Exploring the Microbial Composition of Moonmilk in Wind Cave National Park

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EXPLORING THE MICROBIAL COMPOSITION OF MOONMILK IN
WIND CAVE NATIONAL PARK

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Abstract

Moonmilk is a white substance mainly composed of calcium carbonate (CaCO3) that is often found in limestone caves. It is one of many mineral composites, or speleothems, in Wind Cave National Park South Dakota and is thought to be formed by microbial activity. This cave system is a complex, isolated environment that serves as an analog for similar domains beyond the Earth. Each site has varying degrees of human exposure, from public tour routes to caverns only occasionally visited by park rangers, making it an ideal place of study for pristine samples in extreme conditions. Wind Cave also varies in moisture as well as abundance of moonmilk deposits. By sampling these locations and extracting microbial DNA, we will then be able to measure microbial diversity. The resulting dataset will allow us in the future to estimate how much of the human microbiome transfers to moonmilk, determine whether there is a correlation between microbial diversity and hydration, and offer insights into the microbial composition of moonmilk and their role in its formation. DNA will be extracted and a clone library will be constructed to identify specific microbes in moonmilk. The results of this study will not only add to the larger genetic map of Wind Cave being developed by the other students on my research team, but also contribute to a larger understanding of life in extreme environments.
Introduction

Microbial carbonate precipitation is thought to be carried out by specialized bacteria as a byproduct of metabolic function. A number of bacteria are capable of producing calcium carbonate, such as the gram positive *Sporosarcina pasteurii* (Mitchell and Ferris, 2007). In 2017, animal fat and moonmilk was found in a 2,700 year old jar in China, believed to be an ancient cosmetic face cream (Marshall, 2019). Moonmilk was also used in cave art and as a medicine for acidosis, its basic properties increasing pH. Carbonate precipitation has many modern practical uses as well, including monument repair and concrete improvement, as well as wastewater treatment and soil enhancement (Okwadhi and Li, 2010). Its application in bioremediation stems from the ability of some facultative bacteria, including *Sporosarcina pasteurii*, to precipitate calcium via urea hydrolysis. These bacteria are called ureolytic bacteria, and they are well-studied partly due to their production of a urease enzyme that hydrolyses urea and increases the concentration of carbonate, eventually leading to the production of calcium carbonate (Muynck, et. al, 2008).

Ureolysis can be utilized by organisms in oligotrophic, or nutritionally low, environments. Some of these bacteria have been found in association with calcium deposits in cave systems. Although it is still debated, there is evidence that the metabolic activity of calcium-producing bacteria plays a role in the formation of speleothems. *Macromonas bipunctata*, a bacterium first discovered in caves over a hundred years ago, was found in close association with moonmilk. More recently, a *Streptomyces* strain was isolated from moonmilk in an ancient tomb and formed calcium carbonate onto plates (Cirigliano,
et. al, 2018). The role of bacteria in cave systems could reveal the specific requirements for the growth of moonmilk and produce an overall larger genetic map of Wind Cave.

Wind Cave National Park South Dakota is home to a complex cave system which allows for the study of karst speleothems such as moonmilk. The extremeness and isolation serves as an analog for similar environments in the solar system. There is also evidence that dry and wet moonmilk harbor different bacterial communities (park, et al. 2020). Wind Cave in particular varies in human exposure as well as chamber moisture. By extracting environmental DNA from these samples with our own modified protocol, we will then be able to measure microbial diversity, and compare and contrast these findings based on the differences we know of each sample.

**Research Questions**

Is our modified DNA extraction protocol effective in extracting microbial DNA from moonmilk in Wind Cave? If so, what are the specific microorganisms that are associated with Wind Cave moonmilk?

**Methodology**

**Sample Collection and Description**

Samples were collected into sterile containers and transported back to the lab at room temperature. Each moonmilk sample was sampled from a different location in Wind Cave. Snowdrift Alley and Frostline appeared to be the most pure moonmilk samples, and Frostline in particular had high moisture levels. The other three samples – Boxwork
Chimney, Popcorn Room, and Pyramid Room – were more rocky or crystalline in nature, and therefore appeared less pure. Pyramid Room in particular was along a public tour route, increasing the chances of human contamination. Samples were then distributed into smaller working stocks to mitigate exposure to contamination, and each sample was stored at 4°C. Working stocks were used directly for DNA extractions.

Figure 1. Moonmilk Samples
Each sample is named from the chamber in Wind Cave that it was sampled from. Moonmilk appearance varies depending on its location.

DNA Extraction and Amplification
DNA was extracted directly from 0.5 g moonmilk subsamples using our own modified DNA extraction protocol to recover the highest amount of DNA from Wind Cave samples. Many kit-based DNA extraction protocols are not designed for environmental cave samples with extremely low cell counts, and so may not be the best extraction method to isolate the highest possible amount of DNA. Subsamples were suspended in TEN lysis buffer and bead beaten for 30 seconds. 10% SDS was added after bead
beating and before incubation in order to avoid clumping of DNA. After centrifugation and removal of cellular debris. DNA was precipitated twice as it yielded the best results, first with 95% EtOH and second with salty PEG. After the second rinse with 75% EtOH, DNA was suspended in 15 μL 10 mM Tris pH 8.

DNA extraction was followed by 16S rDNA Endpoint PCR amplification using GoTaq Green MasterMix (Promega, M7122) in 25 μL reactions to determine if the DNA was purified and plentiful enough for further molecular analysis. Each PCR reaction was composed of 12.5 μL GoTaq Green MasterMix, 9.25 μL nuclease free water, 1.25 μL 8mg/mL BSA, 1 μL 10 mM forward primer, 1 μL 10 mM reverse primer, and 1μL of the previously extracted DNA template. The PCR touch-down cycling protocol for the primer pairs 515F-806R was as follows: 95°C for 2 minutes followed by 20 cycles of 95°C for 30 seconds, 62°C (decreasing 0.5°C per cycle) for 30 seconds and 72°C for 90 seconds followed by 25 cycles of 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 90 seconds. A final elongation step of 10 minutes at 72°C was then followed by a 12°C hold. A negative and positive control were also processed through the DNA extraction protocol. The negative controls ensure that any positive results are not caused from contamination during the extraction, and the positive control represents exact methods that have been used previously which yielded positive results. Once PCR products were generated, they were visualized on agarose gel using electrophoresis to confirm microbial DNA was present in the sample, and at the expected size. Most of the environmental DNA samples produced dark bands visible on an agarose gel stained with ethidium bromide (Figure 2). All environmental DNA samples produced
bands that were distinct from the negative control which did not produce a visible band. Some samples showed extra bands that do not match the expected size. We have observed this type of pattern in preliminary tests with these primers when there is a low concentration of target template, i.e. primer dimers.

![Figure 2. Amplification of Moonmilk after Endpoint PCR](image)

*Bands represent amplified DNA, with the brightest bands having higher yields.*

**PCR Cloning and Sequencing**

PCR products were purified via a modified protocol using 95% EtOH, LPA, and 5 M ammonium acetate, which is the best salt to remove small DNA fragments (<100 bp), dNTPs, primers, and dimers. Cloning of the PCR product was performed using the pGEM-T easy vector system (Promega, A1360) following the manufacturer's protocol. In preparation for colony PCR, sterile pipette tips were used to transfer a small amount of each colony into 25 µL of DNA grade water followed by incubation at 95°C for 10 minutes. 1 µL of the incubated colony mixture was used as template for PCR using the previously described protocol with an annealing temperature of 48°C for primer pair
m13F-SP6. A subset of chosen colony PCR products were then purified again via the same modified protocol using salty EtOH in preparation for Sanger sequencing. Sequencing was performed by the Iowa State University DNA facility using their stock universal 21M13 Primer (“U”) on an Applied Biosystems 3730xl DNA Analyzer. Sequences were aligned using BLASTn analysis (https://blast.ncbi.nlm.nih.gov/), which identifies the nucleotide sequence of target DNA. The resulting sequences were then aligned using BLASTn analysis - a nucleotide database that can find regions of similarity between sequences in our samples and those within its own database - which can help us to determine what bacteria is associated with moonmilk in Wind Cave. This process was repeated once more with a 96-well plate, using thirteen colonies from each cloned moonmilk plate. The resulting dataset allowed us to estimate how much of the human microbiome transfers to moonmilk and whether there is a correlation between microbial diversity and the level of hydration in Wind Cave moonmilk.

Results

DNA was able to be extracted from each of the five samples of Moonmilk. Each sample listed below was analyzed using BLASTn analysis, helping to identify the bacterium isolated or its close relatives. No significant differences between moisture levels were found, and some sequences had no significant similarity in the database.

Table 1. BLASTn Analysis Results

<table>
<thead>
<tr>
<th>Database match: Blas, Experimental databases, Prokaryota (bacteria and</th>
<th></th>
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<td>Sequence date</td>
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<td>---------------</td>
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**Discussion**

Caves are extreme and specialized habitats that can serve as analogs for life on other areas outside of Earth. Wind Cave specifically is an isolated subterranean environment, rich in minerals. Due to the findings in other cave systems worldwide, prior research of microbial life in Wind Cave, and the results of this study, it does appear that microbial life exists in moonmilk. Likewise, DNA from Wind Cave can be isolated using these methods. The findings from this study have revealed more of the genetic makeup of Wind Cave, and in the future, the relationship between metabolic activity of bacteria and the formation of moonmilk may tell us more about how life could exist in other regions of the solar system.
Literature Cited


Marshall, Michael (2019-02-26). "2700-year-old face cream was made from animal fat and cave 'milk'". New Scientist. Retrieved 2021-02-08.


