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A COMPARISON OF CAVE MICROBIAL COMMUNITIES TO CAVE ROOSTING BAT MICROBIOTA IN EL MALPAIS NATIONAL MONUMENT, USA

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by

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THESIS

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ABSTRACT

The arrival of white-nose syndrome (WNS), a devastating fungal disease, has highlighted the need to better understand bat microbiota and how bats acquire their microbiota. To address this need, we investigated how bat microbiota compare to microbiota from the cave walls in two El Malpais National Monument caves. The external surfaces of six roosting bats from each cave, representing four different bat species, and their associated microbial mats were sampled. One to three air samples were taken in each cave. Samples were sequenced using Illumina MiSeq sequencing of the 16S rRNA gene for bacterial diversity and the ITS region for fungal diversity. Many bacterial and fungal operational taxonomic units (OTUs) were shared among the sample types. Within the bacterial OTUs, Actinobactria were highest overall in all samples, but were higher in bats than mats. The most prevalent actinobacterial genera recovered were *Rhodococcus*, *Streptomyces*, *Arthrobacter*, and *Rubrobacter*. SourceTracker suggested bat bacterial communities may

originate from mat and air samples. Within the fungal OTUs, Ascomycota were highest overall in all samples, but higher in bats than mats. Prevalent fungal families included Cladosporiaceae, Pleosporaceae, Pseudeurotiaceae, Microascaceae, Leucosporidiaceae, and Mortierellaceae. A top fungal OTU recovered was a close relative of *Pseudogymnoascus destructans*, the cause of WNS. Our results shed light on a relatively understudied area that could have implications for understanding the source of potential natural defenses of bats, which could be important in predicting which western bats species are most vulnerable to WNS.

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Introduction

One of the greatest threats to North American bats today is the lethal disease known as white-nose syndrome (WNS), caused by the fungus *Pseudogymnoascus destructans* (*P. destructans*) (Blehert et al., 2009). *P. destructans* invades the exposed skin of the bats, causing erosions and ulcerations on the wings, uropatagium, ears, and muzzle (Meteyer et al., 2009). Since its discovery in New York in the winter of 2006-2007, this fungal disease has killed over seven million bats and has spread to 33 states and seven Canadian provinces (https://www.whitenosesyndrome.org/, 2019). In the past two years, the fungus or the disease itself has been continuing on its path westward and has been discovered recently in California, Mississippi, North Dakota, Texas, and Wyoming (https://www.whitenosesyndrome.org/, 2019).

In the aftermath of the discovery of WNS, there have been several studies that have focused on many aspects of this disease, including bats themselves and possible reasons for varying susceptibility (Wilder et al., 2011; Davy et al., 2017; Moore et al., 2018). Documented patterns of infection and mortality in bats with WNS show that some bat species may be less vulnerable than others (Turner et al., 2011; Langwig et al., 2012). A possible reason for these differences in vulnerability may be a bat's natural defenses against incoming pathogens, such as a bat's microbiome whose members could inhibit *P. destructans* (Winter et al., 2017). Research into which natural defenses bats may have and where they are acquiring these defenses is important in predicting which western bat species may be most vulnerable to WNS.

In humans, the skin microbiome consists of bacteria, fungi, viruses, and mites (Marples, 1965; Grice and Segre, 2011). This external microbiome can provide a first-line defense and plays an important, yet poorly understood, role in the overall health of humans (Grice and Segre, 2011; Mathieu et al., 2013). It has been shown that these external microbes are a source of antibiotic and antifungal compounds that can help maintain a healthy microbiome (Donia et al., 2014). There has been research done on wild animals, such as amphibians (Fitzpatrick and Allison, 2014; Kueneman et al., 2014) and whales (Apprill et al., 2011, 2014), and their external microbiomes, but very little is known about the external microbiome of bats and the natural defenses they may have (Avena et al., 2016; Lemieux-Labonté et al., 2017; Winter el al., 2017).

Previous studies have shown that bacteria isolated from bats can inhibit the growth of *P. destructans* (Hoyt et al., 2015; Hamn et al., 2017). The bacteria that inhibited *P. destructans* in Hamm et al. (2017) were all from the phylum Actinobacteria. Actinobacteria are one of the highest producers of secondary metabolites, such as antibiotics and antifungals (Bérdy, 2012; de Lima Procópio et al., 2012). In addition, many of these bacteria were isolated from bats caught in caves. Actinobacteria have been found in high abundance and with great diversity in caves (Northup et al., 2011; Riquelme et al., 2015; Rangseekaew and Pathom-aree, 2019); thus, cave microbial mats may be a large source of beneficial Actinobacteria.

Looking at the fungal aspect of the external microbiome, little is known about fungi on bats and what roles they play in natural defenses. Fungi are known to produce secondary metabolites in great numbers (Throckmorton et al., 2015); thus, there is potential for fungi to provide a line of defense against incoming pathogens. In addition, Singh et al. (2018) found

that a fungus, *Trichoderma polysporum*, has the ability to inhibit the growth of *P. destructans* with minimal effect on native soil microbes. Fungi have also been found to be abundant and diverse in caves where bats roost or hibernate (Vanderwolf et al., 2013a), making them an important aspect to consider in the bat microbiota and cave ecosystem.

From the literature cited above, it is evident that bacteria and fungi have the potential to play an important role in bat health and defenses against incoming pathogens. It is particularly important to look at these natural defenses in caves since WNS mortality has only been documented to occur when bats are hibernating in caves or mines (Frick et al., 2010). Winter et al. (2017) and previous unpublished research by the Northup lab group have shown differences in the external bacterial (16S rDNA) and fungal (ITS) communities on cave-caught bats versus surface-caught bats (bats caught outside of caves). Although these differences are not completely understood, we hypothesized that some of the bacteria and fungi found exclusively on cave-caught bats come from bacteria and fungi previously reported from caves.

In this study, we compared the diversity of bacterial and fungal communities on bats versus the cave walls in El Malpais National Monument (ELMA). There have been a few studies that have compared the microbial diversity of bats to their environment (Avena et al., 2016; Lemieux-Labonté et al., 2017), but these studies have only looked at the bacterial diversity and not the fungal diversity. Understanding the fungal diversity provides a more comprehensive view of how a roosting site may impact a bat's microbiome. In addition, no studies thus far have done this type of comparison in lava caves such as the ones in ELMA. The caves at El Malpais are known to host hibernating bats, many of the caves have ideal temperatures and humidity for the growth of *P. destructans* (Torres-Cruz et al., 2019), and it

is currently a WNS-free area, making this an ideal location for this study. With WNS-free areas disappearing rapidly, studying WNS-free areas like ELMA in New Mexico is tremendously important because it establishes a baseline of interactions prior to the introduction of WNS.

Methods

Sample Collection

Samples were collected on March 16-17, 2017 from two ELMA lava caves (Classic Cave and West Cave) located in Cibola County, NM, United States. The two lava caves were chosen based on past surveys showing roosting bats and appropriate temperature and relative humidity for growth of *P. destructans*. In addition, these caves are not open to the public, thus there is very little human activity and contamination.

In each cave, six roosting bats and their associated microbial mats were sampled (n=23). Samples of the air were taken in each of the caves by wetting sterile swabs with sterilized Ringer's solution and leaving them exposed to the air of the room away from human activity for fifteen minutes. One air sample (n=1) was taken in West Cave, as all the bats were roosting in the same room. Three air samples (n=3) were taken in Classic Cave since that cave had multiple rooms where the bat samples were collected. Four species of bats are represented in this data set: *Corynorhinus townsendii* (n=5), *Myotis volans* (n=4), *Myotis evotis* (n=1) and *Myotis thysanodes* (n=2). Multiple dry-bulb temperatures (Digital Thermometer Model 2400 IMC Instruments Inc.) were taken in each cave and averaged. The collecting permit was issued by the National Park Service (#ELMA-2017-SCI-0002) to Dr.

Diana Northup, and work was conducted with an approved IACUC protocol (#16-200508-MC). **Table 1** summarizes all the sample and temperature information.

Table 1. Summary of samples taken in each cave.

	Classic Cave (CLC) (Mean dry-bulb temp. 1.18℃)	West Cave (EST) (Mean dry-bulb temp. 3.22℃)	Total
Corynorhinus townsendii (COTO)	5	0	5
Myotis evotis (MYEV)	0	1	1
Myotis thysanodes (MYTH)	0	2	2
Myotis volans (MYVO)	1	3	4
Microbial Mat	5*	6	11
Air	3	1	4
Total	**14**	**13**	**27**

*One microbial mat sample was paired with two bat samples since the bats were roosting very close together.

Bat samples were collected aseptically following established protocols (Winter et. al., 2017). The body fur, wings, and uropatagium of the bats were swabbed with sterile nylon tipped swabs, which were wetted with sterilized Ringer's solution. The swabs were stored in sucrose lysis buffer (Giovannoni et al., 1990) to preserve the DNA and then put on dry ice until they could be stored at -80℃ to await shipping for sequencing. All bats were caught and handled by an experienced bat biologist, under her New Mexico Game and Fish permit, which ensured the safety of the bats and humans involved.

Microbial mat samples were collected aseptically using a flame sterilized cold chisel to take rock chips from the wall or ceiling near or under the roosting bats. Where mats were not adjacent to sampled bats, microbial mat samples were collected as close as possible to the

roosting bat. These bat and microbial mat samples were separated into pairs and assigned a letter for their pair (**Table 2**). The microbial mat samples collected were also placed in sucrose lysis buffer and kept at 4°C prior to transfer to the laboratory where they were stored at -80°C until they were sent to a facility for DNA extraction and sequencing.

Table 2. Sample pairs of bat and microbial mat samples.

Pair	Bat Species (abbreviation)	Cave (abbreviation)/Microbial mat
Pair_A	COTO	CLC mat
Pair_B	COTO	CLC mat
Pair_C	MYVO	CLC mat
Pair_D1	COTO	CLC mat*
Pair_D2	COTO	CLC mat*
Pair_E	COTO	CLC mat
Pair_Q	MYVO	EST mat
Pair_V	MYTH	EST mat
Pair_W	MYVO	EST mat
Pair_X	MYEV	EST mat
Pair_Y	MYTH	EST mat
Pair_Z	MYVO	EST mat

*Only one microbial mat sample was taken for Pair_D and paired with bat pairs D1 and D2.

16S rRNA Gene Amplification and Sequencing

Bat swabs and microbial mat samples were sent to MR DNA in Shallowater TX (http://www.mrdnalab.com/) for genomic DNA extraction and sequencing. A portion of the prokaryotic 16S ribosomal RNA (rRNA) gene region was amplified using the primer set 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT), with a barcode on the forward primer for a 30 cycle Polymerase Chain Reaction (PCR). The

PCR was done using a HotStarTaq Plus Master Mix (Qiagen, USA) with the following thermocycler conditions: 94℃ for 3 minutes, followed by 28 cycles of 94℃ for 30 seconds, 53℃ for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes. After amplification, PCR products were checked in 2% agarose gel. Samples were pooled together based on their molecular weight and DNA concentrations and then purified using calibrated Ampure XP beads. The pooled and purified PCR product was used to prepare the DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed using the 515F primer at MR DNA on a MiSeq following the manufacturer's guidelines.

ITS Gene Region Amplification and Sequencing

Bat swabs and microbial mat samples were sent to MR DNA in Shallowater TX (http://www.mrdnalab.com/) for genomic DNA extraction and sequencing. After initial extraction at MR DNA, efforts to amplify the fungal gene region for the microbial mats failed. Mat samples were then extracted at the University of New Mexico using the Qiagen DNeasy PowerSoil Kit (Qiagen, USA). After troubleshooting efforts, a method of nested PCR using first ITS 1F-4 primers, then nested PCR with ITS1-4 primers was used. Extracted microbial mat DNA was sent to MR DNA, where nested PCR was performed on both bat and microbial mat samples. The internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) operon was amplified using the primer set ITS 1F (CTTGGTCATTTAGAGGAAGTAA) and ITS 4 (TCCTCCGCTTATTGATATGC) with a barcode on the forward primer. A 30 cycle Polymerase Chain Reaction (PCR) was performed using a HotStarTaq Plus Master Mix (Qiagen, USA) with the following thermocycler

conditions: : 94℃ for 3 minutes, followed by 28 cycles of 94℃ for 30 seconds, 53℃ for 40 seconds and 72℃ for 1 minute, after which a final elongation step at 72℃ for 5 minutes. After amplification, PCR products were checked in 2% agarose gel. Samples were pooled based on their molecular weight and DNA concentrations and then purified using calibrated Ampure XP beads. The pooled and purified PCR product was used to prepare the DNA library following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed using the ITS 1F primer at MR DNA on a MiSeq following the manufacturer's guidelines.

16S rRNA Gene Read Processing and Data Analyses

Reads produced from sequencing were preprocessed in Qiime 1.9.1(Caporaso et al., 2010). A fastq file was created using convert_fastaqual_fastq.py, and index files were created using extract_barcodes.py. The files generated were then used in USEARCH v11.0.667_i86osx32 (Edgar, 2010, 2013) for quality filtering, global trimming, and operational taxonomic unit (OTU) assembly. Samples were de-multiplexed, and barcodes and primers were removed. Raw reads were filtered, sequences that had a greater expected error than 1.0 were removed, and sequences were then trimmed to 270 nucleotides. Sequences were then de-replicated using fastx_uniques to give an abundance basis for clustering OTUs. OTUs were clustered at a 97% identity threshold, singletons were removed to filter out any low-quality sequences, and chimeras were filtered denovo. Using this output data and the reads per sample, an OTU table was created with usearch_global. Taxonomy was assigned using the RDP classifier Version 2.11 (Wang et al., 2007) trained on the RDP 16S training set v16 (access date 4-4-2019; RDP Release 11, Update 5 :: September 30,

2016). OTUs classified as Archaea or chloroplasts were removed from the OTU table. The final OTU table contained 3804 OTUs and 27 samples.

For a majority of the bacterial sequence data analyses, R (Version 3.5.2, R Development Core Team, 2012) was used, and various R packages were implemented on the data. The packages primarily used were the phyloseq package (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2016), and ggpubr (Kassambara, 2018). To assess the species richness of our sampling, a rarefaction curve plot was generated using the ranacapa package (Kandlikar et al., 2018). To observe alpha diversity, the samples were first rarefied without replacement to 13,467 sequences per sample using 'rarefy_even_depth' (set.seed 790) function in phyloseq (v1.26.1). Two Hundred and fifty-one OTUs were removed from the OTU table after rarefaction. Four alpha diversity measures, including the number of observed OTUs, Chao1 richness estimate (Chao, 1984), and Shannon Diversity index (Haegeman et al., 2013) were calculated on both the raw and rarefied data using the 'estimate_richness' function in phyloseq. Normality of both the raw and rarefied alpha diversity measures were visualized with histograms and statistically tested with the Shapiro-Wilks test. An ANOVA or a Kruskal-Wallis test was used to test differences in alpha diversity indices between categorical groups of sample type, caves, and species of bats. If the ANOVA or Kruskal-Wallis had significant results, a Tukey or a Pairwise Wilcoxon test was used to assess which groups were significantly different.

To observe beta diversity, a non-metric multidimensional scaling (NMDS) ordination with a Bray-Curtis dissimilarity matrix was applied to the rarefied data using the 'ordinate' function in phyloseq (v1.26.1). Stress plots were created using the vegan package (Oksanen et al., 2016) to ensure that the stress was not too high (< 0.3) with the chosen number of

dimensions (k=2). To apply 95% confidence ellipses to the NMDS ordination, the 'stat_elipse' function was used in which the calculations were modified from car::ellipse (Fox and Weisberg, 2011). A permutational multivariate analysis of variance (PERMANOVA) through the 'adonis' function in the vegan package was used to examine differences in beta diversity using sample type, caves, and species of bats as explanatory factors. Pairwise PERMANOVAs, using the 'pairwise_adonis' function in the ranacapa package (p_adjust_m= 'fdr'), were used to examine which sample type, caves, and species of bats were driving significant differences. The 'betadispr' function in vegan was used to evaluate differences in dispersion among the groups. In cases where beta dispersion was not equal, an analysis of similarities (ANOSIM) test was used instead of a PERMANOVA.

Community composition was analyzed using a relativised OTU table produced by using the 'transform_sample_counts' command in phyloseq, which divided the read numbers for each OTU in a sample by the total number of reads in that sample giving a relative abundance. The most abundant taxonomic groups were compared using an ANOVA or a Kruskal-Wallis test to determine whether significant differences in relative abundances existed among sample types, caves, and species of bats. Group significance analyses were performed on the top abundant OTUs using the group_signficance.py script in Qiime (http://qiime.org/scripts/group_significance.html) with default parameters. The test was used to compare OTU frequencies of the top OTUs between just the bat and microbial mat sample group types. The OTU table was rarefied before group significance was done per recommendation of the script developers. Air samples were excluded due to their overall low species richness and sequencing depth. Using the numbers generated from the 'venn'

function in gplots (Warnes et al., 2009), a Venn diagram was created using the R package eulerr (Larsson, 2018) to look at shared OTUs between sample types (bat, mat, and air).

The SourceTracker software package (Knights et al., 2011) was used to determine the potential sources of microbial communities on the surfaces of bats with the potential sources being the microbial mat and air samples. The assumptions of the SourceTracker model are that each surface community is a mixture of communities deposited from known and unknown sources. The model uses a Bayesian approach to estimate the proportion of the surface community originating from the different sources. SourceTracker was applied to the rarefied data, treating the microbial mat and air samples as sources and the bat samples as sinks.

ITS Gene Region Read Processing and Data Analyses

Reads produced from sequencing were preprocessed in Qiime 1.9.1(Caporaso et al., 2010). A fastq file was created using convert_fastaqual_fastq.py and index files were created using extract_barcodes.py. The files generated were then used in USEARCH v11.0.667_i86osx32 (Edgar, 2010, 2013) for quality filtering, global trimming, and operational taxonomic unit (OTU) assembly. Samples were de-multiplexed, and barcodes and primers were removed. Raw reads were filtered, sequences that had a greater expected error than 1.0 were removed, and sequences were then trimmed to 245 nucleotides. Sequences were then de-replicated using fastx_uniques to give an abundance basis for clustering OTUs. OTUs were clustered at a 97% identity threshold, singletons were removed to filter out any low-quality sequences, and chimeras were filtered denovo. Using these output data and the reads per sample, an OTU table was created with usearch_global.

Taxonomy was assigned using CONSTAX (Gdanetz et al., 2017), a tool that determines the consensus classification of three common fungal taxonomic assignment tools (RDP classifier, UTAX, and SINTAX). The reference database used was UNITE v7.2 (01-12-2017) FASTA release database (Kõljalg et al., 2013). The default settings of CONSTAX were used with a confidence threshold of 0.8. After assignment with CONSTAX, Basic Local Alignment Search Tool (BLAST) queries of the NCBI database (Altschul et al., 1990) were used to better assign OTUs with incomplete taxonomy and to identify OTUs with high read numbers that were not truly fungal reads. Thirteen OTUs were removed from the OTU table that were closely related to an invertebrate or a bacterium. OTUs classified in the phylum Mortierellomycota were reassigned to the phylum Mucoromycota (Spatafora et al., 2017). The final OTU table contained 425 OTUs and 27 samples.

The data analyses for the fungal sequence data were done the same way as the bacterial data analyses with the exception of the following. To observe alpha diversity, the samples were first rarefied without replacement to 1462 sequences per sample using 'rarefy_even_depth' (set.seed 790) function in phyloseq. Sixty-three OTUs were removed from the OTU table after rarefaction. Originally a relativised OTU table was going to be used to observe the fungal community composition, but on closer inspection of the data, it was revealed that some samples were severely skewing the abundances of certain fungal communities. Thus, the rarefied OTU table was used to look at the fungal community composition. The rarefied OTU table with all the samples contained 362 OTUs and 27 samples. In addition, the FUNGuild annotation tool (Nguyen et al., 2016) was used to attain functional information about some of the fungal OTUs present in this data set. FUNGuild

allows for the illumination and characterization of the ecological guilds present within this data.

Results

16S rRNA Gene Sequencing Summary

After filtering, a total of 1,822,341 bacterial 16S rRNA gene sequences were obtained with a range of 13,467 to 97,498 per sample and a mean of 67,494.11 sequences (standard deviation 22,217.70). A total of 3,804 OTUs were in the final OTU table. Of those 3,804 OTUs, 3,281 were in the bat samples, 3,010 were in the microbial mats, and 549 were in the air samples. Twenty-seven samples, consisting of 12 bat, 11 microbial mat, and four air samples, were in the final data set used for a majority of the data analyses. For part of the analyses, only the bat and microbial mat samples were used (n=23).

Bacterial Community Diversity

Figure 1 shows the rarefaction curves and alpha diversity indices of bacterial communities for each sample type. The rarefaction curves (**Figure 1A)** showed a plateauing in all the samples. In addition, the figure shows that the bat and microbial mat samples have a higher species richness and sample depth than the air samples. Observed OTUs and Chao1 showed similar diversity among the bat and microbial mat samples, but the Shannon diversity index showed some (not significant) difference between bat (3.53 ± 1.28) and mat (4.45 ± 0.44) samples (**Table 3)**. There was no significant difference between the bat and microbial mat samples with respect to any of the alpha indices (ns: $p > 0.05$) (**Figure 1B**). There were significant differences in observed OTUs, Chao1, and Shannon diversity between mat and air samples (**: $p <= 0.01$), and there were significant differences in observed OTUs and Chao1

between bat and air samples (**: p <= 0.01). The bat samples had the highest mean alpha diversity in regard to observed OTUs (678.25 ± 218.70) and Chao1 richness (908.28 ± 202.02). The mat samples had the highest mean Shannon diversity (4.45 ± 0.44). There were not significant differences between the two caves (CLC and EST) with respect to the three alpha indices (p>0.05). A pairwise Wilcoxon test showed that there were not significant differences (p>0.05) between the species of bats (COTO, MYVO, MYTH, MYEV).

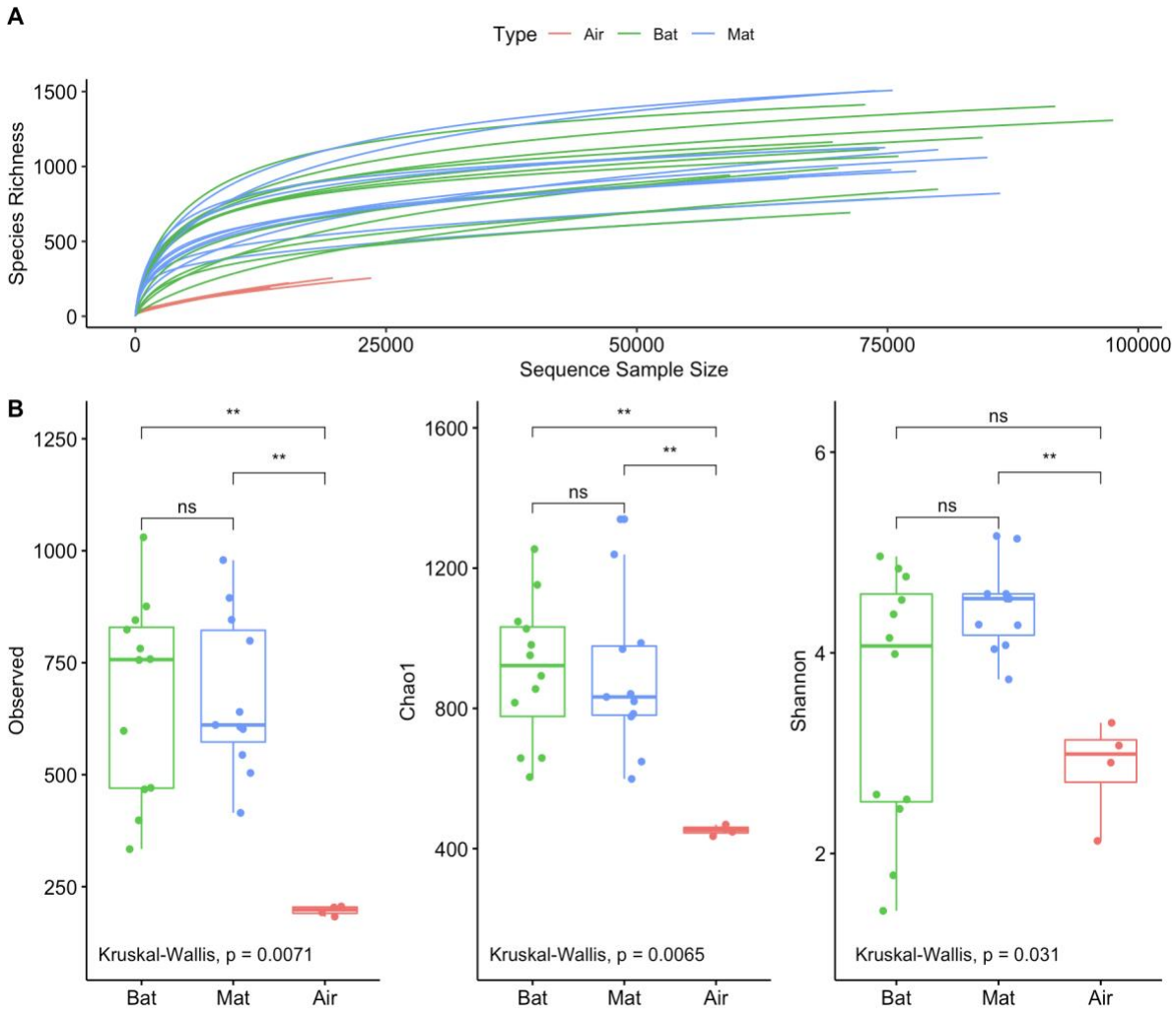

Figure 1. Bacterial richness and alpha diversity **(A)** Rarefaction curve by sample type on raw data. **(B)** Alpha diversity indices (Observed richness, Chao1, and Shannon) on rarefied data. Box plots by sample type. Brackets denote the pairwise comparison p-value significance codes (ns: p > 0.05, *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001, ****: p <= 0.0001). Kruskal-Wallis p-value shown at the bottom of each alpha index box plot.

Table 3. Bacterial alpha diversity by sample type. Values are mean ± 1 Standard Deviation. Values calculated on rarefied data diversity indices.

Diversity Indices	Bat	Mat	Air
Observed OTUs	678.25 ± 218.70	676.55 ± 177.39	196.50 ± 10.66
Chao1	908.28 ± 202.02	894.24 ± 227.31	452.63 ± 13.97
Shannon	3.53 ± 1.28	4.45 ± 0.44	2.85 ± 0.51

An adonis test revealed significant differences between sample type based on the Bray-Curtis dissimilarity metric ($R^2 = 0.30$, $p <= 0.001$). Pairwise adonis test showed significant differences between all sample type pairs ($p <= 0.05$) (**Table 4**). These differences were revealed when the NMDS plots were colored by sample type (**Figure 2 A, B)**. **Figure 2b**, which has had the air samples removed, better demonstrates the differences between the bat and microbial mat samples. 'Betadisper' analyses showed no significant differences in beta dispersion among the sample types and caves ($p> 0.1$), but beta dispersion was significantly different among species of bats ($p <= 0.01$). An adonis test revealed significant differences between caves ($R^2 = 0.14$, $p = 0.001$). A mixed effects model with type and cave as factors showed that the interaction between sample type and cave was significant ($p = 0.001$). An ANOSIM test with species as a factor showed that the samples were similar with some differences based on an R^2=0.26. The "species" factor separates out not only by the species of bat, but also the mat and air sample, thus these differences may be due to differences between sample type.

Table 4. Bacterial pairwise adonis results with false discovery rate (FDR) corrections.

Pairs	F.Model	R^2	p.value	p.adjusted
Bat vs Mat	4.713920	0.1833217	0.002	0.002
Bat vs Air	4.934972	0.2606274	0.001	0.002
Mat vs Air	5.961463	0.3143989	0.002	0.002

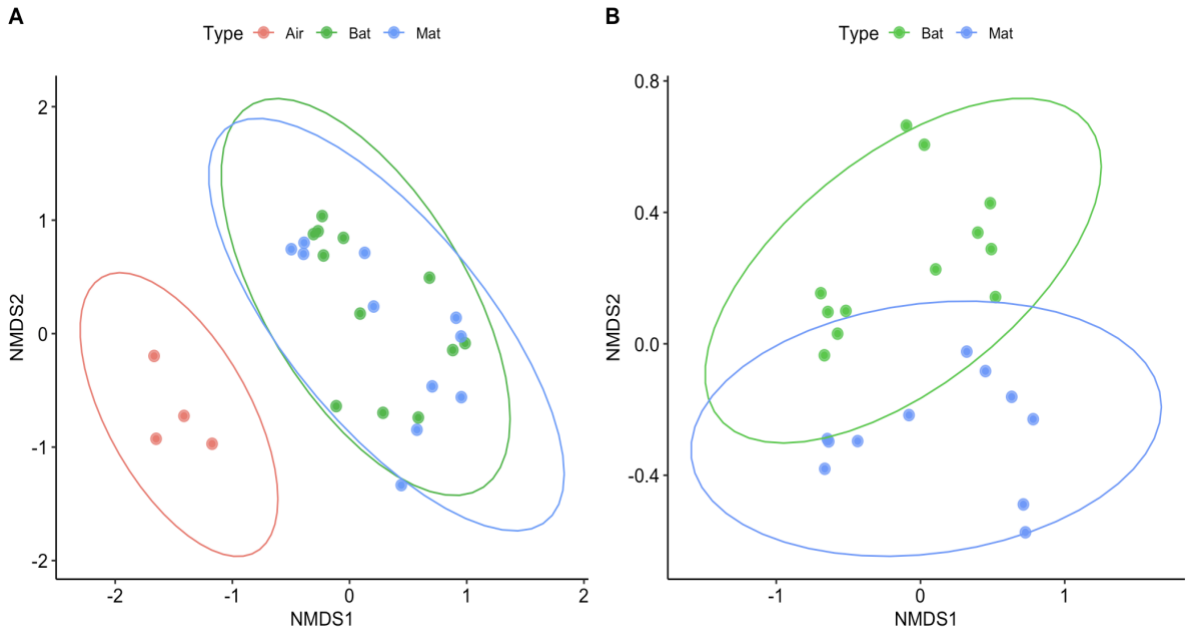

Figure 2. Bacterial NMDS plots. (A) The first two axes of a 3D NMDS (stress=0.0683) colored by sample type with 95% confidence ellipses. (B) The first two axes of a 3D NMDS (stress=0.0628) colored by bat and mat with 95% confidence ellipses.

Bacterial Community Composition

The overall highest relative abundances (>1%) of phyla in the OTU table were Actinobacteria (40.44%), Proteobacteria (29.38%), Acidobacteria (9.61%), Unclassified bacteria (9.05%), Bacteroidetes (3.06%), Nitrospirae (1.66%), Verrucomicrobia (1.59%), Planctomycetes (1.53%), Firmicutes (1.46%), and Gemmatimonadetes (1.22%). These nine phyla and unclassified bacteria made up approximately 98% of all the OTUs. Phyla that were

significantly different in relative abundance between bat and mat microbial mat samples were Actinobacteria (p = 0.00007), Proteobacteria (p = 0.004), Acidobacteria (p = 0.03), Firmicutes (p = 0.003), and Verrucomicrobia (p = 0.001) (**Figure 3**). The relative abundance of unclassified bacteria was significantly higher (p = 0.001) in microbial mat samples than in bat and air samples. The phyla of the air samples that were significantly different from the bat and mat samples were Proteobacteria (p <= 0.001), Firmicutes (p <=0.01), Gemmatimonadetes (p <= 0.05), Planctomycetes (p <=0.01), and Verrucomicrobia (p <=0.01) (**Figure 3**).

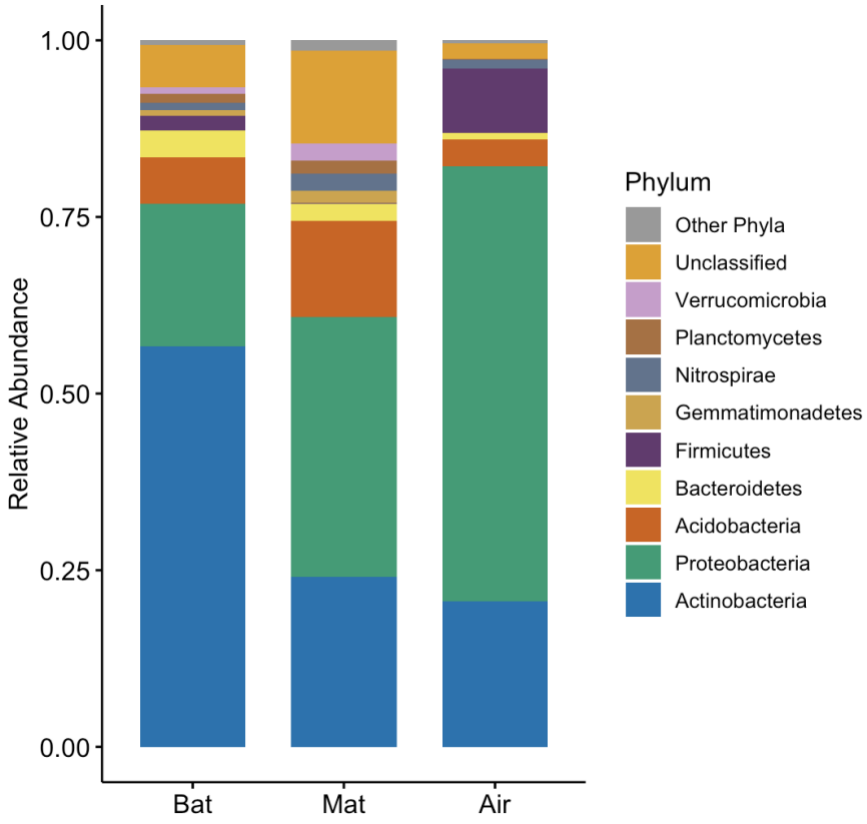

Figure 3 Relative abundance of bacterial phyla. All phyla with 1% or more relative abundances from each sample type are represented by name, and the phyla with less than 1% mean relative abundances are grouped into the "Other Phyla" category.

Group significance on the 20 most abundant OTUs of just the bat and mat samples showed significant differences between the bat and mat in eight OTUs (**Table 5**). Most of the significantly different OTUs had a higher mean abundance in bats than in mat samples. In fact, only one of the significant OTUs was higher in the mats than in the bat samples (OTU34). This OTU could only be classified down to the class level of Gammaproteobacteria (**Table 5**). *Rhodococcus* (OTU1) and *Arthrobacter* (OTU2) are genera in the phylum Actinobacteria that were significantly different in the bat and microbial mat samples, but there were other Actinobacteria species that were among the top 20 OTUs that were not significantly different. They include *Rubrobacter* (OTU7) and *Streptomyces* (OTU3) species, and bacteria in the families *Pseudonocardiaceae* (OTU1106) and *Acidimicrobiaceae* (OTU18) (**Table 5**).

Table 5. Group significance of top 20 bacterial OTUs.

OTU	Test-Statistic	P	FDR_P	Bonferroni_P	Bat_mean	Mat_mean	taxonomy
OTU1	16.5081562	4.84E-05	0.00096883	0.00096883	12599.75	166.09	p__Actinobacteria; g__Rhodococcus
OTU13	13.5438706	0.00023305	0.00158751	0.00466103	612.75	6.27	p__Firmicutes; g__Carnobacterium
OTU21	13.5034411	0.00023813	0.00158751	0.00476253	1230.08	12.55	p__Bacteroidetes; g__Pedobacter
OTU34	8.01515152	0.00463876	0.0231938	0.09277519	88.58	609.82	c__Gammaproteobacteria;o__;f__;g__
OTU9	6.37057176	0.01160282	0.04641128	0.23205641	920.17	20.91	c__Alphaproteobacteria; g__Brevundimonas
OTU2	5.47240073	0.01931907	0.0643969	0.3863814	9230.67	517.36	p__Actinobacteria; g__Arthrobacter
OTU8	4.81891106	0.02814912	0.07808061	0.56298247	768.83	12.18	c__Gammaproteobacteria; g__Hafnia
OTU4	4.64015152	0.03123225	0.07808061	0.6246449	1474.25	7281.55	c__Gammaproteobacteria; o__Chromatiales; f__; g__
OTU16	3.52541605	0.06043466	0.13429924	1	506.25	858.73	p__Nitrospirae; g__Nitrospira
OTU7	2.56187181	0.1094689	0.21893781	1	563.67	665.64	p__Actinobacteria; g__Rubrobacter
OTU14	2.18181818	0.1396494	0.253908	1	642.42	901.45	p__Acidobacteria; g__Aridibacter
OTU3	1.83333333	0.17573434	0.29289056	1	4675.17	7017.27	p__Actinobacteria; g__Streptomyces
OTU15	1.09469697	0.29543285	0.42204693	1	201.83	917.55	c__Acidobacteria_Gp4; o__; f__; g__
OTU5	1.09469697	0.29543285	0.42204693	1	585.67	949.45	c__Betaproteobacteria;o__Burkholderiales;f__;g__
OTU1106	0.74242424	0.38888541	0.51851388	1	367.58	314.82	p__Actinobacteria; f__Pseudonocardiaceae;g__
OTU19	0.5011946	0.4789757	0.59871962	1	166.08	506	c__Acidobacteria_Gp4; o__; f__; g__
OTU18	0.11475342	0.734796	0.86446589	1	442.42	838.09	p__Actinobacteria; f__Acidimicrobiaceae;g__
OTU11	0.01515152	0.90203465	0.94951016	1	197.5	754.55	c__Gammaproteobacteria; g__Luteimonas
OTU12	0.01515152	0.90203465	0.94951016	1	492.92	838.09	c__Alphaproteobacteria; g__Beijerinckia
OTU29	0.00094744	0.97544461	0.97544461	1	526.42	960.55	c__Gammaproteobacteria; o__; f__; g__

Since Actinobacteria have the potential to play an important role in the health of bats, the Actinobacteria in the bat and mat samples were characterized in more detail (**Figure 4**). The relative abundances in **Figure 4** are the relative abundances within the Actinobacteria phylum. It is important to keep in mind that overall the Actinobacteria relative abundance in the bat samples was higher (56.74%) than in the microbial mat samples (24.03%) (**Figure 3**). With respect to the Actinobacteria in **Figure 4**, the bat samples did not seem to share the same proportions or types of Actinobacteria genera. There were eight bat samples that were generally dominated by *Arthrobacter* and *Rhodococcus* species (Pairs B-C and Q-Z). The other four bat samples were generally dominated by *Streptomyces* and *Rubrobacter* species (Pairs A and D1-E). The microbial mat samples did not all share the same portions of Actinobacteria genera, but they all generally contained *Streptomyces* species (**Figure 4)**. Five mat samples had high proportions characterized as "Unclassified Genera of Actinobacteria" (Pairs A-D and Q). Looking at both the bat and mat samples, eight bat samples (Pairs B-C and Q-Z) stood out as being very different, with respect to Actinobacteria genera, from all the mat samples (**Figure 4**).

Figure 5 shows the shared bacterial OTUs between each sample type collectively. There were 526 OTUs out of 3,804 shared among every sample type (Bat, Mat, and Air). The bat samples had more unique OTUs (780) than the other two sample types. The bat and mat samples shared a large percentage of their OTUs with each other, 59.86% of the bat samples' OTUs were shared with the mat samples and 65.25% of the mat samples' OTUs were shared with the bat samples. Collectively, the air samples shared most of their OTUs with the bat and the mat samples (99.45%).

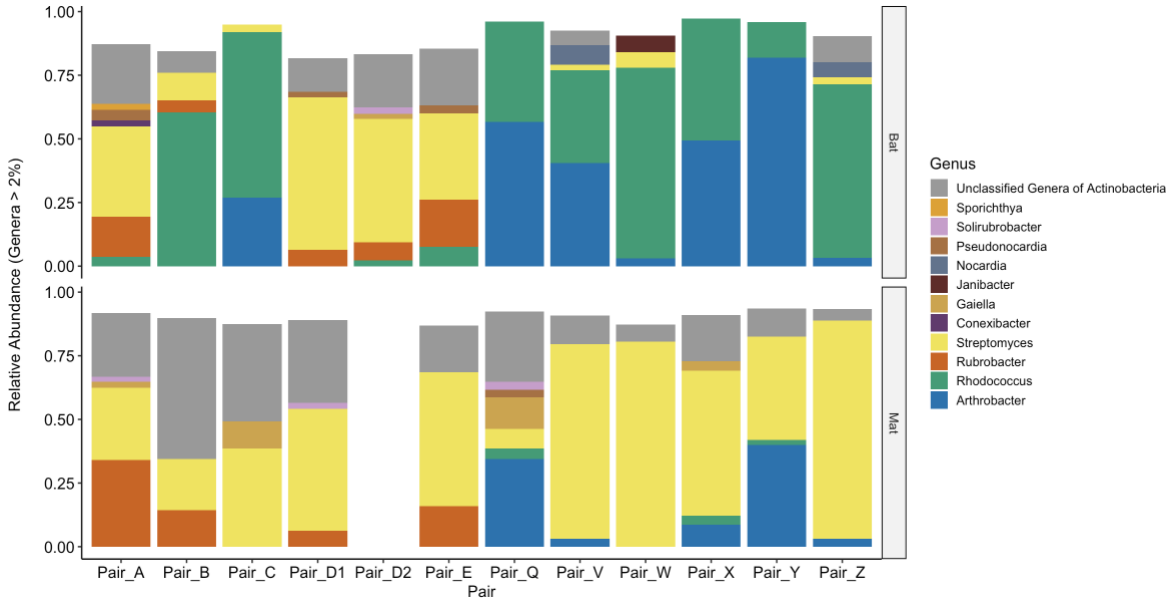

Figure 4. Relative abundance of Actinobacteria genera for bat and microbial mat samples. Represented are the relative abundance at the genus level across each bat and microbial mat samples. All Actinobacteria genera with 2% or more relative abundances from each sample are represented by name. Bat sample pairs D1 and D2 are both paired with one microbial mat sample (Pair_D1).

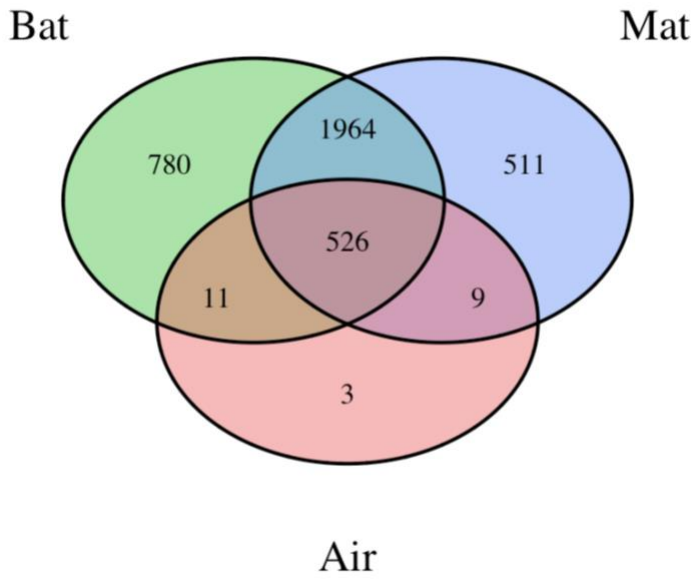

Figure 5. Bacterial Venn diagram. Shows the shared OTUs among the three sample types.

The SourceTracker results shed light on the potential impact that the microbial mats and air of a cave can have on the bats' bacterial communities (**Figure 6**). For the most part, each of the bat samples showed high proportions of their potential source of bacteria coming from their respective cave microbial mats, with the exception of the "Bat_MYVO_CLC_PairC" and "Bat_MYVO_EST_PairW". CLC cave air appeared to be a potential source of bacteria in every bat sample, with a range of 0.05% to 58.85%. EST cave air appeared to be a smaller potential source of bacteria in only five of the bat samples, with a range of 0.03% to 16.37%. The CLC microbial mat appeared to be a high potential source of bacteria in five of the bat samples, with a range of 42.62% to 84.18%. The CLC microbial mat appeared to be a high potential source of bacteria in seven of the bat samples, with a range of 17.81% to 93.45%. The percentage range of the source being "unknown", meaning not from the mat or air samples, was 2.8% to 39.67%.

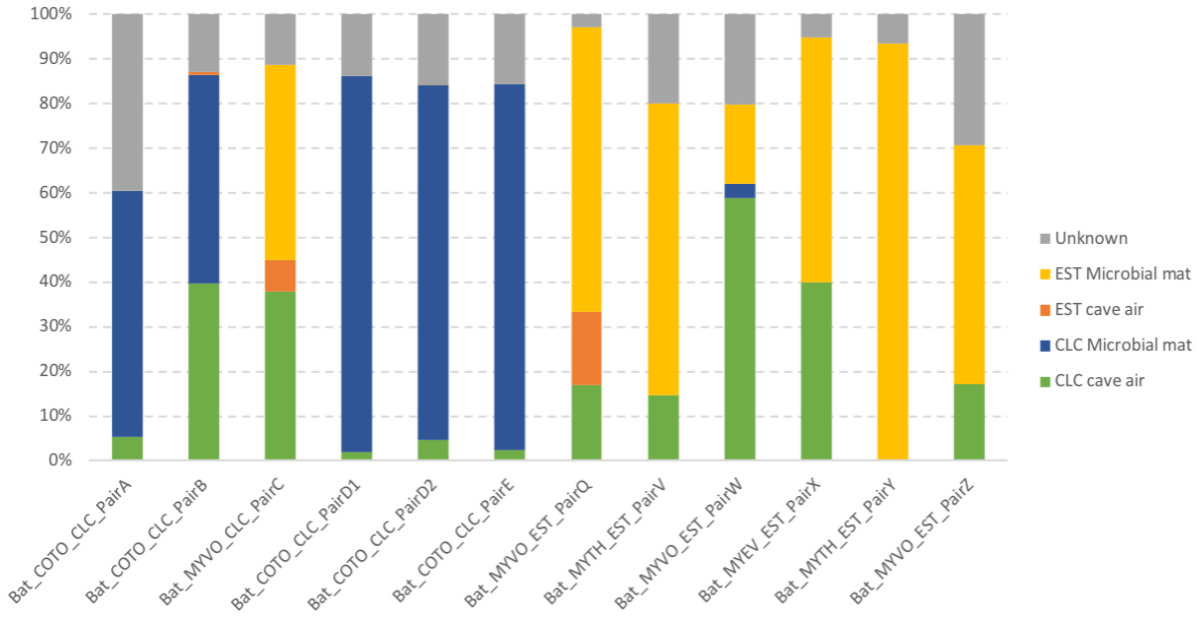

Figure 6. Bacterial SourceTracker source contributions. Shows estimated source environment proportions of potential contribution to the bat samples. Possible source environments are CLC cave air, CLC microbial mat, EST cave air, EST microbial mat, and unknown source.

ITS Gene Sequencing Summary

After filtering, a total of 686,131 fungal ITS gene sequences were obtained with a range of 1,462 to105,540 sequences per sample and a mean of 25,412.26 sequences (standard deviation 19,259.61). A total of 425 OTUs were in the final OTU table. Of those 425 OTUs, 414 were in the bat samples, 185 were in the microbial mats, and 72 were in the air samples. Twenty-seven samples, consisting of 12 bat, 11 microbial mat, and four air samples, were in the final data set used for a majority of the data analyses. For part of the analyses, only the bat and microbial mat samples were used (n=23).

Fungal Community Diversity

Figure 7 shows the rarefaction curves and alpha diversity indices of fungal communities for each sample type. The rarefaction curves (**Figure 7A)** showed a plateauing in all the samples except two. In addition, the figure shows that the bat samples have a higher and more variable species richness than both the microbial mat and air samples. One microbial mat sample had a much higher sequencing depth than all the other samples, and one air sample had a much lower sequencing depth than all the other samples. Observed OTUs, Chao1, and Shannon indices showed very different diversity among the bat and microbial mat samples (**Table 6)**. The means of all the bat alpha indices were higher than all of those of the mat samples. There were significant differences between the bat and microbial mat samples with respect to all of the alpha indices (**: $p <= 0.01$) (**Figure 7B)**. There were significant differences in observed OTUs, Chao1, and Shannon diversity between bat and air samples (**: $p <= 0.01$). There were not significant differences in observed OTUs, Chao1 and Shannon diversity between the mat and the air samples (ns: $p > 0.05$). There were not

significant differences between the two caves (CLC and EST) with respect to the three alpha indices (p>0.05). A pairwise test of species showed that there were not significant differences (p>0.05) between the species of bats (COTO, MYVO, MYTH, MYEV).

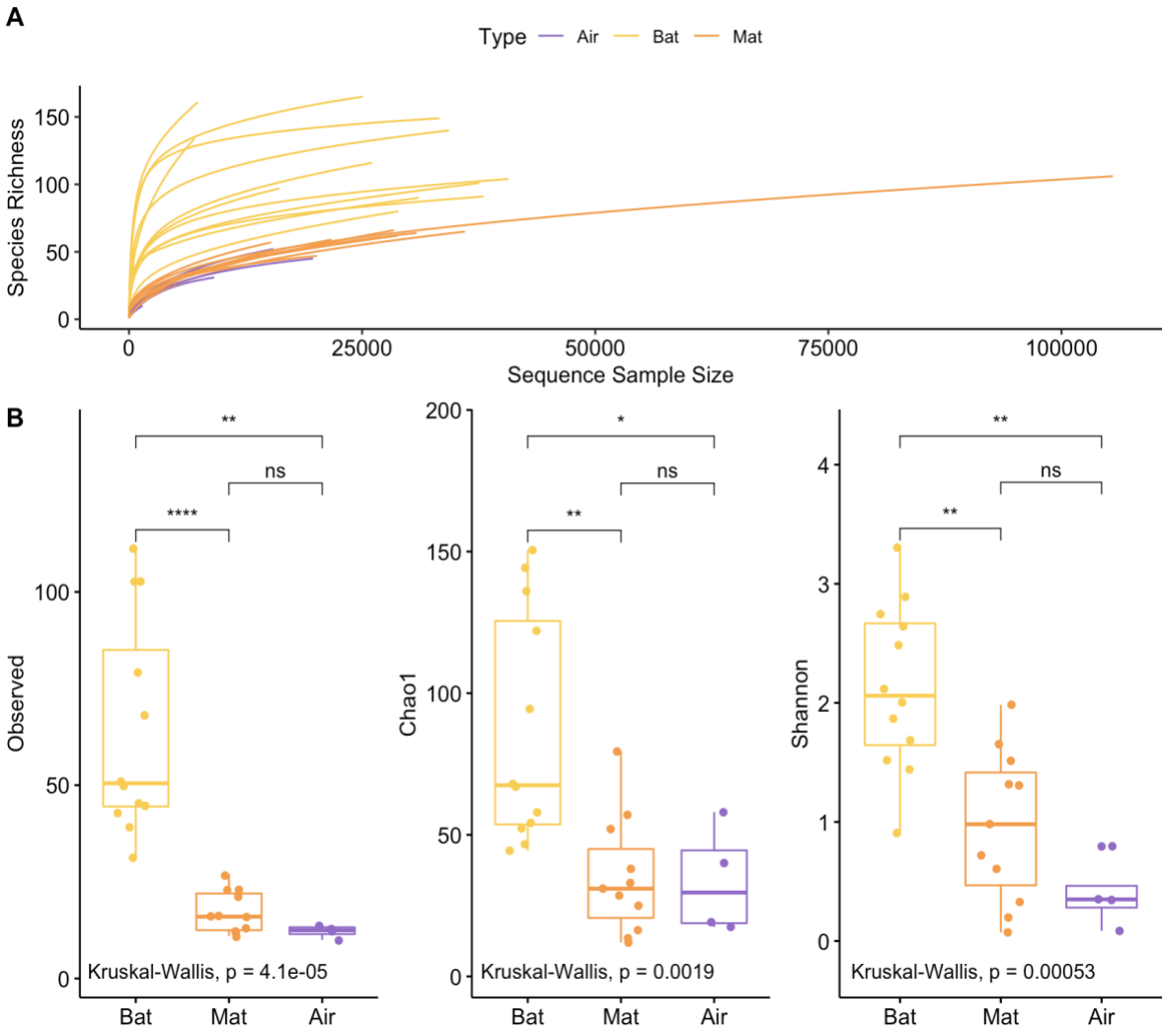

Figure 7. Fungal richness and alpha diversity **(A)** Rarefaction curve by sample type on raw data. **(B)** Alpha diversity indices (Observed richness, Chao1, and Shannon) on rarefied data. Box plots by sample type. Brackets denote the pairwise comparison p-value significance codes (ns: p > 0.05, *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001, ****: p <= 0.0001). Kruskal-Wallis p-value shown at the bottom of each alpha index box plot.

Table 6. Fungal alpha diversity by sample type. Values are mean ± 1 Standard Deviation. Values calculated on rarefied data diversity indices.

Diversity Indices	Bat	Mat	Air
Observed OTUs	64.00 ± 28.18	17.27 ± 5.41	12.25 ± 1.71
Chao1	86.47 ± 40.83	35.08 ± 20.65	33.69 ± 19.16
Shannon	2.13 ± 0.70	0.97 ± 0.64	0.39 ± 0.29

In examining beta diversity, 'Betadisper' analyses showed significant differences in beta dispersion among the sample types ($p <= 0.001$). Since beta dispersion was significantly different between sample types, an ANOSIM test was used in lieu of an adonis test. ANOSIM results showed significant differences between all sample types ($R = 0.44$ $p = 0.0009$) and between bat and mat samples ($R = 0.44$ $p = 0.0009$). These differences were revealed when the NMDS plots were colored by sample type (**Figure 8 A, B)**. **Figure 8B** demonstrates the differences between the bat and microbial mat samples and shows the dispersion pattern. 'Betadisper' analyses showed significant differences in beta dispersion among the species of bats ($p <= 0.001$). Since Beta dispersion was not significantly different between the two caves ($p> 0.1$), an adonis test was used and revealed significant differences between caves ($R^2 = 0.070$ $p = 0.002$). A mixed effects model with type and cave as factors showed that the interaction between sample type and cave was significant ($p = 0.001$). An ANOSIM test with species as a factor showed significant differences ($R = 0.09$, $p = 0.0009$).

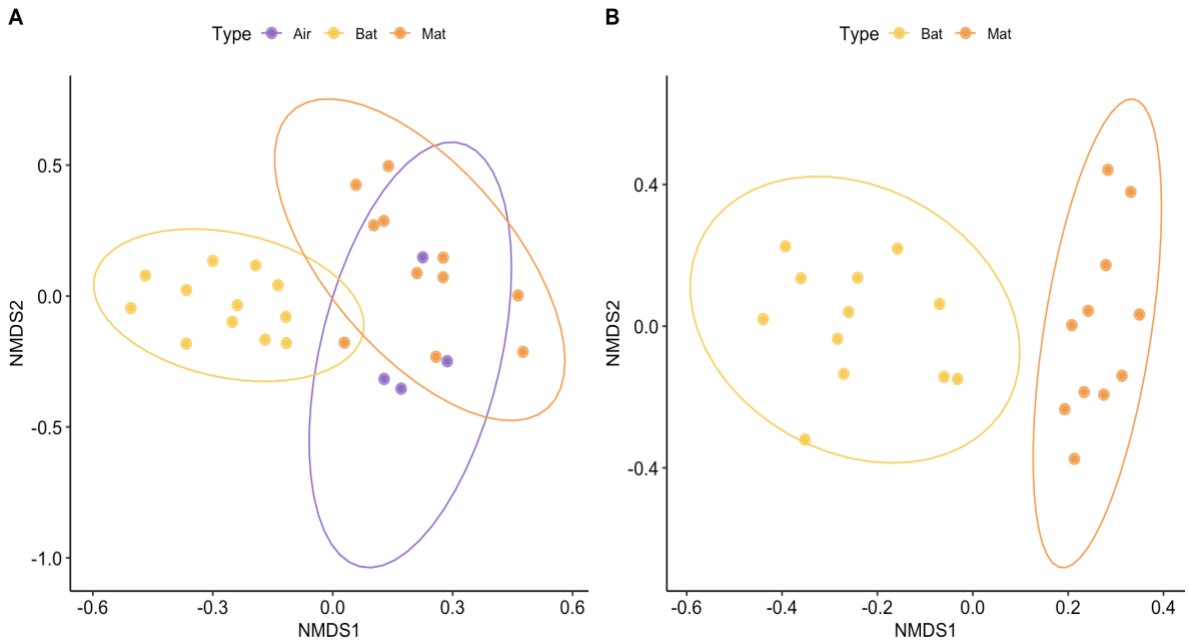

Figure 8. Fungal NMDS plots. **(A)** The first two axes of a 3D NMDS (stress=0.151) colored by sample type with 95% confidence ellipses. **(B)** The first two axes of a 3D NMDS (stress=0.133) colored by bat and mat with 95% confidence ellipses.

Fungal Community Composition

The overall relative abundances of phyla in the rarefied OTU table were 47.80% Ascomycota, 24.73% Basidiomycota, 15.26% Mucoromycota, and 12.88% Unclassified fungi. Looking at **Figure 9A**, there are differences in relative abundances of the three phyla and Unclassified fungi among sample types, but most of the observed differences were not statistically significant. Ascomycota and Basidiomycota abundances were not significantly different between any of the sample types (bat, mat, and air). Mucormycota abundances were significantly different between microbial mat and air samples ($p <= 0.01$) and bat and air samples ($p <= 0.01$). Unclassified fungi abundances were significantly different between microbial mat and air samples ($p <= 0.05$) and bat and air samples ($p <= 0.01$). Unclassified fungi were in higher relative abundance in microbial mat samples (28.55%) than in the other sample types, bat (2.74%) and air (0.22%). The large percentage of unclassified fungi in the

mat samples was due to three mat samples that were mainly composed of unclassified fungi (78-96% of samples sequences are unclassified). All the other mat samples had low abundances of unclassified fungi. There were three unclassified OTUs that made up most of the sequence abundance of the three mat samples (OTU 2, 9, and 24) (**Figure 9C**).

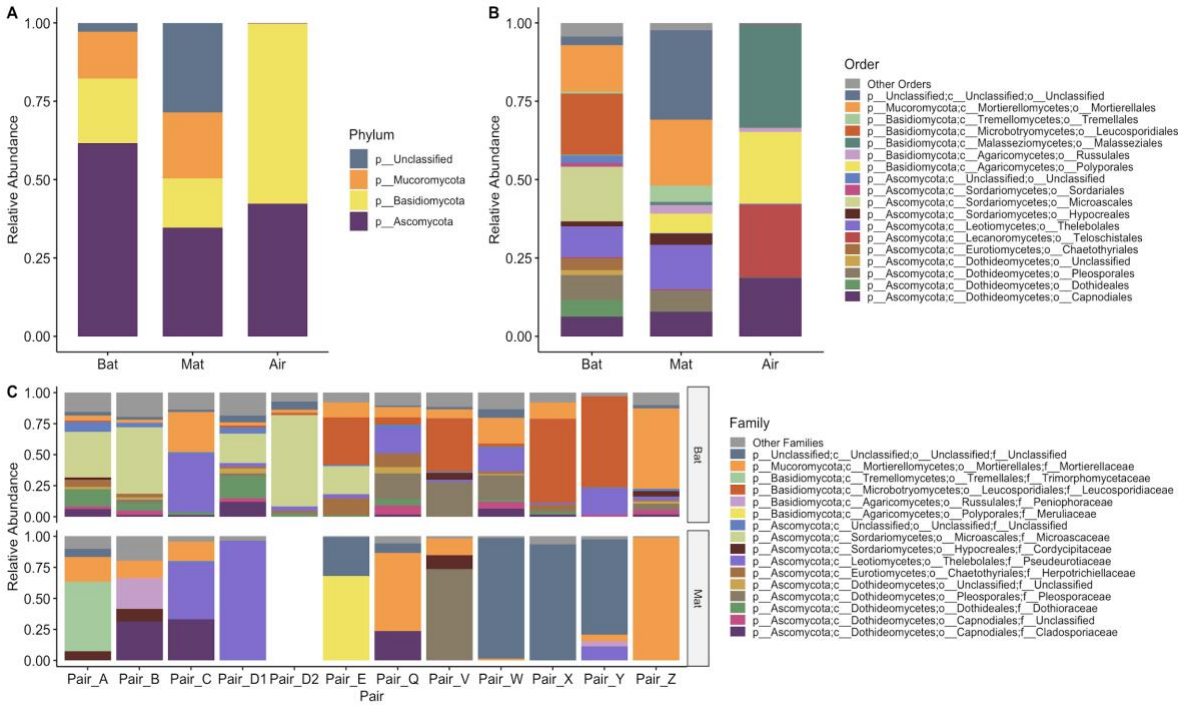

Figure 9. Relative abundance of fungal taxa at the phylum **(A)** and order **(B)** levels across sample types. The relative abundance at the family level across each bat and microbial mat samples **(C)**. Bat sample pairs D1 and D2 are both paired with one microbial mat sample (Pair_D1). All orders and families with 1% or more relative abundances from each sample type are represented by name and the orders and families with less than 1% mean relative abundances are grouped into the "Other Orders" and "Other Families" categories.

At the Order level (**Figure 9B**), the bat and mat samples were fairly diverse in the types of fungi found within them and the air samples were not as diverse. The air samples were mainly dominated by four Orders, Capnodiales (Ascomycota), Teloschistales (Ascomycota), Polyporales (Basidiomycota), and Malasseziales (Basidiomycota). Both bat and mat sample types shared more similarities in terms the fungal Orders found within them, than they did with the air samples. For example, both bat and mat sample types shared fungal

taxa (at 1% relative abundance or higher) within the Orders Capnodiales, Pleosporales, Theleboables, Hypocreales, Mortierellales, and Unclassified fungi. These five Orders and the unclassified fungi made up 43.29% and 82.01% of the bat and mat sample type community composition respectively. Proportions that were driving these differences in overall abundances of these orders were first the high abundance of unclassified fungi in the mat samples (28.55%), Mortierellales in mats (21.07%) as opposed to in bats (14.76%), and Theleboables in mats (14.16%) as opposed to in bats (10.03%).

Figure 9C represents the top 16 families that are in 1% or greater abundance overall in both the bat and mat sample types and each stacked bar represents a sample within the bat or mat sample type. It is apparent looking at **Figure 9C** that many of the samples are different, not only between bat and mat sample types, but also between individual samples within a given sample type. Families that stand out in the samples were Pleosporaceae, Pseudeurotiaceae, Microascaceae, Leucosporidiaceae, Mortierellaceae, and unclassified fungi (**Figure 9C)**. Pleosporaceae was present in higher than 1% abundance in eight of the bat samples with a range of 1.98% to 28.97% relative abundance and was only present in one mat sample at 72.53% relative abundance. Pseudeurotiaceae, the family that contains the genus *Pseudogymnoascus*, was present in ten of the bat samples with a range of 1.41% to 46.73% and in only three of the mat samples ranging from 11.14% to 96.50%. Looking at Microascaceae, it was in relatively high abundance in just five of the bat samples, ranging from 22.48% to 73.50% relative abundance. These five bat samples (Pairs A-B and D1-E) were the five COTO bat species samples taken in this study. Leucosporidiaceae was present in nine of the bat samples and was not present in the mat samples, the nine bat samples ranged from 1.34% to 67.66% relative abundance. Mortierellaceae was present in higher than

1% relative abundance in 19 of the samples. It was present in eleven of the bat samples ranging from 2.08% to 64.64% and in eight of the mat samples ranging from 1.38% to 99.05% relative abundance. One mat sample (Pair Z) was almost completely dominated by the Mortierellaceae family, and its corresponding bat sample (Pair Z) had a high abundance as well.

Table 7. Group significance of top 24 fungal OTUs**.**

OTU	Test-Statistic	P	FDR_P	Bonferroni_P	Bat_mean	Mat_mean	Taxonomy
OTU25	16.88372093	3.97E-05	0.00095378	0.000953783	96.83	0.73	p__Ascomycota; f__Herpotrichiellaceae; g__
OTU8	14.64017221	0.00013011	0.00156134	0.003122672	1084.67	5.18	p__Basidiomycota; f__Leucosporidiaceae; g__Leucosporidium
OTU14	13.68040155	0.0002167	0.00173363	0.005200902	271.5	1.27	p__Ascomycota; f__Dothioraceae;g__Aureobasidium
OTU3	12.59210962	0.00038738	0.00232427	0.009297088	635.42	5.18	p__Ascomycota; f__Pseudeurotiaceae; s__Pseudogymnoascus roseus
OTU5	11.1835918	0.00082524	0.00396115	0.019805727	232.17	447.91	p__Ascomycota; f__Pleosporaceae; g__Alternaria
OTU20	10.20266767	0.00140238	0.00560951	0.033657034	217.917	2.27	p__Ascomycota; f__Pleosporaceae;g__Leptosphaerulina
OTU4	9.345895522	0.00223485	0.00766236	0.053636501	627.08	15.45	p__Mucoromycota; f__Mortierellaceae; s__Mortierella parvispora
OTU115	5.771547109	0.01628769	0.04886307	0.390904544	217.83	0.82	p__Basidiomycota; f__Leucosporidiaceae; g__Leucosporidium
OTU38	3.478395062	0.06217501	0.15227962	1	36.42	60.18	p__Ascomycota; f__Aspergillaceae; g__Penicillium
OTU11	3.444832751	0.06344984	0.15227962	1	165.17	529.82	p__Ascomycota; f__Cladosporiaceae; g__Cladosporium
OTU7	2.278176568	0.13120605	0.28626775	1	51.25	879.36	p__Ascomycota; f__Pseudeurotiaceae; g__Pseudogymnoascus
OTU6	2.134030578	0.14406151	0.28812302	1	1171	4.55	p__Ascomycota; f__Microascaceae; g__Kernia
OTU2	1.230312036	0.26734636	0.49356252	1	63.83	1267.64	p__Unclassified_Fungi
OTU39	1.113621691	0.29129603	0.49936462	1	1.67	86.45	p__Mucoromycota; f__Mortierellaceae; s__Mortierella exigua
OTU9	0.933946941	0.33383941	0.53414306	1	4.25	307.64	p__Unclassified_Fungi
OTU33	0.741640127	0.38913599	0.54385887	1	133.83	1	p__Ascomycota; f__Herpotrichiellaceae; g__Coniosporium
OTU16	0.668558652	0.41355458	0.54385887	1	3.92	177.36	p__Ascomycota; f__Cordycipitaceae; g__Cordyceps
OTU23	0.653171574	0.41898104	0.54385887	1	2.83	412.55	p__Basidiomycota; f__Meruliaceae; s__Phlebiopsis gigantea
OTU17	0.621326072	0.43055494	0.54385887	1	3.25	147.82	p__Basidiomycota; f__Peniophoraceae; g__Peniophora
OTU24	0.496330006	0.48111714	0.56765435	1	2.17	225.18	p__Unclassified_Fungi
OTU15	0.461985392	0.49669756	0.56765435	1	144.5	55.55	p__Mucoromycota; f__Mortierellaceae; s__Mortierella amoeboidea
OTU32	0.27599236	0.59934008	0.64907486	1	17.25	180.73	p__Mucoromycota; f__Mortierellaceae; s__Mortierella antarctica
OTU12	0.2430246	0.62203007	0.64907486	1	63.58	1000.55	p__Mucoromycota; f__Mortierellaceae; s__Mortierella fimbricystis
OTU13	0.199504337	0.65512123	0.65512123	1	3	328.55	p__Basidiomycota; f__Trimorphomycetaceae; s__Saitozyma paraflava

Group significance on the 24 most abundant fungal OTUs of just the bat and mat samples showed significant differences between the bat and mat in eight OTUs (**Table 7**). Most of the significantly different OTUs had a higher mean abundance in bats than in mat samples. In fact, only one of the significant OTUs (classified as *Alternaria*) was higher in the mats than in the bat samples (OTU5). There were two OTUs in the table that were classified

in the genus *Pseudogymnoascus* (OTU3 and OTU7), which is the genus of the white-nose pathogen *P. destructans*. OTU3 was classified to the species *P. roseus* and OTU7 was not able to be classified to the species level. OTU3 was significantly higher in abundance in the bat samples ($p = 0.002$, mean = 635.42) and OTU7 was higher in abundance (mean =879.36) overall in the mat samples, but was not significantly higher. There were five OTUs classified in the genus *Mortierella*, two of which had a higher mean abundance in the bat samples and three of which had a higher mean abundance in the mat samples.

Figure 10 shows the shared fungal OTUs among sample types. There were 64 OTUs out of 425 OTUs shared among every sample type (Bat, Mat, and Air). The bat samples had more unique OTUs (232) than the other two sample types. The mat samples shared 94.60% of their OTUs with the bat samples and the bat samples shared 42.27% of their OTUs with the mat samples. The air samples shared most of their OTUs with the bat and the mat samples (98.61%).

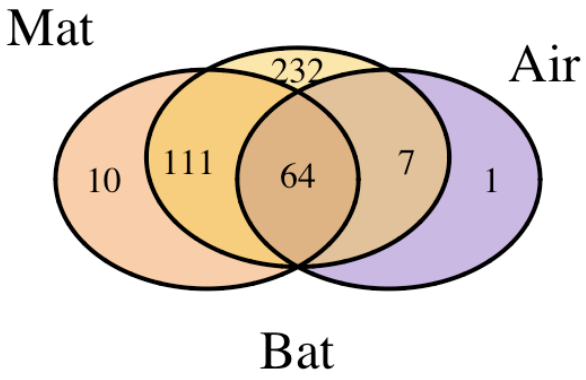

Figure 10. Fungal Venn diagram. Shows the shared OTUs between the three sample types.

The SourceTracker results (**Table 8**) for the fungi appeared that the potential impact of the microbial mats and air of a cave have on the bats fungal communities is very low, if not at all. The percentage range of the source being "unknown", meaning not from the mat or

air samples, was 70.50% to 99.99%. The EST microbial mats appeared to be a potential source of fungi in seven of the bat samples from both caves, with a range of 0.01% to 29.50%. The CLC microbial mats appeared to be a very small potential source of fungi in just two of the bat samples from CLC cave, with a range of 0.002% to 7.46%.

Table 8. Fungal SourceTracker results. Numbers are percentages.

Sample ID	CLC cave air	CLC Microbial mat	EST cave air	EST Microbial mat	Unknown
Bat_COTO_CLC_PairA	0	0	0	0	100
Bat_COTO_CLC_PairB	0	0	0	0	100
Bat_MYVO_CLC_PairC	0	0	0	1.00E-02	99.99
Bat_COTO_CLC_PairD1	0	7.46	0	8.39	84.15
Bat_COTO_CLC_PairD2	0	2.00E-02	0	6.15	93.83
Bat_COTO_CLC_PairE	0	0	0	1.00E-02	99.99
Bat_MYVO_EST_PairQ	0	0	0	7.39	92.61
Bat_MYTH_EST_PairV	0	0	0	29.5	70.5
Bat_MYVO_EST_PairW	0	0	0	3.25	96.75
Bat_MYEV_EST_PairX	2.00E-02	0	0	0	99.98
Bat_MYTH_EST_PairY	0	0	0	0	100
Bat_MYVO_EST_PairZ	0	0	0	0	100

Discussion

Our results shed light on an understudied area that could potentially have implications for the health of bats that roost in caves. We characterized the bacterial and fungal communities across samples from the external microbiomes of varying species of bats, cave microbial mats, and cave air. Looking at both the bacterial and fungal communities of these

samples allowed us to get a more complete view of the microbial communities in the caves and cave roosting bats.

Overall, the bacterial and fungal results showed some similar general trends. In both cases, the bat samples generally had higher species richness than the microbial mat samples. Many of the OTUs were shared among all sample types in both results (**Figures 5 and 10**), and all the top OTUs were shared between the bat and the mat samples (**Tables 5 and 7**). In addition, both results showed significant differences in beta diversity among sample types (**Figures 2 and 8**). They also showed significant differences in beta diversity between the two caves. This suggests that where a sample is taken geographically (e.g. in separate caves) can impact the bacterial and fungal communities. This is consistent with other studies showing geographical impact on bat bacterial communities (Avena et al., 2016; Winter et al., 2017). This result, taken with our finding that there are many shared OTUs among the sample types in each data set, indicates that there are microbial communities potentially being exchanged between bats and the environments in which they roost. Although the sample types shared many OTUs, they were certainly not identical to each other. This indicates that there are certain microbial communities that are favored or regulated in different ways within their respective environments (i.e. the fur of a bat or the wall of a cave). Avena et al. (2016) and Lemieux-Labonté et al. (2017) looked at bat samples versus the environment and found similar findings to ours; however, they did not have fungal data in either of their studies. Our study illuminates the possibility of not only a sharing of bacterial communities between bats and their environment, but also fungal communities as well. We recognize that the sample size for this study is small, and in some instances, we could not

make robust statistical conclusions from the data, but the characterization of these samples will help guide and inform future research in this area.

The air samples in both the bacterial and fungal results had low species richness and diversity in comparison to the bat and mat samples (**Figures 1 and 7**). This is most likely because we only have four air samples in the data set and the swabs for these samples were only exposed to the air for a short period of time. For future studies, a laboratory-grade air sampler would be better suited for sampling the cave air, which has been done in other cave studies, such as those of Man et al. (2018) and Martin-Sanchez et al. (2014). Even though the air samples in this study had low sequencing depth, we kept them in the data set because they showed a potential impact on the bat samples' microbial communities in the bacterial SourceTracker results (**Figure 6**). This indicates that a more in-depth study with higher quality air samples could better illuminate the relationship between the air of a cave and the external microbiome of bats.

16S rRNA Gene Discussion and Implications

The rarefaction curves and the alpha diversity indices for bacterial diversity indicate that we had good sequencing coverage of the bat and microbial mat samples (**Figure 1**). Both bat and mat samples had high richness and diversity, with the exception of a few bat samples. Samples with low diversity did not have a very even mix of bacterial OTUs, meaning they may be dominated by just a few OTUs. Looking at the bacterial abundance and taxa diversity overall, there were evident differences in our results among the three types of samples. **Figure 2** demonstrates these differences in the clustering by sample type with respect to their beta diversity. In **Figure 2A**, the bat and mat samples appear to be clustered together, but

when the air samples are removed from the NMDS, there is a clear separation between the bat and mat samples (**Figure 2B**). From this finding it seems as though the air samples' species composition is so different from the bat and mat samples that in the multidimensional space the bat and mat samples are similar enough that differences are masked when air samples are included. Despite this finding, the adonis test showed that there was a significant difference between the bacterial communities of the bat and mat samples. These samples may be somewhat similar, but overall the bat samples tend to share their own similar homogeneity of bacterial communities, and mat samples also tend to share their own similar homogeneity of bacterial communities. Avena et al. (2016) also found a significant difference between the bat and environmental samples in their data.

Actinobacteria had the highest relative abundance in every sample overall (40.44%). From this result it can be inferred that Actinobacteria are a main component of bacterial communities in the cave environment, including bats that roost there. Actinobacteria was the dominant phylum in the bat samples (**Figure 3**), which is consistent with other studies of bacterial communities on bats (Lemieux-Labonté et al., 2016; Avena et al., 2016; Lemieux-Labonté et al., 2017; Winter et al., 2017). All of the studies mentioned above also found Proteobacteria, Bacteroidetes, and Firmicutes to all be in the top abundant phyla of the bats. Only Winter et al. (2017) found Acidobacteria to be in the top abundant phyla of bats, as we did in this study. Actinobacteria was not the dominant phylum in the mat samples. Since Actinobacteria have been shown to be prevalent and diverse in caves (Northup et al., 2011; Riquelme et al., 2015), we had hypothesized that the abundance would be higher if not equal to the abundance in the bat samples. More comparative samples would need to be taken in order to tell if this is a trend or a coincidence just observed in our data. Proteobacteria and

Acidobacteria were both significantly higher in the mat samples than the bat samples. Both of these phyla were found to be in the top abundant phyla in the lava caves studied by Lavoie et al. (2017). The phylum Firmicutes was in the highest relative abundance in the air samples. Martin-Sanchez et al. (2014) found that culturable bacteria of Firmicutes were in the top highest abundance of the samples they obtained from cave air, along with Actinobacteria and Proteobacteria. In addition, Newman et al. (2018) found four families of Firmicutes that predominated at all different depths in guano piles. Taken together, this could indicate guano as a potential source of Firmicutes in the air samples.

There are some actinobacterial genera that stand out in our data, *Rhodococcus*, *Arthrobacter*, *Rubrobacter*, and *Streptomyces* (**Figure 4**; **Table 5**). The genus *Rhodococcus* was significantly higher in relative abundance in the bats than in the mat samples. A species of *Rhodococcus*, *Rhodococcus rhodochrous*, has been cultured from bats and has shown to inhibit the growth of *P. destructans* (Cornelison et al., 2014; Hamm et al., 2017). Lemieux-Labonté et al. (2017) found that the microbiota of white-nose syndrome positive *Myotis lucifugus* bats was enriched by species of *Rhodococcus*. Although *Rhodococcus* spp. were not highly abundant in the mat samples, they have previously been isolated from cave samples (Groth et al., 1999: Hamedi et al., 2018). The genus *Rubrobacter* was in approximately the same amount in half of the bat and mat samples. *Rubrobacter* spp. have been found to be abundant in arid soils and have been found in 16S rDNA sequencing of cave samples (Holmes et al., 2000; Yasir, 2018). No study thus far has shown the presence of *Rubrobacter* spp. on bats. The genus *Arthrobacter* was significantly higher in relative abundance in the bats than in the mat samples. In Hamm et al. (2017), *Arthrobacter* spp. were isolated from the external microbiome of bats in the Southwest and tested for antifungal

activity against *P. destructans* but showed no inhibition activity against *P. destructans*. The genus *Streptomyces* was in relatively high abundance in every mat sample and was high in abundance in just four of the bat samples. *Streptomyces* is a diverse genus that has been shown to be abundant on bats and in caves (Groth et al., 1999; Hamm et al., 2017; Hamedi et al., 2018). In the study of Hamm et al. (2017), *Streptomyces* spp. were the most abundant bacteria isolated from bats, and many of the species were shown to inhibit the growth of *P. destructans* in vitro. Since *Streptomyces* spp. have been shown to be abundant on bats, the higher abundance of it on mats than bats in this study may indicate that the bats may be getting some of their helpful *Streptomyces* spp. from the caves in which they roost.

The bat and mat sample types share a large majority of their OTUs with each other, but they both still have many of their own unique OTUs. The air samples on the other hand share most of their OTUs with the bat and mat samples. This, however, does not indicate that the air samples shared these OTUs in the same portions, and what makes up the majority of the bacterial communities in the air samples may be completely different from the bat and mats. This is evident in **Figure 2** by how separated the air samples are from the bat and mat samples. SourceTracker results for the bacterial data show that the mat and air samples are potential sources for the bacterial communities on the bats (**Figure 6**). This further indicates the potential impact of the cave environment on the bacterial communities found on bats. This finding, although interesting, would greatly benefit from many more samples of both the bats and different parts of the cave environment and in different caves geographically to see if this potential impact of the microbial mats and air is still influential.

ITS Gene Discussion and Implications

The rarefaction curves and the alpha diversity indices for the fungal diversity indicate that further deep sequencing may be needed to attain a more comprehensive look at the fungal communities in caves and cave roosting bats (**Figure 7**). In addition to the species richness and diversity being low in air samples, as described above, the microbial mat samples also had low species richness and diversity as compared to the bat samples. This low alpha diversity may be attributed to one of two things. First, initial sequencing of the mat samples failed, and a nested PCR was needed to obtain sequences. Second, there may not be very much fungal diversity in cave microbial mats. Northup and Spilde (personal communication) have looked at lava cave microbial mats using a scanning electron microscope in caves all over the world and have found very few structures resembling fungi. In addition, Jurado et al. (2009) found that no fungal DNA could be amplified in certain areas of cave walls where bacterial colonization was high. Bacteria are very ubiquitous in caves, and lava caves have been shown to have extensive visible microbial mats on the cave walls and ceilings (Northup et al., 2011; Lavoie et al., 2017). It is possible that in the lava caves in this study the bacterial communities may be out competing the fungal communities on the microbial mats, possibly through the production of anti-fungal compounds. Thus, other sample types from the cave, such as soil around the roosting bats, may be better suited for a comparison of the fungal communities in caves and cave roosting bats.

Looking at the fungal abundance and taxa diversity of what was present in the microbial mats and air samples as compared with that of the bat samples, there were evident differences in our results between these three types of samples. First of all, **Figure 8** demonstrates these differences in the clustering by sample type with respect to their beta

diversity. The bat and mat samples clustered more closely to their respective sample types, which means the fungal communities overall within each sample type were more similar to each other than they were to samples of a different type. This, however, does not mean the sample types are completely different from each other, nor does it mean the samples within a type are exactly similar to each other. The ANOSIM results revealed that the samples by sample type were different with some overlap. This is exemplified in **Figure 9**, which shows some similar taxa in each sample type, but the sample types were generally different from one another with respect to their fungal taxa and abundances of those taxa.

Many of the fungal taxa found in this study are consistent with other studies of fungi on bats and studies of fungi in caves (Vanderwolf et al., 2013a; Vanderwolf et al., 2013b; Johnson et al., 2013; Vanderwolf et al., 2016; Man et al., 2018). There were only three fungal phyla identified in this data set, Ascomycota, Basidiomycota, and Mucormycota (**Figure 9A**). Vanderwolf et al.'s (2013a) world review of fungi in caves, found that Ascomycota tended to dominate the cave environment. Ascomycota were in high relative abundance in all the sample types. In addition, Basidiomycota was high in the air samples. Some of the high abundant Basidiomycota taxa in the air samples were in the Order Malasseziales (**Figure 9B**). Genera in this order are common inhabitants of human skin and ear mucosa of some animals (including bats) and can be opportunistic pathogens (Dall' Acqua Coutinho et al., 2006; Gandra et al., 2008; Findley et al., 2013). OTUs in this order were not found in higher than 1% relative abundance in any of the bat or mat samples. This may indicate that the Malasseziales taxa are a possible contamination of the air samples by human skin, since they are commonly found on human skin (Findley et al., 2013). More robust air samples could potentially alleviate this showing as a fungus in high abundance in the cave air. Unclassified

fungi made up a large percentage of the mat samples, which was due to three samples that were mainly composed of one to three OTUs and all three sample were from West cave. These OTUs made-up most of the abundance of unclassified fungi. These three OTUs may be new species of fungi that are unique to the cave microbial mats.

The bat samples showed much more diversity than the mat samples in the types of taxa found on them (**Figure 9C**). Many of the mat samples were dominated by just one to three fungal taxa. This is a further indication that the mat samples did not have good sequencing depth. Some families that stood out in the results, that do not dominate just one sample, were Cladosporiaceae, Pleosporaceae, Pseudeurotiaceae, Microascaceae, Leucosporidiaceae, and Mortierellaceae. These families have all been reported to be in caves or on bats, except Leucosporidiaceae (Vanderwolf et al., 2013a; Lorch et al., 2013; Johnson et al., 2013; Out et at., 2016). Leucosporidiaceae is a psychrophilic yeast (Watson et al., 1976), thus it makes sense that it was found to be abundant on samples taken in relatively cold caves (1.18 °C to 3.22 °C mean temperature in our study caves). Mortierellaceae and its genus *Mortierella* were present or prevalent in most of the samples (**Figure 9C**; **Table 7**). *Mortierella* spp. are psychrotolerant or psychrophilic and are primarily associated with soil, and in some cases plant and animal decay and insects in contact with soil (Domsch et al., 2007; Hassan et al., 2016). Interestingly, a species of *Mortierella, M. elongata*, has been shown to have bacterial endosymbionts (Fujimura et al., 2014; Uehling et al., 2017). We did not find that particular species of *Mortierella* in our data, but other species of *Mortierella* found in our data could potentially have bacterial endosymbionts. This may indicate that fungi that are able to persist in the caves may have symbiotic relationships with the very prevalent bacteria found there. Microascaceae were abundant in the five *Corynorhinus*

townsendii samples (bat samples; Pairs A, B, D1, D2, E). This finding is interesting because species in the family Microascaceae have been shown to produce antifungal and immunosuppressant secondary metabolites (Bills et al., 2013). This finding, in addition to the antimicrobial producing bacteria found on these bats may be a strong first line of defense against *P. destructans*. Pseudeurotiaceae and its genus *Pseudogymnoascus* were found in many of the bat samples and a few of the mat samples. One species of *Pseudogymnoascus* found in higher mean abundance on the bats than the mat samples is *P. roseus* (**Table 7**). Minnis and Linder (2013) found in their phylogenetic evaluation that *P. roseus* was the closest relative to *P. destructans*. The other OTU classified in the genus *Pseudogymnoascus* was higher in mean abundance in the mats than the bat samples and was not able to be classified to the species level. This *Pseudogymnoascus* sp. could potentially be a new species and a close relative to *P. destructans*.

Many of the bat OTUs (232) were not shared with either the mat or air samples and a majority of the mat and air sample OTUs were shared with each other and the bat samples (**Figure 10**). It is important to keep in mind that although the mat and air samples share a majority of their OTUs with the bat samples, it does not mean they are sharing them in the same proportions, which is evident from **Figure 9**. SourceTracker results for the fungal data is opposite from the bacterial data in this study, indicating that the mat and air samples were not potential sources for the fungal communities on the bats (**Table 8**). This result could be influenced by the fact that the mat and air samples had very low diversity and sequencing depth. It is possible that with better sequencing coverage and more samples that the environment in the cave (i.e. the walls or air) could show an impact on the bats that roost there.

Conclusions

To our knowledge, this is the first study to look at both bacterial and fungal data in a comparison study of the microbial communities in caves and on cave roosting bats using Illumina MiSeq sequencing. This study highlights the diversity of microbial communities in cave ecosystems and the importance of understanding the influence of the environment on a bat's microbiota. We found that the bats and their environment shared many microbial OTUs but were still different in their respective overall microbial communities. In addition, SourceTracker suggested bat bacterial communities may originate from mat and air samples. We also identified microbes in both the bats and the microbial mats that have the ability to produce antimicrobials, further highlighting the importance of microbial communities to bat health. Further investigation and research into these defenses and how they might be acquiring or sharing these defenses with their environment is needed. For the bacterial side, Actinobacteria were the most abundant overall but higher in the bats than the mats. For the fungal side, the bats had more fungal diversity than the mat samples, showing the need for alternative sample types to reflect the fungal communities in the cave environment. More samples from all types of environments in and around the caves may better illuminate the relationship between bats and their environment for both bacterial and fungal diversity. For both the bacterial and fungal data, some trends emerged that showed differences in the microbial communities among bat species. The results of this study contribute to the first of many steps that need to be taken to fully understand how microbial communities in caves support the comprehensive health of cave-roosting bats. The bacterial and fungal communities characterized in these results could potentially show that knowing the source of

natural defenses for bats against certain pathogens like *P. destructans* is valuable for forecasting which species of bats are more likely to be negatively impacted by WNS.

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