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Evaluation of DESS as a storage medium for microbial community analysis

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Microbial ecology research requires sampling strategies that accurately represent the microbial community under study. These communities must typically be transported from the collection location to the laboratory and then stored until they can be processed. However, there is a lack of consensus on how best to preserve microbial communities during transport and storage. Here, we evaluated DESS (Dimethyl sulfoxide, Ethylenediamine tetraacetic acid, Saturated Salt) solution as a broadly applicable preservative for microbial ecology experiments. We stored fungus gardens grown by the ant *Trachymyrmex septentrionalis* in DESS, 15% glycerol, and phosphate buffered saline (PBS) to test the ability of these preservatives to maintain the structure of fungus garden microbial communities. Variation in microbial community structure due to differences in preservative type was minimal when compared to variation between ant colonies. Additionally, DESS preserved the structure of a defined mock community more faithfully than either 15% glycerol or PBS. DESS is inexpensive, easy to transport, and effective in preserving microbial community structure. We therefore conclude that DESS is a valuable preservative for use in microbial ecology research.

Evaluation of DESS as a storage medium for microbial community analysis

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Abstract

Microbial ecology research requires sampling strategies that accurately represent the microbial community under study. These communities must typically be transported from the collection location to the laboratory and then stored until they can be processed. However, there is a lack of consensus on how best to preserve microbial communities during transport and storage. Here, we evaluated DESS (Dimethyl sulfoxide, Ethylenediamine tetraacetic acid, Saturated Salt) solution as a broadly applicable preservative for microbial ecology experiments. We stored fungus gardens grown by the ant *Trachymyrmex septentrionalis* in DESS, 15% glycerol, and phosphate buffered saline (PBS) to test the ability of these preservatives to maintain the structure of fungus garden microbial communities. Variation in microbial community structure due to differences in preservative type was minimal when compared to variation between ant colonies. Additionally, DESS preserved the structure of a defined mock community more faithfully than either 15% glycerol or PBS. DESS is inexpensive, easy to transport, and effective in preserving microbial community structure. We therefore conclude that DESS is a valuable preservative for use in microbial ecology research.

Introduction

Microbial ecologists frequently use culture-independent methods to study the structure of microbial communities (Hugerth and Andersson, 2017). Such experiments face many design challenges that must be considered so that data they produce matches the structure of the original microbial community (Vandeputte et al., 2017; Pollock et al., 2018). Sample collection, handling, and storage are the first steps in any culture-independent study, and decisions made during this phase of a study can strongly affect downstream analyses (Hugerth & Andersson, 2017; Vandeputte et al., 2017). Ideally, DNA should be extracted from samples immediately following collection (Rochelle et al., 1994; Cardona et al., 2012). However, sampling typically occurs outside of the laboratory and samples can therefore experience prolonged and sometimes poorly controlled transportation and storage conditions that permit nucleic acid degradation and/or microbial growth (Amir et al., 2017). Either of these processes will alter the structure of the sampled microbial community such that obtained experimental data does not match the original structure of that community.

Cold storage (at -80°C, -20°C, or 4°C, in order of preference) is the accepted gold standard for protecting samples from potentially adverse conditions that compromise microbial community structure (Rissanen et al., 2010; Williamson et al., 2011;; Choo, Leong & Rogers, 2015; Hale et al., 2016; Kia et al., 2016; Song et al., 2016, but see also Bahl, Bergström & Licht, 2012). One disadvantage of cold storage is that cold chains are fragile, especially when collection sites are remote (Vandeputte et al., 2017). Electrically powered cooling requires substantial and robust physical infrastructure. Alternatively, refrigerants such as ice, dry ice, and liquid nitrogen can be difficult to obtain, must be refreshed routinely, and (in the case of dry ice and liquid nitrogen) are regulated or prohibited on many modes of transportation. These issues limit the deployment and robustness of cold storage for preserving microbial community samples, despite its effectiveness.

Many studies have used preservative media as an alternative solution to overcome the logistical difficulties of cold transport and storage. These include proprietary media such as DNAgard, RNAlater, OMNIgene.GUT, and LifeGuard, and less-expensive non-proprietary media such as Tris-EDTA, ethanol, and phenol-chloroform (Rissanen et al., 2010; Gaither et al., 2011; Gray, Pratte & Kellogg, 2013; Tatangelo et al., 2014; Choo, Leong & Rogers, 2015). As with cold storage, these buffers attempt to preserve the structure of the microbial community being sequenced. Many studies have compared the effectiveness of preservative media for a variety of sample types (Rissanen et al., 2010; Vlčková et al., 2012; Gray, Pratte & Kellogg, 2013; Tatangelo et al., 2014; Choo, Leong & Rogers, 2015; Anderson et al., 2016; Blekhman et al., 2016; Amir et al., 2017; Vogtmann et al., 2017), and typically conclude that most preservatives alter microbial community structure to a greater or lesser degree. Some preservatives such as ethanol and phenol-chloroform are also hazardous and therefore face travel restrictions, limiting their use for field collections. There is therefore an ongoing need to identify storage media that are effective, inexpensive, and well-suited to field collections.

Dimethyl sulfoxide (DMSO)-ethylenediamine tetraacetic acid (EDTA)-saturated salt solution (DESS, also known as SSD) is a non-proprietary, non-hazardous storage medium that shows strong potential for use as a preservative in microbial ecology studies. In this medium, DMSO permeates cells and facilitates the rapid entry of EDTA that suppresses nuclease activity by chelating divalent cations. Sodium chloride salt further suppresses enzymatic activity and contributes sodium ions that stabilize the negatively charged phosphate backbone of DNA (Seutin, White & Boag, 1991). DESS was first used to preserve avian blood samples by Seutin et al. (1991) and has since been used to preserve the morphology and DNA of nematodes (Yoder et al., 2006), corals, and small marine invertebrates (Gaither et al., 2011). DESS did not strongly alter microbial community structure when used to preserve mock microbial communities created from environmental isolates (Gray, Pratte & Kellogg, 2013) or soil and water samples, with and without cold storage (Tatangelo et al., 2014). Other DMSO-based preservatives have similar properties (Kerckhof et al., 2014). DESS therefore shows promise as a broadly applicable preservative that can overcome the fragility or lack of cold chains during sample transport between collection sites and the laboratory.

Here, we evaluated DESS as a preservative using the microbial communities found in fungus gardens grown by the ant *Trachymyrmex septentrionalis* as a model system studied by our research group. We typically collect these samples in hot and humid locations that are far from the lab, meaning that our cold chain is susceptible to failure. We further validated our field-based observations using a mock microbial community with a defined structure. Our results suggest that DESS is an excellent preservative of microbial community structure that is useful for field collections where cold transport and storage are challenging.

Materials and Methods

Sample Collection

T. septentrionalis colonies were collected in New Jersey, Florida, and North Carolina during 2014 and 2015. Permits for collecting samples were obtained from the corresponding state department:

State of New Jersey Department of Environmental Protection Division of Parks and Forestry State Park Service Unnumbered Letter of Authorization; North Carolina Division of Parks and Recreation Scientific Research and Collecting Permit 2015_0030; Florida Department of Agriculture and Consumer Services unnumbered Letter of Authorization. Colonies were identified by their distinctive "half-moon" mound shape and the presence of *T. septentrionalis* worker ants. An initial ~25cm deep trench was dug beside the colony entrance and then expanded until the fungus garden chamber was gently breached. After expanding this opening, the fungus garden was removed using a flame-sterilized spoon. Fungus gardens were naturally homogenized during collection by crumbling due to their fragility. Approximately 200mg of each fungus garden was subsampled into DESS (20% DMSO (v/v), 250mM EDTA, saturated with sodium chloride), 15% (v/v) glycerol, or PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH = 7.4), frozen immediately on dry ice, and then transferred to -80°C storage upon return to the laboratory. Glycerol was chosen for comparison based on its potential use as a cryoprotectant that would allow cells to be cultured upon return to the lab. PBS served as a baseline that was not expected to facilitate sample preservation beyond maintaining osmotic balance.

Sample Processing

***T. septentrionalis* fungus gardens:** Samples were warmed to -20°C overnight and then thawed at 4°C just prior to processing (2-5 hours). Replicate subsamples were pooled to obtain ~1g wet mass of fungus garden (equivalent to 250-500mg dry mass). Preliminary experiments using DNA extracted directly from *T. septentrionalis* fungus garden inconsistently amplified 16S rRNA genes using PCR (data not shown). This was likely due to the high amount of fungal biomass in these samples compared to bacterial biomass and the presence of inhibitory chemical compounds (Ishak et al., 2011). We therefore adapted the protocol of (Apajalahti et al., 1998) to enrich for bacterial cells prior to DNA extraction, using medium speed vortexing to homogenize samples instead of rotational shaking and a final centrifugation at 15,000g for 15 minutes instead of 30,000g to pellet bacterial cells. DNA was extracted from the resulting cell pellets using a CTAB/bead beating protocol modified from Cafaro & Currie, 2005 by using three two-minute cycles of bead beating (Biospec Minibeadbeater) separated by 2.5 min cooling on ice for cell lysis, and 24:1 chloroform:isoamyl alcohol for DNA extraction. DNA extracts were resuspended in nuclease-free water and quantified spectrophotometrically using a BioSpec EON plate reader with a Take3Trio plate. Negative DNA extraction controls that contained water instead of sample were included alongside each extraction batch. DNA extracts with $A_{260/280}$ or $A_{260/230}$ ratios ≤ 1.5 were purified using Agencourt XP magnetic beads (Beckman Coulter) following the manufacturer's protocol.

Mock Community: A ZymoBIOMICS Microbial Community Standard (Zymo Research Corporation, comprised of five Gram positive bacteria, three Gram negative bacteria, and two fungi – hereafter the "mock community") was split into 18 equal aliquots. Each aliquot was centrifuged at 5000g for 5 minutes to pellet the cells. The supernatant containing the proprietary Zymo preservative was removed and the cells were resuspended in 1mL of DESS, PBS, or 15% glycerol by vortexing. DNA was extracted from two samples resuspended in each preservative as described above either immediately (before freezing; t_0) or after one (t_1) or two (t_2) months of storage at -80°C. These DNA extracts were quality checked as described above but none required

magnetic bead cleaning. Negative controls were not used for this experiment because of the known composition of the mock communities.

PCR Screening: All quality-checked DNA extracts were screened by PCR amplification of the V4 region of the 16S rRNA gene before sequencing on an Illumina MiSeq. Each 25µl reaction used 1X GoTaq reaction buffer (Promega), 0.3µM each of primers 515F and 806R (Caporaso et al., 2011; Invitrogen), 1.25 units of GoTaq DNA polymerase (Promega), 300ng/µl bovine serum albumin (New England BioLabs), 50ng of template DNA, and nuclease-free water (Thomas Scientific). PCR reactions were run on a T-100 Thermal Cycler (BioRad) for 3 min at 95°C, 30 cycles of: 30 sec at 95°C, 30 sec at 50°C, and 60 sec at 72°C, followed by a single 5 min cycle at 72°C. Bands were visualized using agarose gel electrophoresis. PCR reactions lacking the expected 350bp product were re-cleaned with magnetic beads as described above and screened by PCR a second time.

Community Amplicon Sequencing

Quality-checked, PCR-screened DNA extracts were submitted to the University of Connecticut Microbial Analysis, Resources, and Services (MARS) facility for sequencing. Submitted DNA samples were quantified fluorometrically using a PicoGreen (Invitrogen) assay in 384-well plates read on a Synergy plate reader (BioTek). After quantification, 30ng of sample DNA was added to 1x Phusion High Fidelity master mix (New England Biolabs) containing 1µM indexed sequencing primers with Illumina adapters (Kozich et al., 2013); Invitrogen), 4nM non-Illumina primers (515F/806R; Caporaso et al., 2011; Invitrogen) to a final volume of 50µl. These reactions were split into three equal aliquots and PCR amplified using settings: 94°C for 3 minutes initial denaturation, 30 cycles of 94°C for 45 seconds denaturation, 50°C for 1 minute annealing, 72°C for 1.5 minutes extension, 72°C for 10 minutes final extension. These PCR reactions were re-pooled and quantified using a QIAxcel instrument (Qiagen). Sample libraries with a PCR product concentration > 0.5ng/µl and peak(s) at 400bp (+/- 15%) were pooled by adding equal masses of PCR product from each sample. These pooled libraries were cleaned using Mag-Bind RXNPure Plus beads (OMEGA bio-tek), resuspended in 25µl molecular biology grade water, quantified using a Qubit assay (Invitrogen), and adjusted to 4nM (1.5ng/µl when using MARS adaptors). Amplicon libraries were diluted to 6pM in Illumina HT1 buffer with 30% PhiX phage DNA added and sequenced using a V2 (2x250) cartridge on an Illumina MiSeq instrument.

Bioinformatic Analysis

16S rRNA gene sequences were processed in R v3.4.3 (R Core Team, 2017) using the DADA2 v1.7.0 pipeline (Callahan et al., 2016) following the guidelines at https://benjjneb.github.io/dada2/tutorial_1_7.html (accessed 7/2/2018). Processed sequences and accompanying metadata were imported into phyloseq v1.22.3 (McMurdie & Holmes, 2013) and screened for potential contaminants using decontam v0.20.0 (Davis et al., 2017) using the decontam "prevalence protocol" with the P* threshold set to 0.5. Amplicon Sequence Variants (ASVs) that were not classified as bacteria were removed, as were bacterial sequences that were not classified to at least the phylum level. Decontam identified 36 fungus garden ASVs as contaminants (ranging from 0-10.2% of the sequences in each sample, mean 1.4%), which were

removed from this dataset. After excluding four samples with low read counts, the final fungus garden dataset contained 26 samples from 10 *T. septentrionalis* colonies (minimum: 11,379 reads; mean: 46,125 reads). Instead of using decontam, seven ASVs (ranging from 0.4 – 3.4% of the sequences in each sample, mean 1.6%) were manually detected and removed from the mock community dataset because they did not match known members of that community. All 16 mock community samples were included in the final mock community dataset (minimum: 24,891 reads; mean: 36,445 reads). The raw sequence data have been deposited in the National Center for Biotechnology Information database under BioProject ID PRJNA479679.

The resulting phyloseq-compatible R data object was subsampled to the lowest read count among all libraries and ASV counts were converted to relative abundances. Weighted UniFrac distances were calculated, ordinated, and visualized using the distance, ordinate, and plot_ordination functions in phyloseq, respectively. Phyloseq was also used to calculate Shannon's diversity, Chao1 diversity, and the observed diversity metrics using the estimate_richness function. Permutational Analysis of Variance (PERMANOVA) tests were done using Adonis in the vegan R package v2.4-5 (Oksanen et al., 2017). The aov, kruskal.test, and TukeyHSD commands implemented in the base R package were used to perform Analysis of Variance (ANOVA), Kruskal-Wallace and Tukey's Honest Significant Difference testing, respectively. The code and metadata files to reproduce these analyses are available in Supplemental File 1: DESS_manuscript_code_and _metadata.zip.

Results

T. septentrionalis fungus gardens

T. septentrionalis ants tend symbiotic fungus gardens as their food source, which also host distinct bacterial microbiomes. Our taxonomic profiles of *T. septentrionalis* fungus garden microbial communities suggest these communities are highly variable, regardless of preservative (Figure 1A). Of the 10 sampled fungus gardens, four were dominated by Firmicutes, two were dominated by Proteobacteria, and one was dominated by Planctomycetes. The remaining three colonies had high amounts of both Firmicutes and Proteobacteria (Figure 1A). Acidobacteria, Actinobacteria, Verrucomicrobia, and (except for colony JKH095) Planctomycetes had low abundances in most fungus gardens.

The type of preservative in which samples were stored only minimally correlated with variation in microbial community structure. This can be seen in the PCoA of Weighted UniFrac distances, where samples from the same colony group together but samples stored in different media are distributed throughout the plot (Figure 1B). Colony of origin accounted for >75% of the between-sample variation in β-diversity (PERMANOVA, $F = 52.496$, $R^2 = 0.799$, $p = 0.001$) with storage medium accounting for a further ~4% of this variation (PERMANOVA, $F = 11.78$, $R^2 = 0.039$, $p = 0.006$) that was linked to colony of origin (colony-by-storage medium interaction: PERMANOVA, $F = 8.37$, $R^2 = 0.156$, $p = 0.010$). Weighted UniFrac distances between samples from the same colony that were stored in different preservatives did not significantly differ from each other (Kruskal-Wallace, $Df = 2$, $\chi^2 = 4.779$, $p = 0.090$), implying that one preservative type did not alter microbial community structure more than the others. Like

β-diversity, α-diversity did not vary between preservative types (Figure 1C; Kruskal-Wallace, Shannon: Df = 2, $\chi^2 = 0.934$, p = 0.627; Chao1 and Observed: Df = 2, $\chi^2 = 1.127$, p = 0.569). Collectively, these data indicate that preservative type had at most a minor effect on the observed structure of *T. septentrionalis* fungus garden microbial communities.

Mock Community Samples

Our experiments with *T. septentrionalis* fungus gardens suggested that preservative type may have a small effect on community structure. However, the lack of a reference standard prevented us from determining which samples best represented the true structure of *T. septentrionalis* fungus garden microbial communities and which represented artificial community structures that were biased by the effects of storage. We therefore used a mock community of known composition to isolate the effect of preservative on microbial community structure. Storage at -80°C for 1 or 2 months changed the relative abundances of all taxa in all preservatives when compared to the corresponding unfrozen (t_0) samples (Figure 2A). The PCoA of the Weighted UniFrac distances showed three distinct clusters, containing either: (1) all t_0 samples; (2) t_1 and t_2 DESS samples; or (3) t_1 and t_2 glycerol and PBS samples (Figure 2B). Variation in mock community structure correlated weakly with preservative type (PERMANOVA, $F_{(2,17)} = 163.8$, $R^2 = 0.133$, p = 0.001). Instead, variation in mock community structure correlated strongly to storage time (PERMANOVA, $F_{(2,17)} = 977.5$, $R^2 = 0.794$, p = 0.010). However, the mean Weighted UniFrac distances between t_1 and t_2 samples frozen in the same preservative were very small (DESS = 0.054, glycerol = 0.055, PBS = 0.036) and not significantly different from each other (ANOVA, $F_{(2,18)} = 1.959$, p = 0.175). These small distances imply that "time" actually represents changes induced by freezing and/or any amount of frozen storage.

Mock community samples frozen in DESS changed less during storage at -80°C compared to the samples that were stored in either PBS or glycerol (Figure 2C). *Bacillus*, *Enterococcus*, *Listeria*, and *Staphylococcus* were all overrepresented in mock communities stored in all preservatives relative to the t_0 baseline samples. Similarly, *Escherichia*, *Lactobacillus*, *Pseudomonas*, and *Salmonella* were all underrepresented in mock communities stored in all preservatives relative to the t_0 baseline samples. The relative abundance of six out of eight taxa differed from the t_0 baseline samples less when frozen in DESS compared to glycerol and PBS (Figure 2C). The exceptions were *Lactobacillus,* whose relative abundance changed equally in all preservatives, and *Bacillus*, whose relative abundance changed more in DESS compared to the glycerol and PBS. Changes in taxon relative abundance compared to the t_0 baseline did not differ between taxa stored in glycerol or PBS (Tukey's Honest Significance with Bonferroni correction, p > 0.050), except for *Staphylococcus* (Tukey's Honest Significance with Bonferroni correction, p = 0.016). Collectively, these data show that the structure of mock community samples stored in DESS were more similar to that of unfrozen t_0 baseline samples compared to samples stored in glycerol or PBS.

Discussion

T. septentrionalis fungus garden microbial communities were more diverse than what has been reported for fungus gardens from related ant species. Fungus-growing ants from the genera *Atta*

and *Acromyrmex* raise fungus gardens that contain low microbial diversity and are dominated by Gammaproteobacteria, as are fungus gardens grown by the only other *Trachymyrmex* species studied thus far, *T. zeteki* (Suen et al., 2010; Aylward et al., 2012, 2014) However, our results agree with the limited data available for other *T. septentrionalis* fungus gardens (Ishak et al., 2011) and some other ant genera (Kellner et al., 2015). These differences could be due to species-specific differences in habitat and foraging behavior (De Fine Licht & Boomsma, 2010), which might lead to different microbes entering fungus gardens via different substrates. Understanding how these dynamics contribute to the diversity of the *T. septentrionalis* fungus garden microbial community is an active and ongoing area of research in our lab.

Preservative type had only a small impact on *T. septentrionalis* fungus garden microbial community structure. The ~4% of variation in fungus garden microbial community structure attributed to preservative by PERMANOVA was dwarfed by the >75% of variation attributed to the colony of origin (Figure 1B). Preservative type also did not correlate with changes in the alpha-diversity of *T. septentrionalis* fungus garden microbial communities (Figure 1C). This minimal effect of preservative parallels other studies where between-host differences in microbial community structure greatly exceeded differences caused by variation in storage strategy (Bai et al., 2012; Hill et al., 2016; Song et al., 2016).

Because our *T. septentrionalis* fungus gardens were all frozen in the field, we lack a reference community against which to compare the slightly different microbial community structures of the fungus gardens stored in DESS compared to those stored in glycerol or PBS. We therefore used a mock community to more precisely assess changes in microbial community structure caused by each preservative. In these experiments, all mock communities changed relative to t_0 samples, likely indicating either a consistently negative effect of storage for 1-2 months at -80°C (Bahl, Bergström & Licht, 2012; Kia et al., 2016) and/or the consistently negative effect of the additional freeze/thaw cycle undergone by the t_1 and t_2 samples but not the t_0 samples (Cardona et al., 2012; Gorzelak et al., 2015). Four of the five Firmicutes strains in the mock community were overrepresented after storage at -80C. Such increases in Firmicutes following cold storage has been reported previously (Bahl, Bergström & Licht, 2012; Anderson et al., 2016; Hill et al., 2016; Song et al., 2016). However, these changes were less severe for samples stored in DESS compared to those stored in glycerol or PBS, confirming previous results (Gray, Pratte & Kellogg, 2013; Tatangelo et al., 2014). These results suggest that changes caused by storage are less severe for samples stored in DESS compared to other preservatives.

Conclusions

In summary, our results suggest that DESS is an excellent storage medium for microbial ecology samples. Storage in DESS did not obscure the ecological differences between the microbial communities of fungus gardens collected from different ant colonies. DESS also preserved the community structure of a mock community more faithfully than either glycerol or PBS. Future research should focus on how microbial community structure changes when samples stored in DESS are repeatedly frozen and thawed, as might occur in a broken cold chain. DESS is inexpensive and non-hazardous, making it easy to transport. Together, these attributes suggest

that DESS is a versatile and economical preservative that is suitable for the transport and storage of microbial communities.

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Figure 1(on next page)

Preservative type does not alter the community structure of *T. septentrionalis* fungus gardens

A) Relative abundances of phyla in *T.* septentrionalis fungus gardens, grouped by colony. Phyla with relative abundances ≤ 15% in all samples are grouped into a single "Other" category. B) PCoA of Weighted UniFrac distances between *T. septentrionalis* fungus garden bacterial communities. Colors indicate preservative types, and shapes indicate samples from different colonies. C) Alpha-diversity (Shannon's, Observed, and Chao1) in *T. septentrionalis* fungus gardens. Center bars in the box plot indicate the median, top and bottom of boxes indicate the 25^{th} and 75^{th} quartile, respectively, and whiskers indicate +/- 1.5 times the standard error. Single points represent outliers.

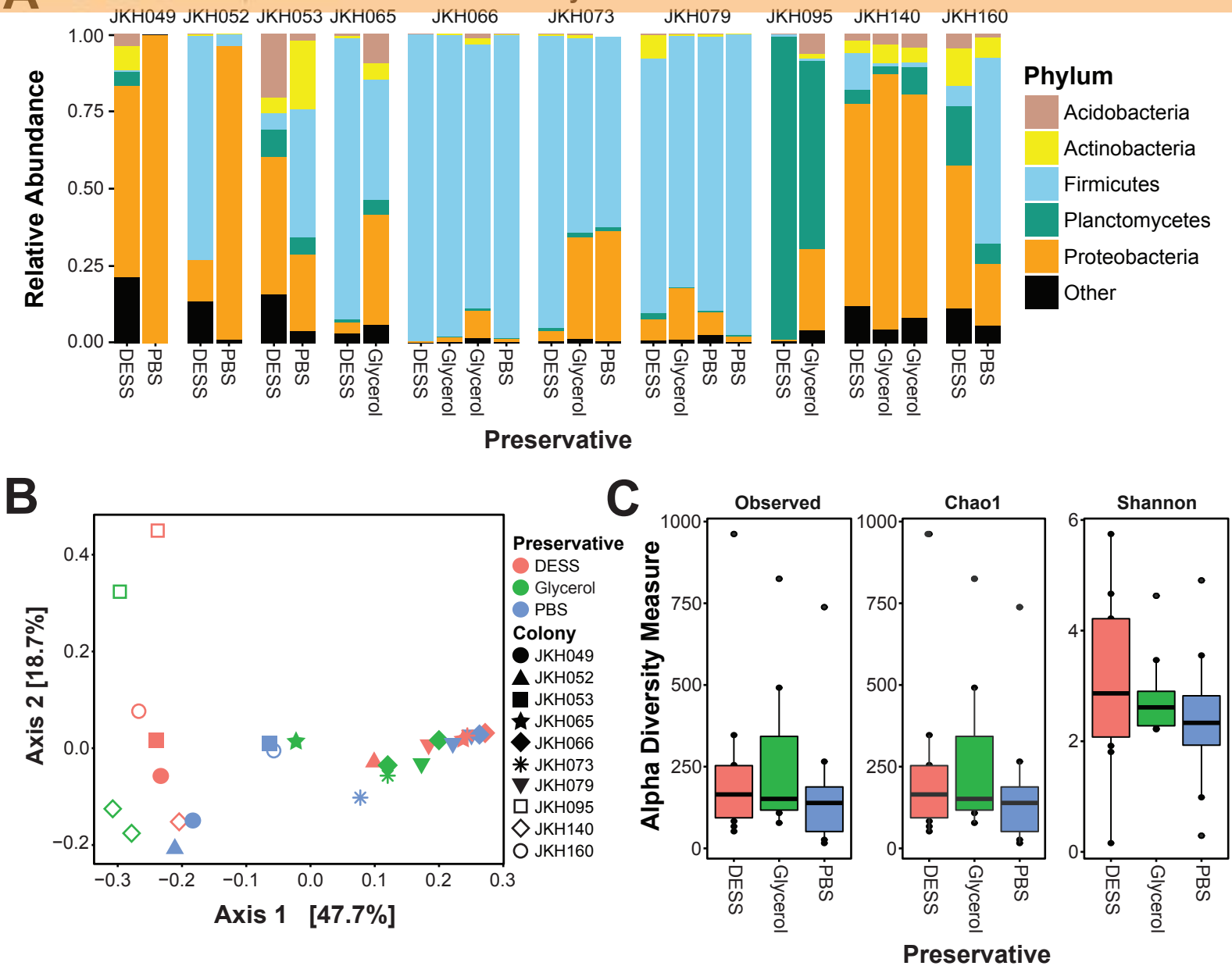

Figure 2 (on next page)

DESS preserves microbial mock community structure better than PBS or glycerol

A) Relative abundances of genera in mock community samples. B) PCoA of Weighted UniFrac distances between mock communities stored in DESS, PBS, or glycerol, indicated by different colors, for 0, 1, or 2 months, indicated by different shapes. C) Heatmap showing $\log_2$ fold changes in the relative abundance of genera in the mock community compared to the t_0 samples. Asterisks (*) indicate Bonferroni corrected p-values (* = $p < 0.01$, *** = $p < 0.001$)