2023

Exploring the Microbial Composition of Moonmilk in Wind Cave National Park

Emma Pellegrino

EXPLORING THE MICROBIAL COMPOSITION OF MOONMILK IN WIND CAVE NATIONAL PARK

A Thesis Submitted

In Partial Fulfillment

of the Requirements for the Designation

University Honors with Distinction

Emma Pellegrino

University of Northern Iowa

May 2023

This Study by: Emma Pellegrino

Entitled: Exploring the Microbial Composition of Moonmilk in Wind Cave National Park

has been approved as meeting the thesis or project requirements for the Designation

University Honors with Distinction

Approved by:

Dr. Marek Sliwinski, Honors Thesis Advisor, Department of Biology

Dr. Jessica Moon Asa, Director, University Honors Program

Abstract

Moonmilk is a white substance mainly composed of calcium carbonate ($CaCO_3$) 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℃. Working stocks were used directly for DNA extractions.

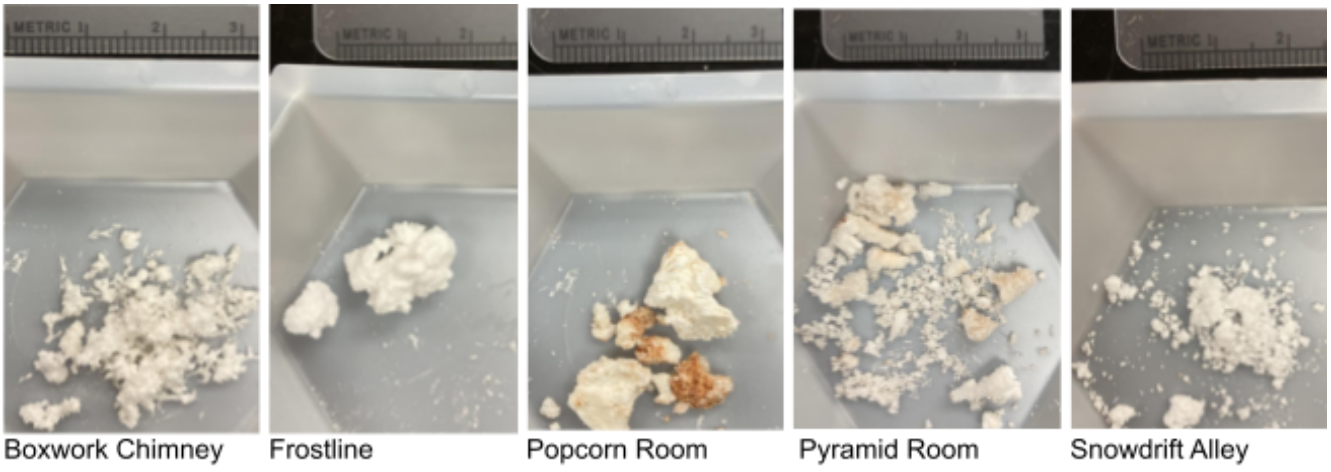

Figure 1. Moonmilk Samples

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 beated 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℃ for 2 minutes followed by 20 cycles of 95℃ for 30 seconds, 62℃ (decreasing 0.5℃ per cycle) for 30 seconds and 72℃ for 90 seconds followed by 25 cycles of 95℃ for 30 seconds, 52℃ for 30 seconds and 72℃ for 90 seconds. A final elongation step of 10 minutes at 72℃ was then followed by a 12℃ 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.

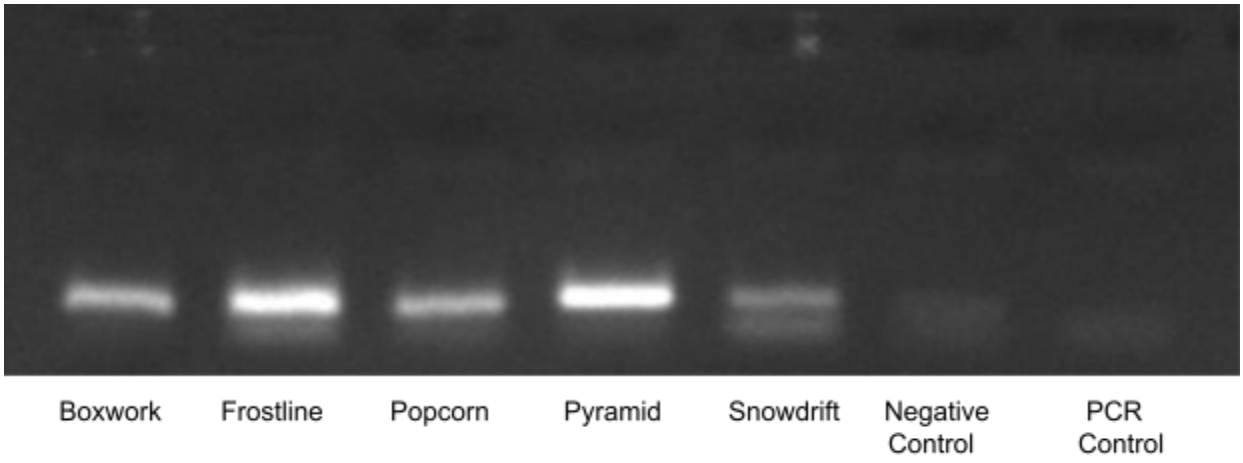

Figure 2. Amplification of Moonmilk after Endpoint PCR
Bands represent amplified DNA, with the brightest bands having higher yields.

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

			Database match: Blastn, Experimental databases, Prokaryota (bacteria and			

			archaea) nt (nt_prok), Highly similar sequences (megablast)			
moonmilk sample name	sequence date	sequence file name	Sequence ID	percent identity	Description	Isolation source
frostline	041923	H01_1_U_1238340.seq	FJ448804.1	91	Uncultured bacterium clone D5_KR_250507A_F06_46_12 16S ribosomal RNA gene, partial sequence	soil
popcorn	041923	A02_2_U_1238341.seq	MN157257.1	83	Uncultured bacterium clone A702 16S ribosomal RNA gene, partial sequence	anaerobic digester
pyramid	041923	D01_3_U_1238342.seq	KY943180.1	99	Uncultured bacterium clone Bact_IS_OTU985 16S ribosomal RNA gene, partial sequence	acid mine drainage contaminated sediments
snowdrift	041923	C02_4_U_1238343.seq	JF147750.1	94	Uncultured bacterium clone ncd1676b12c1 16S ribosomal RNA gene, partial sequence	skin, antecubital fossa
boxwork	041923	F01_5_U_1238344.seq	n/a	n/a	No significant similarity found	n/a
Boxwork	040523	H01_8_1721759.seq	JF153564.1	96	Uncultured bacterium clone ncd1766e09c1 16S ribosomal RNA gene, partial sequence	skin, popliteal fossa
Boxwork	040523	D02_12_1721763.seq	KF263979.1	84	Uncultured Firmicutes bacterium isolate DGGE gel band J14-12 16S ribosomal RNA gene, partial sequence	textile wastewater
Frostline	040523	C04_27_1721778.seq	KT308472.1	96	Uncultured Actinomycetes bacterium clone K2DN249 16S ribosomal RNA gene, partial sequence	sludge
Frostline	040523	D04_28_1721779.seq	n/a	n/a	No significant similarity found	n/a
Frostline	040523	E05_37_1721788.seq	n/a	n/a	No significant similarity found	n/a
Popcorn	040523	A09_65_1721816.seq	n/a	n/a	No significant similarity found	n/a
Popcorn	040523	E09_69_1721820.seq	OW751130.1	93	Patescibacteria group bacterium DNA containing 16S-23S	desert soil

					intergenic spacer region, clone 43	
Pyramid	040523	C11_83_1721834.seq	MH313040.1	97	Uncultured bacterium clone OTU_1034 16S ribosomal RNA gene, partial sequence	river sediment
Snowdrift	040523	F12_94_1721845.seq	MF453700.1	95	Uncultured bacterium clone OTU3907 16S ribosomal RNA gene, partial sequence	sediments
Snowdrift	040523	G12_95_1721846.seq	n/a	n/a	No significant similarity found	n/a

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

16S Illumina Amplicon Protocol: Earth Microbiome Project. (n.d.). Retrieved March 25, 2021, from https://earthmicrobiome.org/protocols-and-standards/16s/

Chelius, M., Beresford, G., Horton, H., Quirk, M., Selby, G., Simpson, R., Horrocks, R., & Moore, J. (2009). Impacts of Alterations of Organic Inputs on the Bacterial Community within the sediments of Wind Cave, South Dakota, USA. International Journal of Speleology, 38(1), 1-10. http://dx.doi.org/10.5038/1827-806X.38.1.1

Cirigliano, Angela, et al. “Calcite Moonmilk of Microbial Origin in the Etruscan Tomba Degli Scudi in Tarquinia, Italy.” *Scientific Reports*, U.S. National Library of Medicine, 26 Oct. 2018, https://pubmed.ncbi.nlm.nih.gov/30367083/.

Geerdes, Nicole. “The Search for Life in Extreme Environments: Measuring Diversity and Quantity of Archaea in Wind Cave National Park.” *University of Northern Iowa*, Scholarworks, 2021, pp. 1–24.

Hershey, O. S., Kallmeyer, J., Wallace, A., Barton, M. D., & Barton, H. A. (2018). High Microbial Diversity Despite Extremely Low Biomass in a Deep Karst Aquifer. Frontiers in Microbiology, 9. https://doi.org/10.3389/fmicb.2018.02823

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

Martens-Habbena, W., Berube, P. M., Urakawa, H., de la Torre, J. R., & Stahl, D. A. (2009). Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature, 461(7266), 976–979. https://doi.org/10.1038/nature08465

Muynck, Willem De, et al. “Bacterial Carbonate Precipitation as an Alternative Surface Treatment for Concrete.” *Construction and Building Materials*, Elsevier, 20 Feb. 2007, https://www.sciencedirect.com/science/article/pii/S0950061806003667.

Okwadha, George D.O., and Jin Li. “Optimum Conditions for Microbial Carbonate Precipitation.” *Chemosphere*, Pergamon, 13 Oct. 2010, https://www.sciencedirect.com/science/article/pii/S0045653510010933.

Park, Shinae, et al. “Microbial Diversity in Moonmilk of Baeg-Nyong Cave, Korean Czo.” *Frontiers in Microbiology*, U.S. National Library of Medicine, 23 Apr. 2020, https://pubmed.ncbi.nlm.nih.gov/32390967/.

Sanchez-Moral, S., et al. “The Role of Microorganisms in the Formation of Calcitic Moonmilk Deposits and Speleothems in Altamira Cave.” *Geomorphology*, Elsevier, 4 Nov. 2011, https://www.sciencedirect.com/science/article/pii/S0169555X11005563.