALDI-TOF, DNA extraction, microbial load, pathogenic species

1. Introduction

Romania is home of well-developed karst systems, with almost 12.000 caves (Goran, 2019), which provide an essential habitat for bats. To date, 32 bat species have been documented in Romania, of which 24 are trogloxenes, using caves as transitional habitats (EUROBATS, 2022). Bat guano represents a significant source of organic matter in caves (Ferreira & Martins, 1999). The bat guano microbiome is mostly composed of Proteobacteria and Firmicutes phyla (Tomova *et al.*, 2013; Vandžurová *et al.*, 2013; Borda *et al.*, 2014; Li *et al.*, 2018; Newman *et al.*, 2018; Gerbáčová *et al.*, 2020; Lundberg & McFarlane, 2024). In the less acidic upper layers of guano, bacterial diversity is higher compared to the deeper ones (Newman *et al.*, 2018; Lundberg & McFarlane, 2024), including bacteria from guanophilic invertebrates, and partially digested insect prey (Lundberg & McFarlane, 2024). Pathogenic agents are normally present in these layers, they spread between bat communities, often over long distances, being thus facilitated by bats' aggregation and migratory behavior (Newman *et al.*, 2018; Smythe *et al.*, 2002). A better understanding of the guano microbiome can provide valuable tools not only for bat conservation, but also for biotechnology, as a source of biologically active compounds (e.g., enzymes, pigments, antimicrobial substances etc.) (Rahmawati *et al.*, 2016; Suryavanshi *et al.*, 2016; Jamil *et al.*, 2023). Despite its scientific significance, the bat guano microbiome remains relatively unexplored, with very few studies having been conducted in this area, especially in Romania (Borda *et al.*, 2014; Manolachi *et al.*, 2023), one of the European countries with the highest bat diversity (EUROBATS, 2022). To fill this gap, the aim of this study was to explore the microbiome of insectivore bat guano deposits from Isverna Cave, Romania, by bridging culture-based, Next Generation Sequencing and flow cytometry as an emerging method for estimating the total number of bacterial particles.

2. Materials and Methods

2.1. Study Site

The targeted area of interest, Isverna Cave, is located in a protected zone, with an elevation of 450 meters at the base of the Mehedinți Mountains, Romania (44°58′40″N 22°38′29″E), marking a site of biological, ecological, and geomorphological importance. The cave is 4 km long, but only the first section is accessible over a distance of 200 meters, more than half being submerged (Baciu *et al.*, 2019). The initial section of the cave, houses a hibernation colony of *Rhinolophus ferrumequinum*, as well as a few *R. hipposideros*, *R. blasii* and *R. euryale* (Bücs *et al.* 2024). The maternity colony, formerly located close to the entrance, composed of several hundred *Myotis myotis* and *M. blythii* was wiped out by an unknown agent in 2015 (most probably a pesticide used in nearby agriculture), destroying the whole colony (Bücs *et al.* 2017), with no recovery since then (Bücs *et al.* 2024).

2.2. Sample collection and bacterial strains isolation

The bat guano samples (*n*=9) were collected in the summer of 2024 from Isverna Cave under optimal conditions to prevent contamination. Based on the location of bat colonies and the presence of guano deposits, samples were collected in biological triplicates from designated sampling points: The Main Gallery - Maternity Colony, The Ceramics Gallery - Hibernation Colony (Wall), and The Ceramics Gallery - Hibernation Colony (Floor). The samples were transported to the laboratories of the Department of Microbiology and Immunology at the Research Institute of the University of Bucharest, where they were stored at 4°C (for the culture-dependent approach) and -80°C (for the culture-

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independent approach) until processing.

About 1 g of each sample was homogenized in 9 mL of sterile physiological saline solution and gently agitated at 250 rpm for 30 minutes at room temperature. The suspensions were allowed to settle by vertical gravitational sedimentation for approximately 60 minutes, after which the supernatant was used to prepare tenfold serial dilutions (10^{-1}–10^{-3}). Volumes of 100 μL from each dilution were spread onto two different culture media, i.e. TSA (*Tryptic Soy Agar*) and Blood Agar for microbial isolation. Bacterial counts were expressed as colony-forming units (CFU) per gram at 24, 48, and 72-hour intervals. Following the three-day incubation period at 37 °C, bacterial colonies with different morphologies were randomly selected for subcultivation on the same media and taxonomically identified by MALDI-TOF MS (*Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry*).

2.3. DNA extraction and 16S rRNA gene amplification

The performance of five DNA extraction protocols was assessed according to the manufacturer's instructions: (1) *DNeasy PowerSoil Kit* (QIAGEN), (2) *DNeasy PowerSoil Pro Kit* (QIAGEN), (3) *QIAamp Fast DNA Stool Mini Kit* - Isolation of DNA from Stool for Human DNA Analysis (QIAGEN), (4) *QIAamp Fast DNA Stool Kit* - Isolation of DNA from Stool for Pathogen Detection (QIAGEN), and a modified *DNeasy PowerSoil Pro Kit* (QIAGEN) protocol. As outlined in the *DNeasy PowerSoil Pro Kit* Handbook, the modifications included extending step 2 to 30 minutes; adding 500 μL of 70% ethanol after step 14; performing step 15 as recommended; repeating steps 14 and 15; eluting DNA in 45 μL of Solution C6 in step 16; and storing the eluted DNA at 4°C for 2 days, followed by storage at -20°C (step 17).

The efficiency of the tested protocols for extracting bacterial DNA was evaluated through *16S rRNA* gene amplification. Each PCR reaction (25 μL) included 1 μL of DNA, 12.5 μL of DreamTaq PCR Master Mix, 1 μL of 8F primer at 10 μM (5′-AGAGTTTGATCCTGGCTCAG-3′), 1 μL of 1492R primer at 10 μM (5′-GGTTACCTTGTTACGACTT-3′), and 9.5 μL of H_2O.

The thermocycler settings were as follows: initial denaturation (95°C, 5 minutes), followed by 35 cycles of denaturation (95°C, 40 seconds), annealing (54°C, 60 seconds), and extension (72°C, 90 seconds), ending with a final extension (72°C, 5 minutes).

The quality and concentration of DNA extracts and PCR products were measured spectrophotometrically using a NanoDrop ND–1000, and their integrity was evaluated by agarose gel electrophoresis.

Flow cytometry (FC) analysis

Sample preparation for FC involved homogenizing guano material in sterile saline solution, followed by removal of coarse particles and fluorescent staining of the microbial fraction with

Syto-9™ (Invitrogen, Thermo Fisher Scientific), and analysis on a BD FACSCalibur cytometer.

3. Results

3.1. Culture-dependent approach

The CFU values varied depending on the culture medium used, ranging from 1.3 x 10^6 to 3.01 x 10^6 on TSA and from 1.6 x 10^6 to 1.78 x 10^7 on Blood Agar, after 72h. Regarding hemolytic activity, analysis of bacterial colonies cultured on Blood Agar showed that 62.5% exhibited α-hemolysis, 4.68% β-hemolysis, and 32.81% γ-hemolysis.

The cultivable microbiome consisted of 3 phyla, 13 families, and 21 species, mostly Gram-positive bacteria (50 bacterial strains were identified through mass spectrometry out of approximately 300 isolates). MALDI-TOF analysis of the bacterial colonies revealed a dominance of the phylum Firmicutes (59.62%), followed by Pseudomonadota (26.92%) and Actinomycetota (13.46%). At the family level, *Bacillaceae* (19.23%), *Yersiniaceae* and *Streptococcaceae* (15.38%), and *Micrococcaceae* (11.54%) were the most common.

At the species level, only eight taxa were common on both culture media: *L. lactis, B. mycoides, E. faecalis, S. fonticola, C. maltaromaticum, H. alvei*. The relative abundance of bacteria varied with the culture media used. For instance, *S. fonticola* was significantly more abundant on Blood Agar (28.57%) compared to TSA (3.33%). Pathogenic and potentially pathogenic Gram-negative bacteria were also detected: *S. fonticola, H. alvei, Moellerella wisconsensis*, and *Escherichia coli.*

3.2. Culture-independent approach

A comparative analysis of DNA extraction protocols revealed that the modified method based on the *DNeasy PowerSoil Pro Kit* (QIAGEN) outperformed in terms of purity (Figure 1) and concentration (Figure 2). The target gene amplification was only successful after diluting the DNA extracts to a final concentration of 40 ng/µL and using a PCR Master Mix instead of individual components for the PCR reaction.

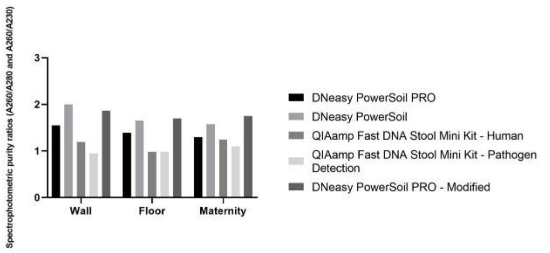

Figure 1 Spectrophotometric purity ratios (A_{260}/A_{280} and A_{260}/A_{230}

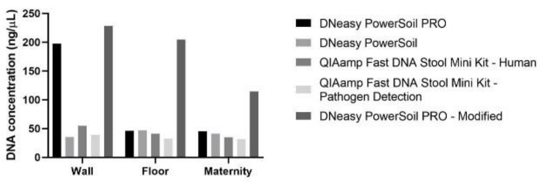

Figure 2 DNA concentration (ng/µL)

FC revealed the complexity of bat guano by identifying both microbial cells and non-microbial particles. Particle abundance was lower at the Ceramics Gallery - Maternity Colony sampling point compared to the Hibernation Colony, where it was higher on the floor and wall.

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4. Discussion

Our study provides a comprehensive characterization of the bat guano microbiome using both culture-dependent and culture-independent methods. The bacterial load in insectivorous bat species was similar to that reported by Gerbáčová *et al.* (2020), being significantly lower compared to frugivorous bat species. The higher bacterial load obtained on TSA from the Ceramics Gallery - Hibernation Colony (Floor) sampling point may also be due to invertebrates on the floor of Isverna Cave, which can influence its microbiome through their tegument microbiota. Conversely, the higher bacterial load on Blood Agar from The Ceramics Gallery - Hibernation Colony (Wall) sampling point might be linked to the cave's shape and has correlated with an increased DNA concentration. This sampling location is situated at the base of a sloped surface and acts as a collection point for materials transported by gravity, such as infiltrating water or debris, each affecting the microbiome of this site. Additionally, *Yersiniaceae* family that requires specific nutrients, like iron from hemoglobin in the culture media, to grow effectively, was dominant here (Weakland *et al.*, 2020).

The cultivable microbiome of bat guano from Isverna Cave is relatively stable, consistent with previously reported data (Manolachi *et al.*, 2023). The presence of Firmicutes, Pseudomonadota, and Actinomycetota at the phylum level reflects their origin from the normal digestive tract microbiota of bats (Hatta *et al.*, 2016; Sun *et al.*, 2019; Imnadze *et al.*, 2020). The dominance of Firmicutes suggests a period of intense feeding, corresponding to the active phase of bats (Mekuchi *et al.*, 2018), indicating that the bat guano microbiome is closely linked to the host's nutritional status and activity. Moreover, members of the *Enterobacteriaceae* and *Staphylococcaceae* families are known to produce chitinase (Park *et al.*, 1997; Konagaya *et al.*, 2006), and their presence in bat guano also suggests they may originate from the bats' intestinal microbiota (Newman *et al.*, 2018). The identified species such as *H. alvei*, *S. fonticola*, *C. maltaromaticum*, *E. faecium*, *E. faecalis*, *L. lactis*, *S. quinivorans*, *P. anaericanus*, *B. mycoides*, *E. coli* (Borda *et al.*, 2014; Newman *et al.*, 2018; Dimkić *et al.*, 2020; Gerbáčová *et al.*, 2020), have been also reported in other studies, suggesting an ecological and microbiological stability of guano deposits and indicating that these bacterial species may be residents of bats' intestinal microbiota. The exclusive presence of *L. lactis* on the floor of The Ceramics Gallery - Hibernation Colony may be linked to the tegument microbiota of guanophilic invertebrates (Newman *et al.*, 2018).

However, the cultivable portion of the fecal microbiome represents only a small subset, as one of the main challenges is the inability to replicate the nutritional and ecological conditions of the natural environment in the lab. For example, the diversity in one gram of fecal matter ranges from 10^7 to 10^{12} species (Bilen *et al.*, 2018). The bacterial load measured after cultivation was consistently lower than that determined by FC, confirming that culture-based methods are inadequate for accurately reflecting the true microbial population (El Mujtar *et al.*, 2022). FC also showed that bat guano from The Hibernation Colony contains more particles and has greater complexity than that from The Maternity Colony.

Based on these considerations, culture-independent approaches can complement the microbiological profile obtained through cultivation followed by MALDI-TOF identification. However, the main challenge remains to obtain DNA of adequate quality and quantity for downstream molecular applications. The failure of PCR amplification in undiluted samples may be attributed to either an excessive amount of DNA or the presence of PCR inhibitors, such as humic acids, which can inhibit the enzymatic activity of DNA polymerase (Lear *et al.*, 2017).

5. Conclusion

This study employed a multi-methodological approach to elucidate the complexity of bat guano, highlighting the importance of both culture-dependent and culture-independent approaches in comprehending microbial community dynamics and ecological interactions.

Our future research will consist of an integrated metagenomics and metabolomics analysis of bat guano in order to characterize its microbial communities and their functional outputs.

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