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Evaluation of Fecal Matter Preservation Methods and their Effect on Composition of Microbiota Assessed through DNA Sequencing and Analysis

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By

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Evaluation of Fecal Matter Preservation Methods and their Effect on Composition of Microbiota Assessed through DNA Sequencing and Analysis

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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 restrictions, which label them as "experimental". This study aimed to compare microbial preservation methods to determine the method with the least detrimental effect on the composition of stool microbes. Fecal matter samples, collected 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. DNA extraction was also performed on samples partitioned and stored at -80°C after 3 weeks and after 10 months of storage. All extracted DNA was then subjected to PCR amplification and sequenced. A Phred score was used to assess quality of DNA sequencing. Based on alpha and beta diversity analysis, the water and 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.

Keywords: Fecal matter transplant, *Clostridium difficile*, preservation methods

Introduction

The human body is covered both externally and internally with bacteria. There are an estimated 100 trillion microbial cells in the human body, 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 metagenomic 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 homeostasic community in which there are species that occur in high abundance and other species that occur in low abundance (Manimozhiyan et al., 2013). An alteration of this natural balance, which can be induced by antibiotic therapy, a diet change, enviornmental effects, and other causes, can lead to disease.

Antibiotic treatment rapidly alters the composition of the gut microbiota. This may lead to a dysbiosis of gut microbes, which can lead to an

infection of *Clostridium difficile* that is characterized by life-threatening diarrhea (CDC). 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* although 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., 2019).

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 FDA considers fecal microbiota transplantation an investigational drug but permits physicians to utilize them upon obtaining informed consent (OpenBiome). The FDA places no restrictions on the route of delivery and does not require donors to be known (OpenBiome). Stool banks such as OpenBiome assess donors through a 200-question Clinical Evaluation, serological testing, and stool-based assays that screen for infectious pathogens (Quality and Safety). The current storage protocol for OpenBiome requires samples to be stored in a glycerol buffer at -80°C (Quality and Safety). 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.

2. Materials and Methods

Fecal matter samples collected from dogs (under 40 lbs.) were used for this study. Samples were combined and weighed, resulting in 80.57g worth of original untreated sample. The sample was divided in half and homogenized. A flow chart demonstrating the partitioning of samples has been provided (Figure 1). One half of the samples were homogenized by adding 120 mL of 85% NaCl to 40.0g of fecal matter. Vortex beads were added to the sample container and the sample was vortexed for 20 minutes. Samples were left to settle for 10 minutes. Five mL of 85% NaCl was added to wash the walls of the container as the sample was poured

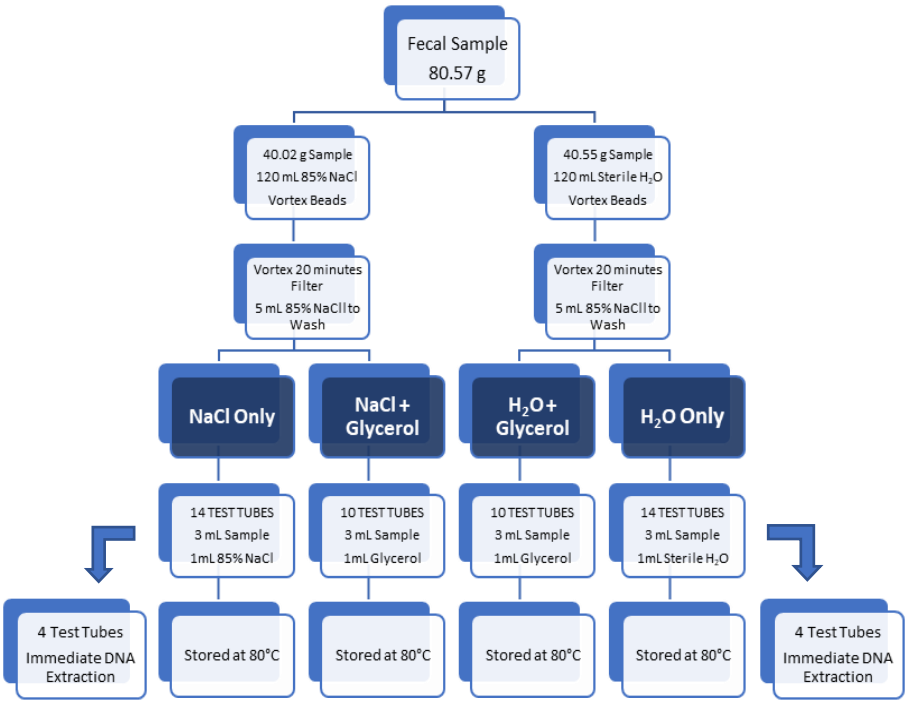


Figure 1. Flow chart demonstrating methodology of sample partitioning and treatment.

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 for immediate DNA extraction or for preservation with or without 25% glycerol prior to -80°C storage. The rest of the tubes were then stored at -80°C. Tubes were removed after 3 weeks and after 10 months to perform DNA extraction.

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). Quanitative 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, and perform alpha and beta analyses and comparisons (programs adapted from Metagenomics Workshop Tutorial 2015, Juniata College).

3. 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 access quality of data (Figure 2). The samples were truncated at 249 bp and average expected error of 0.5%, which retained 602,305 reads (88.26%).

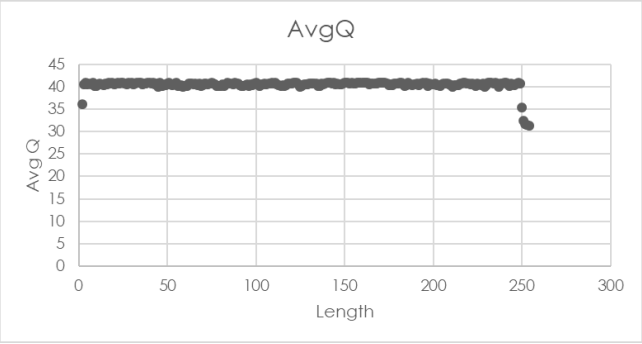
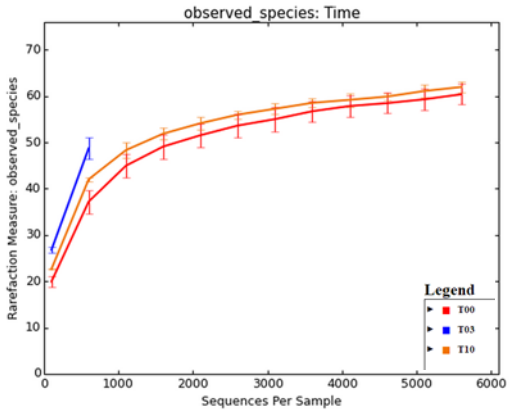


Figure 2. Average Phred quality score determines the probability of a correct base. A Phred score of 30 represents a 99.9% probability that the base is correct. Cut-off made at 249 base pairs.

After filtering, data was clustered into OTUs, chimeras were removed, and taxonomy was assigned.

Alpha Diversity. The OTU tables were rarified creating a series of subsampled OTU tables. The data was collated, and rarefaction plots were generated for observed species (Figure 3). Minimum number of sequences per sample was set at 500. The step number was set to 6,000 sequences per sample.



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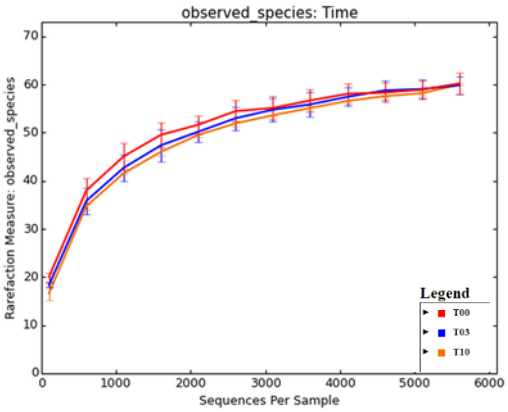

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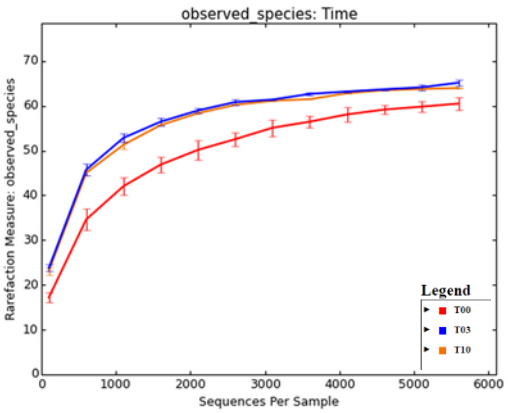

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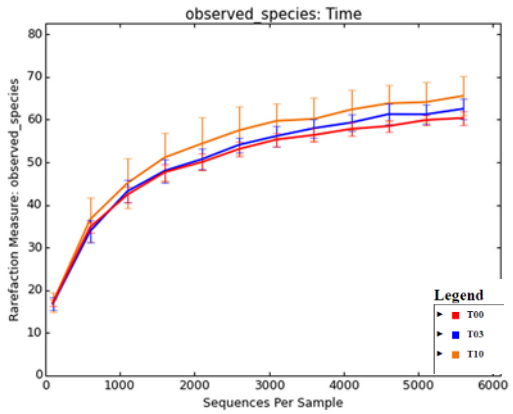

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Figure 3. Rarefaction plots for observed species at sampling times of 0 minutes (T00), 3 weeks (T03), and 10 months (T10). Levelling out indicates sufficient depth of sequencing. a) Water Only b) Saline Only c) Water and Glycerol d) Saline and Glycerol

All rarefaction plots (Figure 3), with the exception of the water only treatment, demonstrated a levelling out as the sequences per sample increased, indicating sufficient depth of sampling. Two-sample T-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 & T03, T00 & T10, or T03 & 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. 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. However, the sample size was too small, likely due to insufficient DNA extraction.

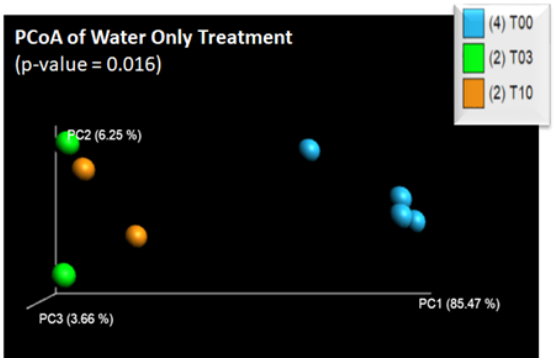
Table 1. Two-sample T-test comparing species richness at each time interval for a given treatment.

	Group 1	Group 2	Group1 mean	Group1 std	Group2 mean	Group2 std	p-value
Water Only	T10	T00	61.95	1.2	60.375	2.19	0.593
	T10	T03	61.95	1.2	nan	nan	None
	T03	T00	nan	nan	60.375	2.19	None
Saline Only	T03	T00	65.15	0.6	60.4875	1.36	0.198
	T03	T10	65.15	0.6	63.975	0.075	1
	T10	T00	63.975	0.075	60.4875	1.36	0.213
Water & Glycerol	T03	T00	59.85	1.9	60.2125	2.19	1
	T03	T10	59.85	1.9	60	0.15	1
	T10	T00	60	0.15	60.2125	2.19	1
Saline & Glycerol	T10	T00	65.575	4.625	60.3875	1.61	0.438
	T10	T03	65.575	4.625	62.55	2.35	1
	T03	T00	62.55	2.35	60.3875	1.614	1

Beta Diversity. Beta diversity reveals diversity across samples. Unrarified OTU tables and their subsequent weighted Unifrac results were used. Principle Coordinate Analysis graphs were generated (Figure 4).

Both the water only treatment and the saline only treatment exhibited clustering, with notable distance between T0 and T03/T10 (Figure 4a & 4b). These differences, tested with a non-parametric multivariate statistical test, analysis of variance using distance matrices (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 treatments.

The water and glycerol treatment demonstrated a lack of clustering and p-value of 0.684 (Figure 4c). This suggests there was no significant difference in diversity across samples over time. The saline and glycerol treatment produced a similar graph with minimal clustering (Figure 4d). The p-value was just under the cut-off at 0.049. Statistically speaking, the saline and glycerol treatment led to significant changes in diversity over time. However, it is important to note that this graph exhibited less clustering than either the water only treatment or the saline only treatment.



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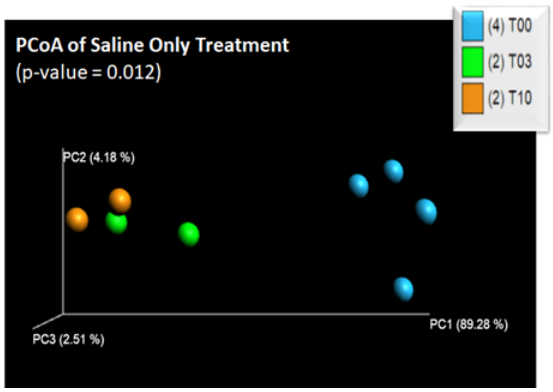

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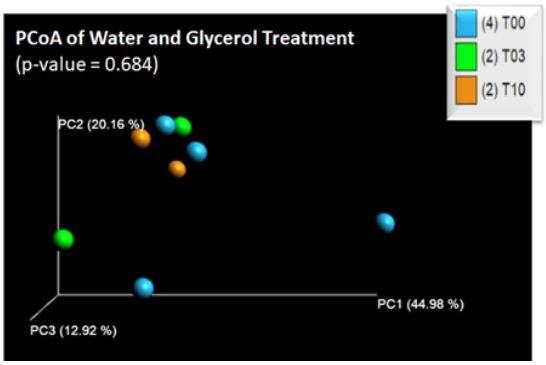

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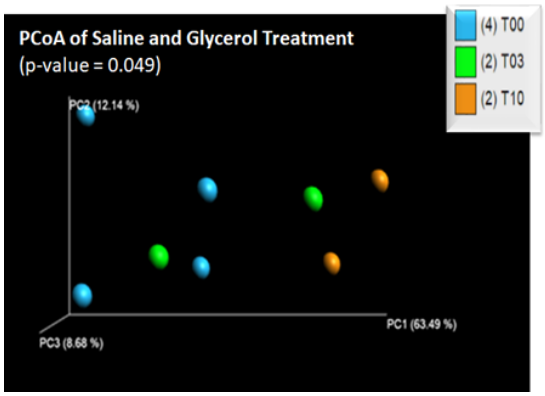

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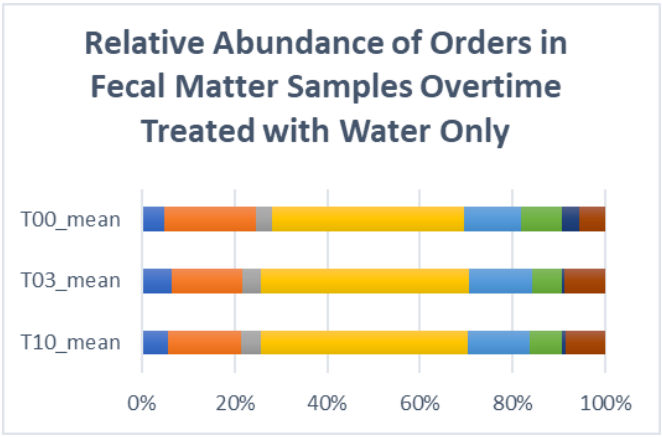
Figure 4. Principle Coordinate Analysis graphs for each treatment at time zero (T00), 3 weeks (T03), and 10 months (T10). Clustering of data points indicates differences in diversity across samples. The lack of clustering of data points indicates similarities in diversity across samples. a) Water Only b) Saline Only c) Water and Glycerol d) Saline and Glycerol

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 (Figure 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 and 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 and 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.



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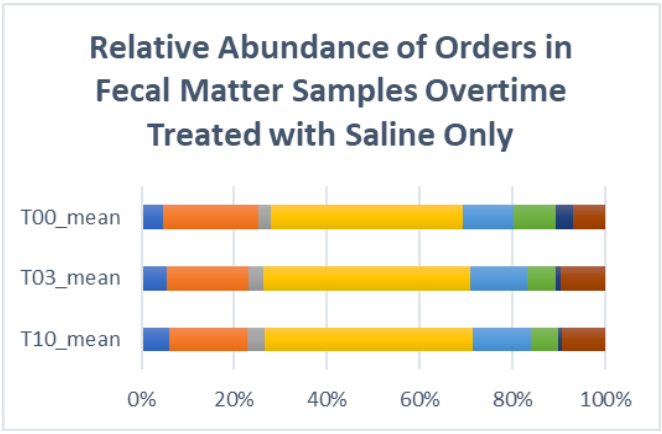

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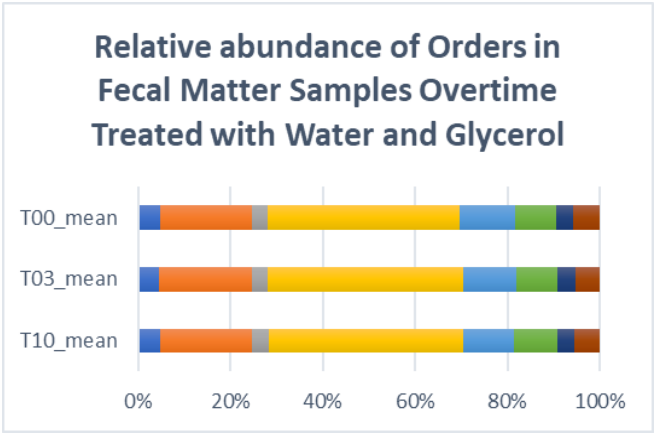

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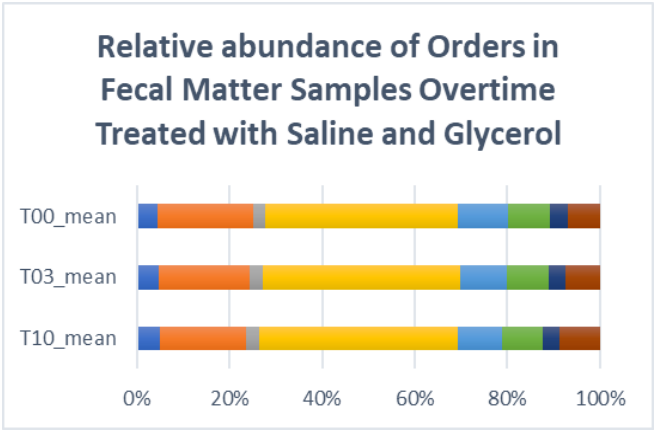

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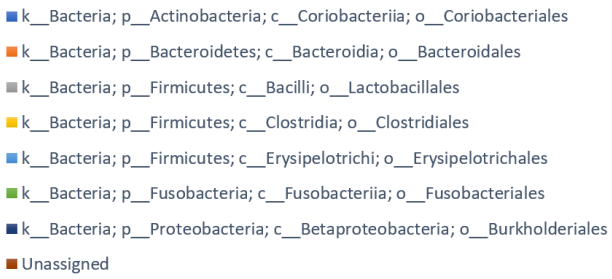

Figure 5. This figure displays the phylogenetic relatedness of OTUs grouped by order between the samples from 0 days (T00), 3 weeks (T03), and 10 months (T10) for different treatments. a) Water Only b) Saline Only c) Water and Glycerol d) Saline and Glycerol.

3. 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 and glycerol treatment as the least detrimental fecal matter preservation method on sample composition of microbiota.

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 was observed in all of the observed species rarefaction plots with the exception of the water only treatment (Figure 3). Again, due to the small size of this sample, there was 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 run. 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 sufficient sampling size were obtained. These results demonstrated that the number of species in each sample was not decreasing significantly with time for all treatments. These results are promising because they indicated 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 done 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, Principle Coordinate Analysis (PCoA) graphs were generated for each treatment. PCoA graphs allow the visualization of similarities and dissimilarities of data. Clustering indicates similarities between samples with greatest distances indicating greatest dissimilarity. The PCoA graph for the water only treatment resulted in samples clustered together based on time, and the statistical test, ADONIS, resulted in a p-value of 0.016 (Figure 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 timepoints. This same trend was observed for the saline only treatment (Figure 4b).

The water and glycerol PCoA graph showed a different trend. There was a lack of clustering of samples, indicating similarities between samples (Figure 4c). The p-value was 0.684, indicating that there was no significant difference between phylogenetic relatedness of samples over time. Thus far, the water only treatment was considered a promising preservation method, for it not only had no significant difference in species richness, but also no significant difference in phylogenetic relatedness. The saline and glycerol treatment visually expressed a lack of clustering, but statistical analyses suggested that the phylogenetic difference was statistically significant (Figure 4d). It is important to note, however, that the p-value was right at the cut-off (alpha of 0.05) at 0.049. Based upon the PCoA graphs, treatments supplemented with glycerol appeared to experience less change over time.

After finding significant differences in phylogenetic relatedness, analysis was done in order to observe the relative abundance of orders in the samples over time 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 three notable decreases in the orders Bacteroidales, Fusobacteriales, and Burkholderiales (Figure 5a and 5b). The samples supplemented with glycerol did not result in any notable changes in orders over time (Figure 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 that the water and glycerol treatment was the least detrimental preservation method on the composition of fecal matter over time. The water and glycerol treatment had no significant difference in alpha diversity or beta diversity and had no notable changes in the relative abundance of orders over time. The saline and glycerol treatment also showed favourable results. These results suggest that the water and glycerol treatment or potentially the saline and glycerol treatment were the best preservation methods tested in terms of conserving fecal matter composition over time. These findings could be used to improve 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 the composition opens up the possibility of using autologous stool samples for FMTs. This would allow individuals to store their own fecal matter before starting an intense antibiotic regiment, chemotherapy, or other therapy that is 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 potentially increase the efficacy of fecal matter transplants and could 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 that the viability of the microbial cells was not tested 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 within the gut. Another limitation of this study is that it was using a model organism (i.e., dog). In order to confirm our findings and change the protocol for fecal matter storage, a similar study would need to be performed using human samples.

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. This study evaluated preservation methods at the order level. Thus, analysis at a finer resolution may reveal other important information regarding changes. Future investigations should include using human samples and looking at the viability of cells after storage.

4. Conclusion

The least detrimental fecal matter preservation method tested was the water and glycerol treatment, followed by the saline and 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.

Author contribituions

Cecelia Martin performed all research and analysis mentioned and was the main writer. Dr. Debra Wohl was involved in experimental design and analysis of data and was the main editor. Dr. Jane Cavender was the second reader.

Acknowledgements

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