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Research Article

Evaluation of fecal matter preservation methods and their effect on composition of microbiota assessed through DNA sequencing and analysis

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Abstract. Fecal matter transplants (FMT) are an effective, yet underutilized, treatment for potentially life-threatening *Clostridium difficile* infections. Following antibiotic treatment, an imbalance between the types of colonic microbiota naturally present in a person's gut may occur, allowing the opportunistic pathogenic bacterium *C. difficile* to proliferate and reach virulent levels. Despite a 90% success rate, and patient reports of immediate improvement, FMTs are approved only as a last resort due to strict US Food and Drug Administration (FDA) restrictions. This study aimed to compare microbial preservation methods to determine the method with the least detrimental effects on the composition of stool microbes. Fecal matter samples, from dogs, were homogenized with either sterile deionized water or 0.85% NaCl. The homogenized mixtures were then partitioned for immediate DNA extraction or for preservation with or without 25% glycerol prior to -80°C storage. After 3 weeks, and again after 10 months, DNA extraction was performed on stored samples. All extracted DNA was subjected to PCR amplification and sequenced. After pairing and filtering, 88.3% of data were retained. Changes in taxa richness over time for each treatment were not significant; changes in taxonomic composition over time were detected in water only and saline only treatments ($p = 0.016$, $p = 0.049$ respectively). The water-glycerol treatment resulted in the least amount of change in taxonomic composition and proportions when compared to the sample prior to preservation. Information gained from this study could be used to further improve FMTs and help fuel FMT related research in hopes of attenuating FDA restrictions.

Introduction

The human body is covered both externally and internally with an estimated 100 trillion microbial cells, which is ten times as great as the number of human cells (Qin et al., 2010). The region on the body where most microbes reside is the gut (Qin et al., 2010). These microbes establish relationships with their human hosts that may be commensal, ammensal, mutualistic, parasitic, and/or pathogenic (Liang et al., 2018). The interactions between hosts and their microbes is complex. Due to coevolution, many of the activities that are essential for the host such as metabolism, detoxification, immune system maturation, and disease mediation involve these microbes (Liang et al., 2018).

Within the mammalian gut, a great diversity of microbes can be found. Firmicutes and Bacteroidales represent roughly 90% of the currently identified microbes present in the gut (Liang et al., 2018). A study utilizing meta-

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genomic sequencing of gut microbes found evidence that, although there is a high degree of variability between individuals, there may be an underlying core community (Qin et al., 2010). The microbes present in the gut are under constant selective pressure from the host as well as other microbes, usually resulting in a homeostastic community in which there are species that occur in high abundance and other species that occur in low abundance (Le Chatelier et al., 2013). An alteration of this natural balance, which can be induced by antibiotic therapy, a diet change, environmental effects, and other causes, can lead to disease.

Antibiotic treatment rapidly alters the composition of the gut microbiota. This may lead to a dysbiosis or imbalance of gut microbes, which can lead to an infection of *Clostridium difficile* that is characterized by life-threatening diarrhea (CDC, 2019). This hospital acquired infection affects nearly 500,000 patients in the United States each year, with roughly 29,000 deaths resulting in the 30 days following a diagnosis (Shogbesan et al., 2018). *C. difficile* infections are a substantial cause of infectious disease death in the United States and place a great burden on the healthcare system. According to the Mayo Clinic, antibiotics continue to be the standard treatment for *C. difficile* even though 25% of patients suffer a recurrent *C. difficile* infection within 60 days of initial treatment (Shogbesan et al., 2018). Recurrence approaches 60% for those treated after a third episode of *C. difficile* (Ramai et al., 2018).

Fecal Matter Transplantation (FMT) as a mechanism for gut diversity restoration has emerged as an effective alternative treatment with a success rate of 80-90% for patients with recurrent disease after initial treatment with antibiotics (Shogbesan et al., 2018). The Food and Drug Administration (FDA) considers fecal microbiota transplantation an investigational drug, but permits physicians to utilize them upon obtaining informed consent (OpenBiome, 2018). The FDA places no restrictions on the route of delivery and does not require donors to be known (OpenBiome, 2018). Stool banks, such as OpenBiome, assess donors through a 200-question Clinical Evaluation, serological testing, and stool-based assays that screen for infectious pathogens (OpenBiome, 2018b). The current storage protocol for OpenBiome requires samples to be stored in a glycerol buffer (i.e., 12.5% glycerol and a 0.90% w/v NaCl in water) at -80°C (OpenBiome, 2018b). There is no bacterial analysis or culturing involved following storage of the sample, thus it remains unknown if the stool sample is affected by the preservation.

This study aimed to evaluate fecal matter preservation methods and their effect on composition of microbiota to determine the least detrimental preservation method. Information gained from this study could be used to further improve FMTs as well as to fuel FMT related research in the hopes of attenuating FDA restrictions.

Materials and Methods

Fecal matter samples were donated to the lab by dogs (under 40 lbs.) for this study. All donated samples were from pets and naturally passed fecal samples collected in accordance with standard practices. Samples were combined and weighed, resulting in 80.57 g worth of original untreated sample. The sample was divided in half and homogenized. A flow chart demonstrating the partitioning of samples has been provided (Fig. 1). One half of the samples were homogenized by adding 120 mL of 0.85% NaCl to 40.0 g of fecal matter. Vortex beads were added to the sample container and the sample was vortexed for 20 min. Samples were left to settle for 10 min. Five mL of 0.85% NaCl was added to wash the walls of the container as the sample was poured through a U.S.A. Standard Testing SIEVE A.S.T.M. E-11 Specification (opening micrometer 250, No. 60, opening in inches 0.0098) to aid in filtering of large matter. This homogenization process was repeated with sterile deionized water for the other half of sample. The homogenized mixtures were then partitioned either for immediate DNA extraction or for preservation with or without 25% glycerol prior to -80°C storage. Tubes were removed after 3 weeks and after 10 months to perform DNA extraction.

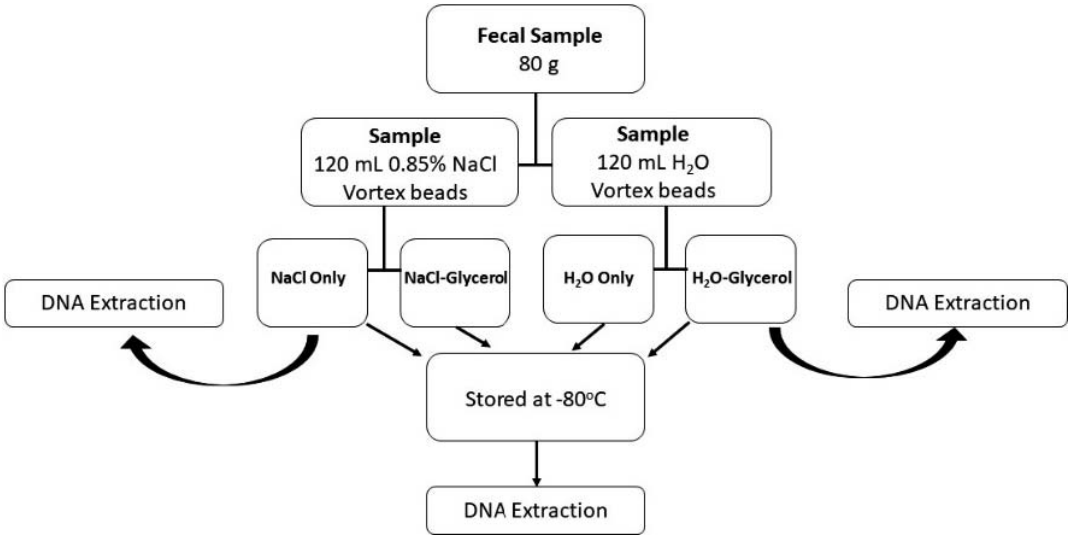

Figure 1. Flow chart demonstrating methodology of canine stool sample partitioning and treatment. Sample treatments were set up in a 3:1 ratio. For example, NaCl Only was 3 parts sample and 1 part 0.85% NaCl, whereas NaCl-Glycerol was 3 parts sample and 1 part glycerol. DNA extractions were performed immediately on NaCl Only and H_2O Only treatments. After storage at -80°C, DNA extractions were performed on all 4 of the treatments.

DNA extraction was performed using the Biostatic Bacteremia DNA Isolation Kit (Qiagen, Germantown, MD) experienced user protocol - extraction of bacterial DNA from cultured blood. Q-Bit was used to assess DNA levels in samples before performing PCR. The V4 region of the 16S ribosomal RNA was amplified via PCR (PTC-200 Peltier Thermal Cycler, Bio-Rad Laboratories, Inc., Hercules, CA) and sequenced using Illumina sequencing performed by Wright Labs, LLC (Huntingdon, PA). Quantitative Insights into Microbial Ecology open-source bioinformatics platform (QIIME) was used to analyze sequence data. Cyberduck and Putty were used to pair reads, perform quality filtering, perform metadata organization/creation, cluster Operational Taxonomic Units (OTUs), remove chimeras, assign taxonomy, as well as perform alpha and beta analyses and comparisons.

To measure alpha diversity, within sample variation, OTU tables were rarified creating a series of subsampled OTU tables. For subsampling, the minimum number of sequences per sample was set at 500 and the step number was set to 6,000 sequences per sample. Beta diversity, a measure of diversity across samples, was examined using a Principle Coordinate Analysis (PCoA) with weighted Unifrac distances. A non-parametric multivariate statistical test, analysis of variance using distance matrices (ADONIS), was used to determine significance between sample times for each treatment.

Results

Data quality and filtering

There was a total of 682,408 reads with an average length of 252.2 base pairs. Phred scores were generated to assess quality of data (Fig. 2). The samples were truncated at 249 bp and average expected error of 0.5%, which retained 602,305 reads (88.26%) of the original reads. After filtering, data was clustered into OTUs, chimeras were removed, and taxonomy was assigned.

Alpha diversity

The data were collated, and rarefaction plots were generated for observed species (Fig. 3). All rarefaction plots (Fig. 3), with the exception of the water only treatment, demonstrated a levelling out as the species per sample

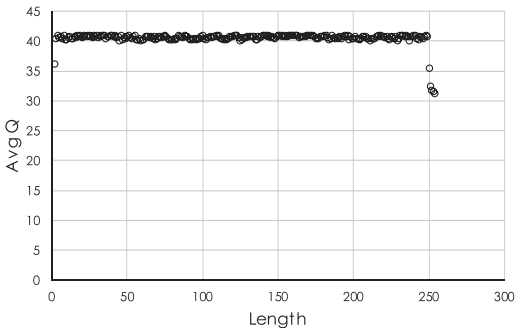

Figure 2. Average Phred quality score determines the probability of a correct base. A Phred score of 30 represents a 99.9% probability that the base is correct, while a Phred score of 40 represents a 99.99% probability that the base is correct. Cut-off made at 249 base pairs.

increased, indicating sufficient depth of sampling. Based on the population parameters, nonparametric Wilcoxon Rank Sum tests were run comparing species richness at each time interval for a given treatment (Table 1). There was no significant difference in species richness between T00 and T03, T00 and T10, or T03 and T10 for any treatment. All *p*-values were greater than an alpha of 0.05, suggesting species richness was similar over time. The sample size from the water only treatment at 3 weeks was too small to run comparative statistical analyses; the small sample size was likely due to insufficient DNA extraction. Given that there was no significant difference in species richness at time T00 and T10 for the water only treatment, it is suspected T03 would have had similar species richness.

Beta diversity

Beta diversity reveals diversity across samples. Using unrarefied OTU tables and their weighted Unifrac results, PCoA graphs were generated (Fig. 4). Both the water only treatment and the saline only treatment exhibited clustering, with notable distance between T0 and T03/T10 (Fig. 4a and 4b). These differences, tested with the non-parametric multivariate statistical test ADONIS, were statistically significant with *p*-values of 0.016 and 0.012, respectively. This indicated that there was a significant difference between the diversity of the samples over time for these two

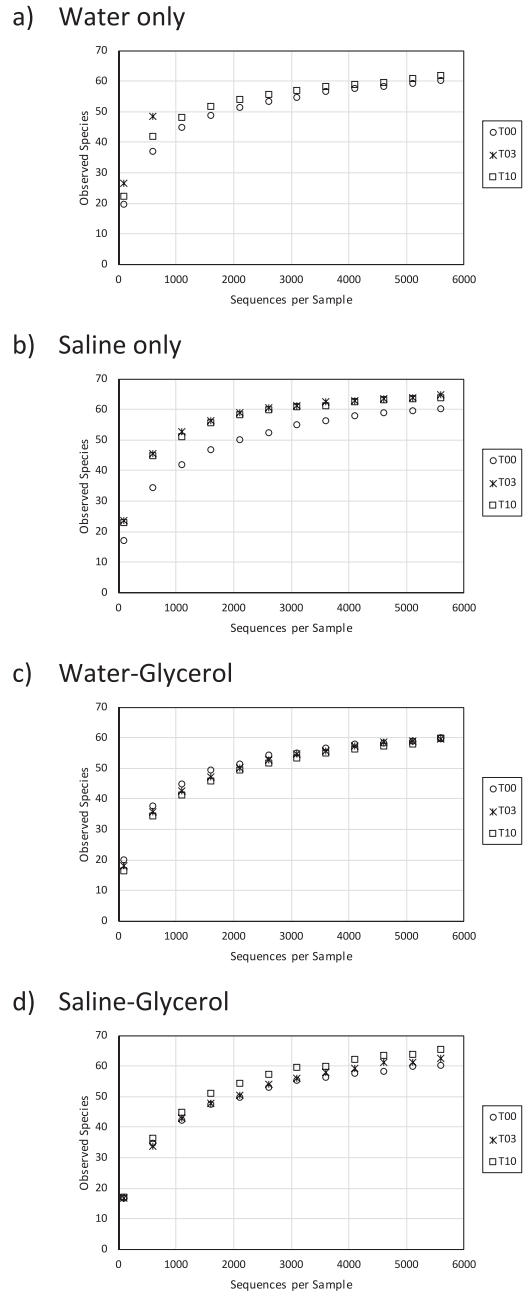

Figure 3. Rarefaction plots for observed species from fecal samples at sampling times of 0 minutes (T00; open circle), 3 weeks (T03; criss-cross), and 10 months (T10; open square). Levelling out indicates sufficient depth of sampling. Treatments are: a) Water only b) Saline only c) Water-Glycerol d) Saline-Glycerol.

Table 1. Nonparametric Wilcoxon rank sum test comparing species richness in fecal samples at each time interval for a given treatment ('nan' is used to indicate these data were not analyzed).

	Group 1	Group 2	Group 1 mean	Group 1 std	Group 2 mean	Group 2 std	*p*-value
Water only	T10	T00	61.95	1.20	60.38	2.19	0.59
	T10	T03	61.95	1.20	nan	nan	None
	T03	T00	nan	nan	60.38	2.19	None
Saline only	T03	T00	65.15	0.60	60.49	1.36	0.20
	T03	T10	65.15	0.60	63.98	0.08	1
	T10	T00	63.98	0.08	60.48	1.36	0.21
Water & glycerol	T03	T00	59.85	1.90	60.21	2.19	1
	T03	T10	59.85	1.90	60.00	0.15	1
	T10	T00	60.00	0.15	60.21	2.19	1
Saline & glycerol	T10	T00	65.58	4.63	60.39	1.61	0.44
	T10	T03	65.58	4.63	62.55	2.35	1
	T03	T00	62.55	2.35	60.39	1.61	1

treatments. The water-glycerol treatment demonstrated a lack of clustering and *p*-value of 0.684 (Fig. 4c). This suggests there was no significant difference in diversity across samples over time. The saline-glycerol treatment produced a similar graph with minimal clustering (Fig. 4d). The *p*-value was just under the cut-off at 0.049.

Relative abundance

All orders that made up less than 2% of all samples were filtered out so that the most abundant taxa grouped by order were represented. The relative abundance of these orders was represented graphically (Fig. 5). Both the water only and saline only treatment resulted in a notable reduction of the Bacteroidales, Fusobacteriales, and Burkholderiales as time progressed. The water-glycerol treatment did not lead to notable changes in these orders. As time progressed, the relative abundance of each of the orders appeared to remain very similar to the original sample. The saline-glycerol treatment did not lead to notable changes, however, there was a slight reduction in the number of Bacteroidales as time progressed. Both treatments supplemented with glycerol appeared to maintain the relative abundance of orders over time.

Discussion

In this study, fecal matter preservation methods were evaluated by species richness, phylogenetic relatedness, and relative abundance of orders over time. The results from this study indicate the water-glycerol treatment as the least detrimental fecal matter preservation method on sample composition of microbiota followed by the saline-glycerol method.

Rarefaction plots were generated to ensure sufficient depth of sampling. Rarefaction curves are generated by randomly re-sampling a pool of N samples multiple times and then plotting the average number of species found in each sample. This generates the expected number of species in a small collection of n samples drawn at random from a large pool of N samples. Rarefaction plots that level out suggest sufficient sampling depth has been achieved. Levelling out at approximately 60 species was observed in all of the observed species rarefaction plots with the exception of the water only treatment (Fig. 3). Again, due to the small size of this sample, there were not sufficient sequence data to generate a complete graph. Based upon the fact that T00 and T10 level out, it can be assumed that if sufficient sampling size of T03 were obtained, it would also level out.

Alpha diversity analysis demonstrated no significant difference in species richness over time (Table 1). However, due to a low sample size of the water only treatment at three weeks, data analysis could not be performed. The number of species at T00 and T10 was not significantly different, leading to the assumption there would not have been a significant difference in the species richness at T03 if a

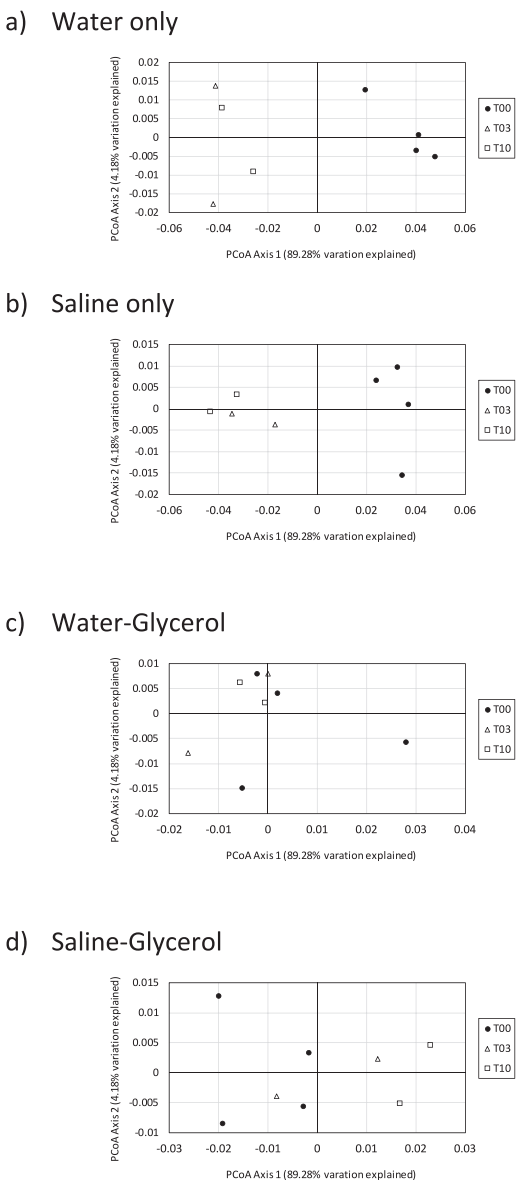

Figure 4. Principle Coordinate Analysis graphs for each treatment at time zero (T00; solid circle), 3 weeks (T03; open triangle), and 10 months (T10; open square). Clustering of data points indicates similarities in phylogenetic diversity of fecal samples. The lack of clustering of data points indicates dissimilarities in diversity across samples. Treatments are: a) Water only b) Saline only c) Water-Glycerol d) Saline-Glycerol.

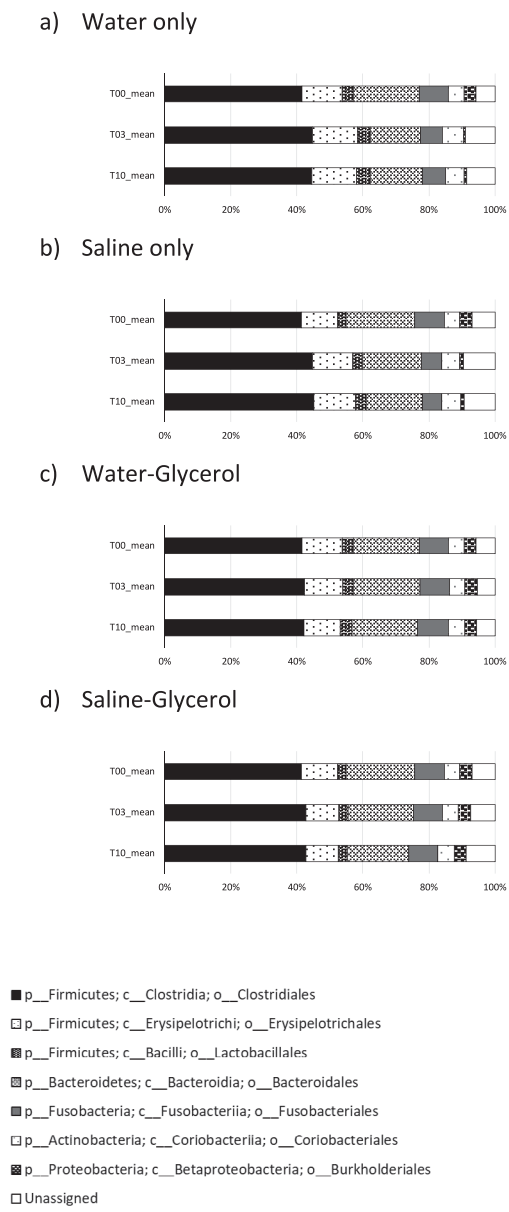

Figure 5. This figure displays the phylogenetic relatedness of OTUs grouped by order between the analyzed fecal samples from 0 days (T00), 3 weeks (T03), and 10 months (T10) for different treatments. Treatments are: a) Water only b) Saline only c) Water-Glycerol d) Saline-Glycerol.

sufficient sampling size were obtained. These results demonstrate that the number of species in each sample was not decreasing significantly with time for all treatments. These results are promising because they indicate that all the preservation methods used in this study maintained species number over time.

After determining that the number of species in the samples remained similar over time for

each treatment and sufficient sampling depth was achieved, analysis was performed to assess the phylogenetic relatedness of the samples over time. At this point, it remained unknown whether the frequencies of the species within the samples were affected by the preservation method. In order to analyze beta diversity, PCoA graphs were generated for each treatment. PCoA graphs allow the visualization of similarities and dissimilarities of data. Clustering of data indicates similarities between samples, whereas samples with the greatest distances between them suggest greater phylogenetic dissimilarity. The PCoA graph for the water only treatment resulted in samples from the same sampling date clustered closely together, and the statistical test, ADONIS, resulted in a p-value of 0.016 (Fig. 4a). This indicated that, within a given timepoint the samples were similar to each other, but that there were significant differences in the phylogenetic relatedness of the samples across time. This same trend was observed for the saline only treatment (Fig. 4b).

The water-glycerol PCoA graph showed a different trend; all samples were clustered closely together, with no apparent clustering by time (Fig. 4c). This clustering indicated the samples, regardless of sample date, shared similarity (Fig. 4c). The *p*-value was 0.684 for the water-glycerol treatment, indicating that there was also no significant difference between phylogenetic relatedness of samples over time. The water-glycerol treatment is considered a promising preservation method, for over time these samples had no significant difference in species richness and no significant difference in phylogenetic relatedness. Like the water-glycerol treatment, the saline-glycerol treatment were all clustered closely and visually expressed a lack of clustering by time. However, statistical analyses suggest significant differences in the phylogenetic composition in the saline-glycerol samples (Fig. 4d). While the *p*-value is right at the cut-off (alpha of 0.05) at 0.049, this indicates some change in sample composition over time. Overall, based upon the PCoA graphs and statistics, treatments supplemented with glycerol appear to experience less change over time than samples preserved with only water or saline.

After finding significant differences in phylogenetic relatedness, analysis was performed in order to observe the relative abundance of orders in the samples in an effort to identify changes in the composition of fecal matter over time. The samples not supplemented with glycerol, water only and saline only, experienced notable decreases in the orders Bacteroidales, Fusobacteriales, and Burkholderiales (Fig. 5a and 5b). The samples supplemented with glycerol did not result in any notable changes in orders over time (Fig. 5c and 5d). These findings suggested that the treatments supplemented with glycerol experienced less change, at least at the order level, over time.

Considering all the analyses performed, it was determined the water-glycerol treatment was the least detrimental preservation method on the microbial composition of fecal matter over time. The water-glycerol treatment had no significant difference in alpha diversity or beta diversity and no notable changes in the relative abundance of orders over time. The saline-glycerol treatment also showed favorable results with only negligible differences. These results suggest that the water-glycerol treatment, or potentially the saline-glycerol treatment, were the best preservation methods tested in terms of conserving fecal matter composition over time.

While not standardized for bio-stool banking, the acquired results are unsurprising since previous studies have shown the importance of using cryoprotectants when preserving cells for extended periods of time (Butler et al., 2019; De Paoli, 2005; Pegg, 1976). One way cryoprotectants such as glycerol are believed to prevent cryoinjury is by binding water found intracellularly. This results in an alteration of the formation of ice crystals during freezing; normal ice crystal formation without cryoprotection could lead to the disruption of cell membranes (De Paoli, 2005). Another benefit of using cryoprotectants is their ability to regulate osmotic pressures. During the freezing point depression, glycerol is able to enter the cell in order to shift the osmotic balance to avoid cell shrinkage; during thawing the shift is opposite

to avoid swelling and lysis (Pegg, 1976). In our samples we saw a decrease in Burkholderiales. Burkholderiales have been noted to thrive in warmer temperatures, and are not well adapted to freezing (Butler et al., 2019). The cryoprotection provided by the glycerol could explain why the freeze-sensitive Burkholderiales were not as affected in the samples containing glycerol.

These findings could be used to standardize bio-stool banking, thereby improving fecal matter preservation methods in order to increase efficacy of fecal matter transplantations. The ability to store fecal matter long-term with minimal detriment to its microbial composition opens up the possibility of using autologous stool samples for FMTs. This would allow individuals to store their own fecal matter earlier in life or even before starting an intense antibiotic regiment or other therapies known to rapidly and drastically alter the gut microbiome. This would enable individuals to receive a self-donation of fecal matter if needed after such interventions. This in itself could increase the efficacy of fecal matter transplants and reduce the risks associated with FMTs, potentially making them a more common and acceptable treatment option. There is less risk associated with self-donation due to reduced infectious disease and immune response concerns associated with transplanting fecal matter.

One of the limitations of this study was the lack of testing for viability of the microbial cells after freezing. The results from this study provide information about the intactness of the DNA present in the samples after the freezing period, but do not provide information about the ability of the microbial cells to grow, reproduce, and continue to survive if re-transplanted within the gut. Another limitation of this study is that it relied on a model organism (i.e., dog). Although previous studies have shown significant similarities in the composition of microbial communities of co-habiting dogs and owners, a study performed using human fecal samples would further authenticate the validity of the acquired results (Coelho et al., 2018).

The results from this research spawned a curiosity into why certain orders (Bacteroidales, Fusobacteriales, and Burkholderiales) decreased in relative abundance over time. This serves as an area that could be further researched. In addition, it would be beneficial to identify the biological significance of these specific orders and other orders that may play an important role in competing with *C. difficile*. Future research could also examine the effect of the different treatments at a higher resolution, such as the genus or species level since this study evaluated preservation methods at the order level. Thus, analysis at a finer resolution may reveal other important information regarding species changes. Future investigations should include using human samples and looking at the viability of cells after storage.

The least detrimental fecal matter preservation method tested was the water-glycerol treatment, followed by the saline-glycerol treatment. Samples that were not supplemented with glycerol resulted in significant differences in beta diversity and notable losses in three orders. Information gained from this study could be used to further improve FMTs and help fuel FMT related research in hopes of attenuating FDA restrictions.

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