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Microbiome Integrity of Unprocessed Canis familiaris Stool Samples prior to Storage for Fecal Microbiota Transplants

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Microbiome integrity of unprocessed *Canis familiaris* stool samples prior to storage for fecal microbiota transplants

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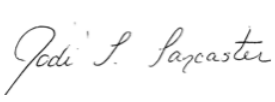
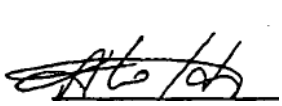
Research Advisor Signature: 

Reader 1 Signature: 

Reader 2 Signature: 

Abstract

Fecal microbiota transplants (FMT) are used for patients with dysbiosis of their gut microbiome or with endogenous pathogens. While FMTs are promising, it has become apparent that the gut microbiome varies between individuals and in the same individuals with diet and age. Therefore, it is difficult to establish a microbiome baseline and assess the compatibility of donor stool since the gut microbiome contains numerous bacteria that facilitate metabolic processes as well as prevent the growth of exogenous pathogens. A recent approach to address this issue is the storage of an individual's stool samples for later. This storage would prove useful for patients prone to dysbiosis pathologies. The primary goal of this experiment was to analyze changes in the stool microbiome overtime prior to stool sample storage. The hypothesis was that as time passes, endogenous bacteria that are poorly adapted for the external environment would decrease while opportunistic bacteria would increase. The study was performed with stool samples passively obtained from *Canis familiaris*. Samples were set out in room temperature for 0 hours, 1 hour, 2 hours, 3 hours, and 2 days. Once the allotted time passed, the samples were homogenized and underwent DNA extraction and PCR amplification of the V4 16S DNA region. Findings show that there was a substantial change in the microbiome for the samples left out for 2 days, notably the Bacteroidales order decreasing and Clostridiales order increasing. Some minor changes were observed between 1 hour and 3 hours which could be critical in a FMT setting, therefore immediate processing would be necessary. Consumer end processing and proper banking infrastructure can help mitigate this delay.

Introduction

A fecal microbiota transplantation (FMT) is a procedure where a donor's microbiome is extracted through their stool and transferred into the large intestine of a patient experiencing dysbiosis. With the healthy bacteria reintroduced into the gut microbiome, the gut should be able to establish a new equilibrium. FMTs are used for patients with dysbiosis of their gut microbiome. Some indications for this procedure include irritable bowel syndrome (IBS) as well as endogenous pathogens such as *C. diff* colitis. The procedure has fairly good success rates at around 90% (Kelly et al., 2020).

While FMTs are promising, the traditional approach utilizes stool donated by another individual, which can lead to failure due to host rejection of the donor microbiome through the host's secretory immunoglobin A, an innately formed mucosal antibody (Mantis et al., 2011). It has become apparent that the gut microbiome varies between individuals and in the same individuals with diet and age, which could be a contributing factor to failed transplants. Therefore, a recent approach to address this issue is stool banking (i.e., the storage of one's own stool samples). The benefit of banking one's own stool is that the microbiota would be very similar to what the stool recipient had prior to the dysbiosis, which would reduce the risks of failure and allow the newly established equilibrium to be similar to that patient's baseline gut microbiome. There are several established procedures for storing the stools as homogenized samples at low temperatures (Burz et al., 2019). However, there is also concern of the sensitivity of the stool microbiome prior to stool sample processing and storage, such as in instances where the sample is collected at home and shipped or if there is a backlog at the lab for sample processing (Abrahamson et al., 2017). Since these samples would remain unprocessed for a prolonged period of time, one would presume that the microbiome present would change. The

primary goal of this experiment was to analyze these changes in the stool microbiome prior to stool sample storage.

It was hypothesized that as time progresses, the number of bacteria accustomed to the internal gut environment would decrease while the more opportunistic bacteria already present within the stool that can survive in ambient conditions would increase in their composition. This is due to the sudden change from the gut environment to the external environment with regards to temperature, oxygenation, and organic materials present. While the bacteria accustomed to body temperature have reduced metabolic rates, the opportunistic bacteria would grow at a faster rate since their metabolic rate would not be as hindered as the others.

Method

Lab procedure

Stool samples from *Canis familiaris* were passively obtained on the day of DNA processing. The samples were equally divided and massed into four treatment groups, identified based on time processed: 0 hours, 1 hour, 3 hours, and 48 hours. Stool samples were placed at room temperature, exposed to room air, until they were processed. Once the desired time elapsed, the samples were placed in a 1:3 dilution of sterile water and vortexed for 20 minutes. The homogenized mixture was filtered through a U.S.A Standard Testing SIEVE A.S.T.M. E-11 Specification (open micrometer 250, No. 60) and the filtrate underwent DNA extraction with a QIAmp PowerFecal Pro DNA kit per its assigned protocol for a total of 3 samples per trial. DNA was stored at –20C until amplification of the V4 16S region of DNA and Illumina Mi-Seq sequencing was performed by Wright Labs, Inc (Juniata, PA).

Data Analysis

Sequence data were analyzed using the QIIME bioinformatics platform. The data were pre-processed to maintain high quality sequences. The individual reads were quality filtered prior to being paired. Paired data were then filtered, and Phred quality scores were used to evaluate the accuracy of base determinations in the sequences. These data were then analyzed to remove chimeras (Lamendella et al., *unpublished*).

Once high-quality sequence data were obtained, the chimeras were removed to prevent redundant results from complimentary DNA sequences from artificially inflating the data. Taxonomic assignments were applied to these data with open reference operational taxonomic unit (OTU) clustering. Following this, low sequence size DNA samples were removed from the dataset. This resulted in the removal the negative control groups which underwent DNA extraction with sterile water because their sequence sizes were much lower than the samples that contained fecal matter (less than 10,000 sequences).

Finally, QIIME was utilized to perform statistical analysis including rarefaction and PCoA on the groups by calculating alpha diversity and beta diversity. The taxonomic data were analyzed visually and plotted using Excel.

Results

A total of 1,099,402 Illumina sequences were obtained. The data were trimmed at 250 base pairs in length which maintained average Phred scores above 30 as visualized in figure 1. After filtering of the data, 922,496 high quality sequences remained with an average of 77, 409 sequences per treatment (Table 1). These remaining sequences were used to assess changes in the stool microbiome when exposed to room air at room temperature at 0 hours, 1 hour, 3 hours, and 48 hours prior to DNA extraction.

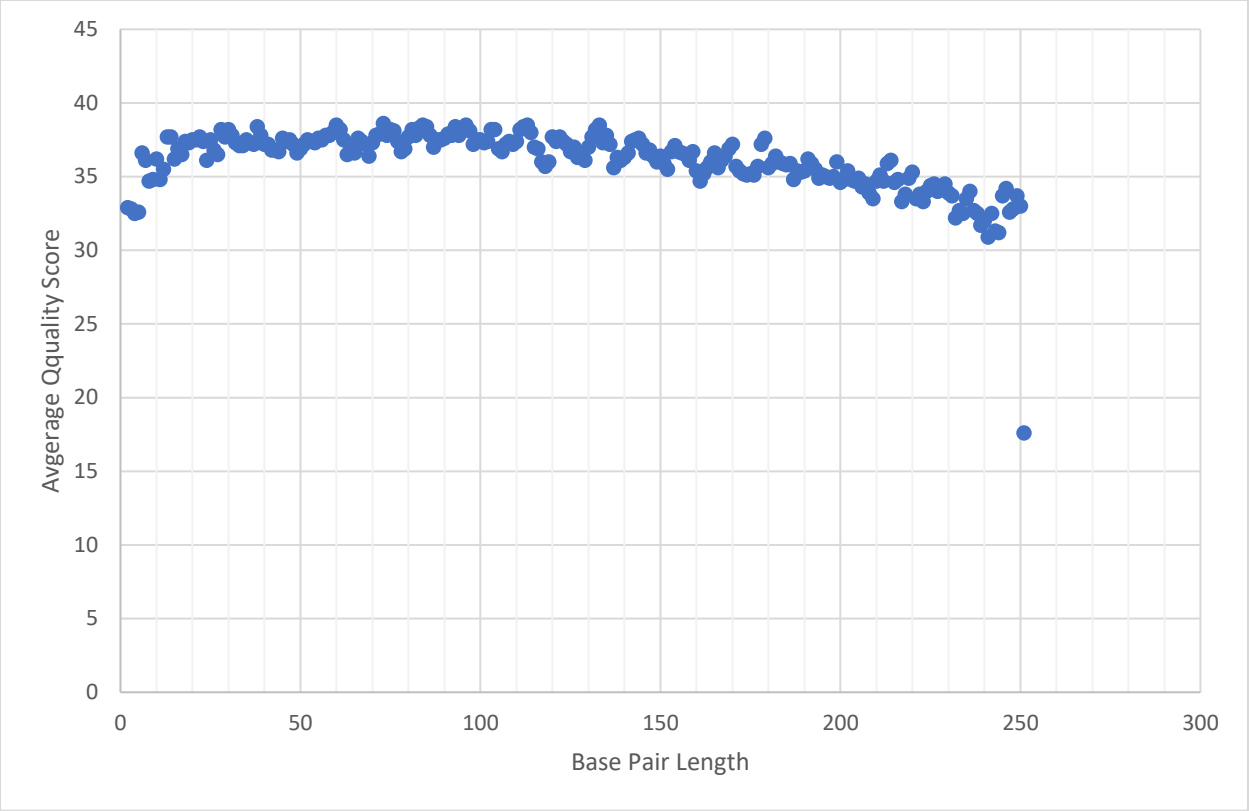


Figure 1: Average quality score of merged sequences of 16S V4 region DNA at varying base pair lengths.

Table 1: Average number of high-quality sequences obtained for each time of processing after data filtering. (n = 3)

Time of Processing	Average Number of Sequences
0 hours	66,059
1 hour	101,750
3 hours	82,180
48 hours	59,648

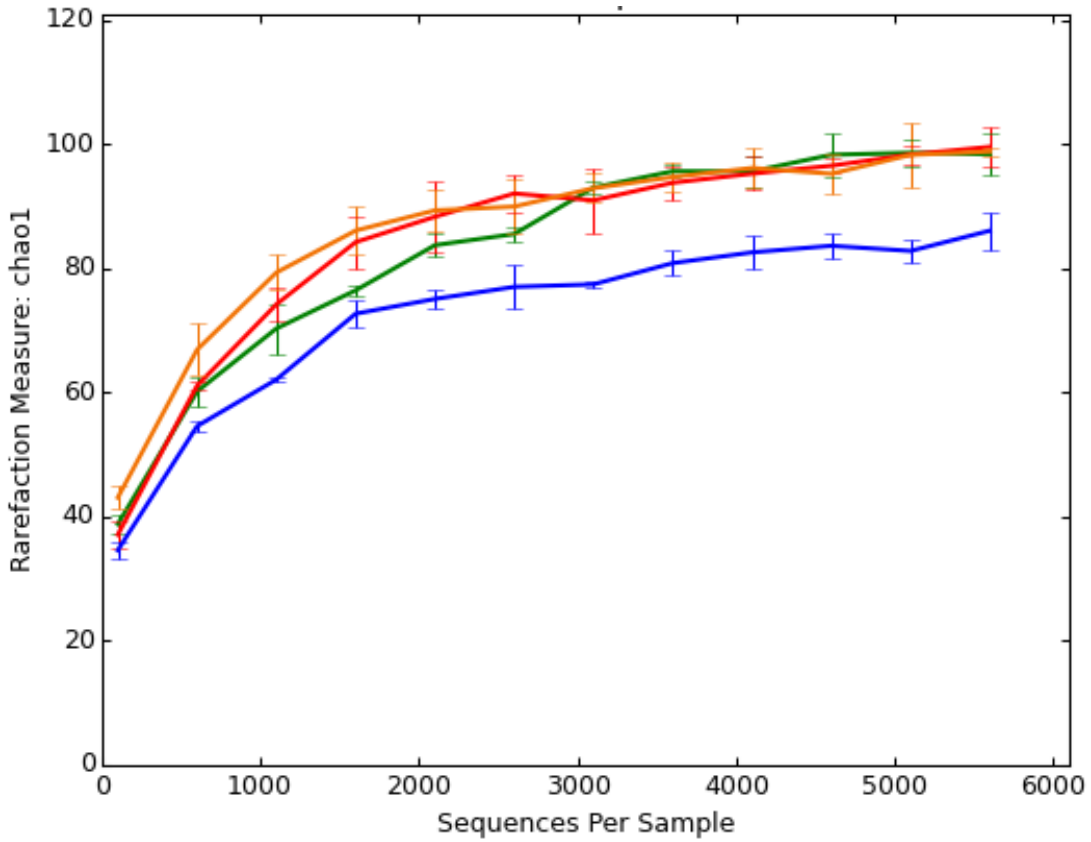


Figure 2: Rarefaction plot of samples by sample time.

A rarefaction plot was used as a measure of alpha diversity, within sample variation, to show that the fecal matter samples had undergone an ample amount of sample depth. Diversity estimates of the rarefied data indicate the observed OTUs are stagnant at around 4000 sequences per sample (Figure 2). As shown in Table 1, all of the samples exceed 4000 observed OTUs, meaning that the data obtained accurately and proportionally represented the microbiome community present.

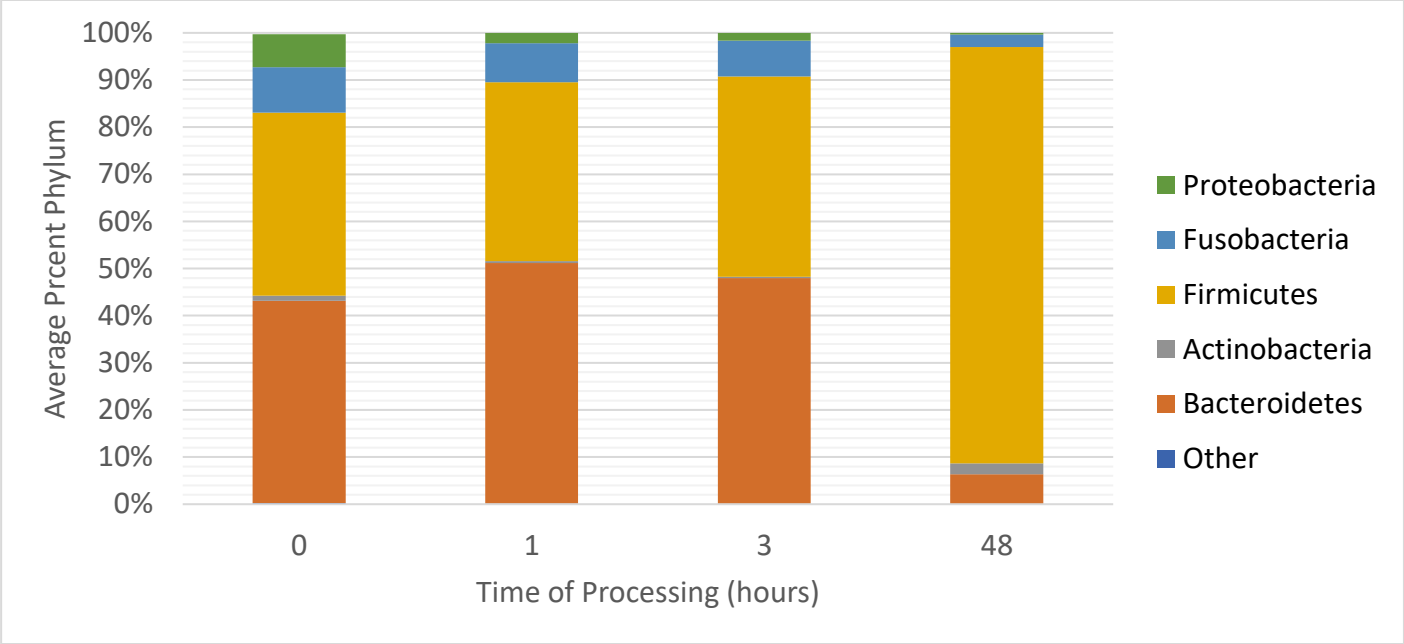


Figure 3: Average percentage of bacteria phyla present in *Canis familiaris* stool samples after 0 hours, 1 hour, 3 hours, and 48 hours spent in room temperature. (n=3)

The relative proportion of sample comprised by the Firmicutes phyla substantially increased from around 40% to 88% while the Bacteroidetes phyla substantially decreased in samples processed 48 hours after receipt as compared to the 0, 1 hr, and 3 hr treatments from a range of 50% of the bacterial microbiome composition to 6% (Fig. 3). A gradual decrease in the Proteobacteria and Fusobacteria phyla were also observed throughout this experiment with a sharp decline between the 3 hr and 48 hr treatments. The Actinobacteria phyla had a slight decrease in its relative proportion of the sample, but it increased when compared to the 3 hr and 48 hr treatments.

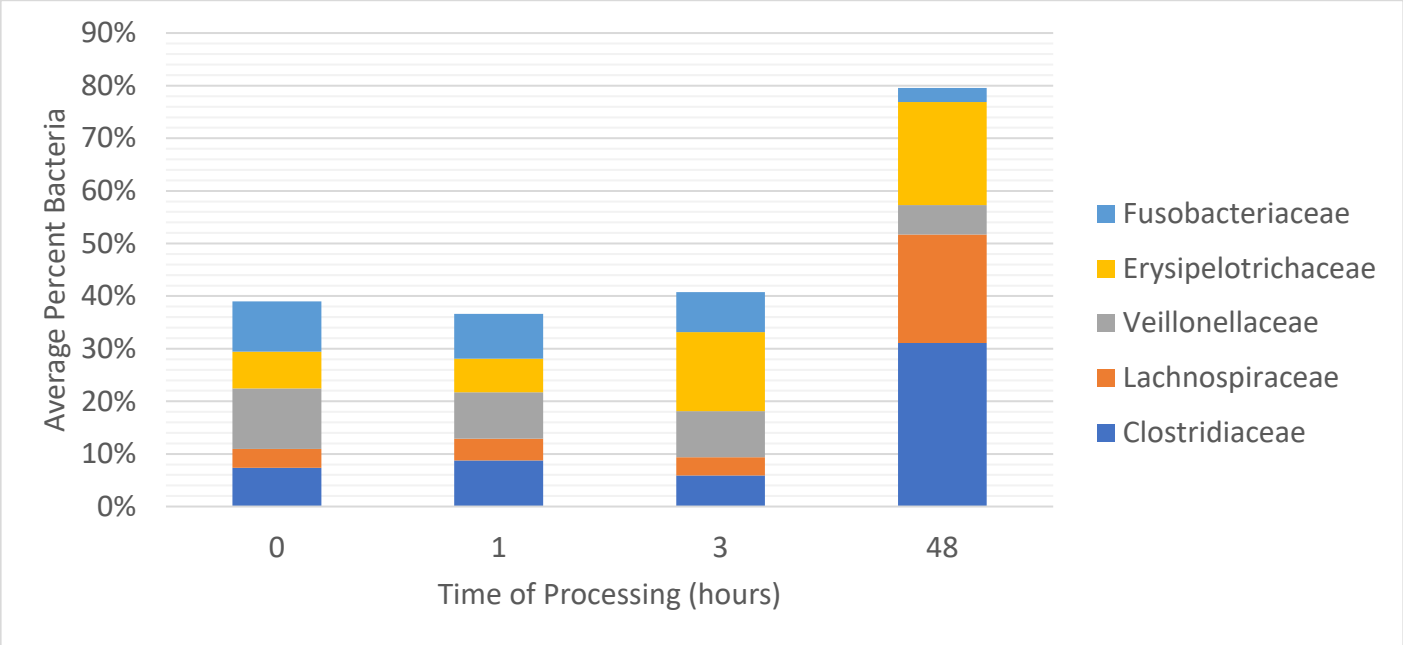


Figure 4: Average percentage of Clostridiales orders present in *Canis familiaris* stool samples after 0 hours, 1 hour, 3 hours, and 48 hours spent in room temperature. (n=3)

Within the Firmicutes phyla, we examined the notable changes that occurred in the dominant order Clostridales (Fig. 4). The primary families within the Clostridales that increased in overall microbiome presence were the Clostridiaceae (increase from 6% to 31%), Lachnospiraceae (increase from 3% to 21%), and Erysipelotrichaceae families (gradual increase from 7% at 0 hours, 15% at 3 hours, and 20% at 48 hours). The relative amount of Fusobacteriaciae decreased over sample times from 10% to 3% (Fig 4).

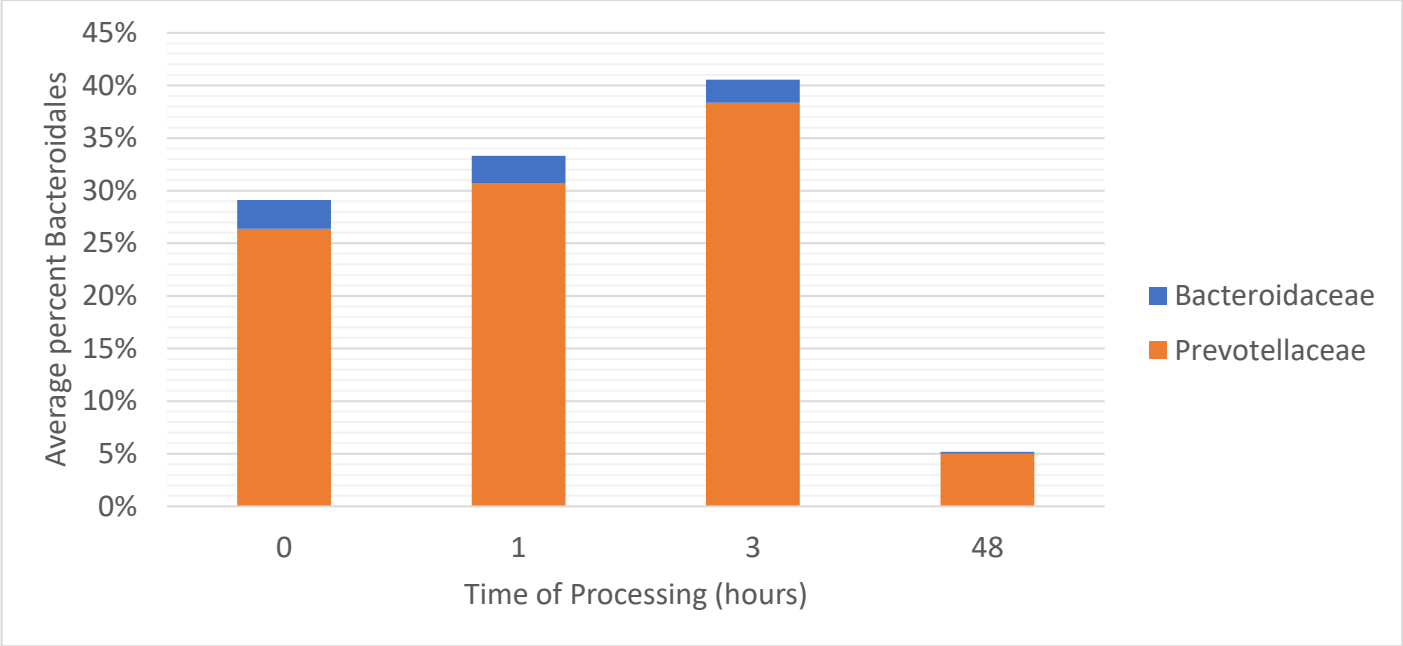


Figure 5: Average percent of significant Bacteroidales orders present at varying times of processing. (n =3)

The relative community abundance decrease was noted in the Bacteroidetes phyla between the 3 hr and 48 hr treatments. The primary constituents of this phyla that were most affected were Bacteroidaceae and Prevotellaceae of the Bacteriodales order. Both families had an increase in their relative proportion in the stool sample when 0 hr was compared to 1 hr and 3 hr, but a decrease was observed between the 3 hr and 48 hr treatments. Bacteroidaceae decreased from around 2.4% to 0.18%. Prevotellaceae initially increased from 26% at 0 hours to 38% at 3 hours, but it decreased to 5% in the 48-hour treatment (Fig. 5)

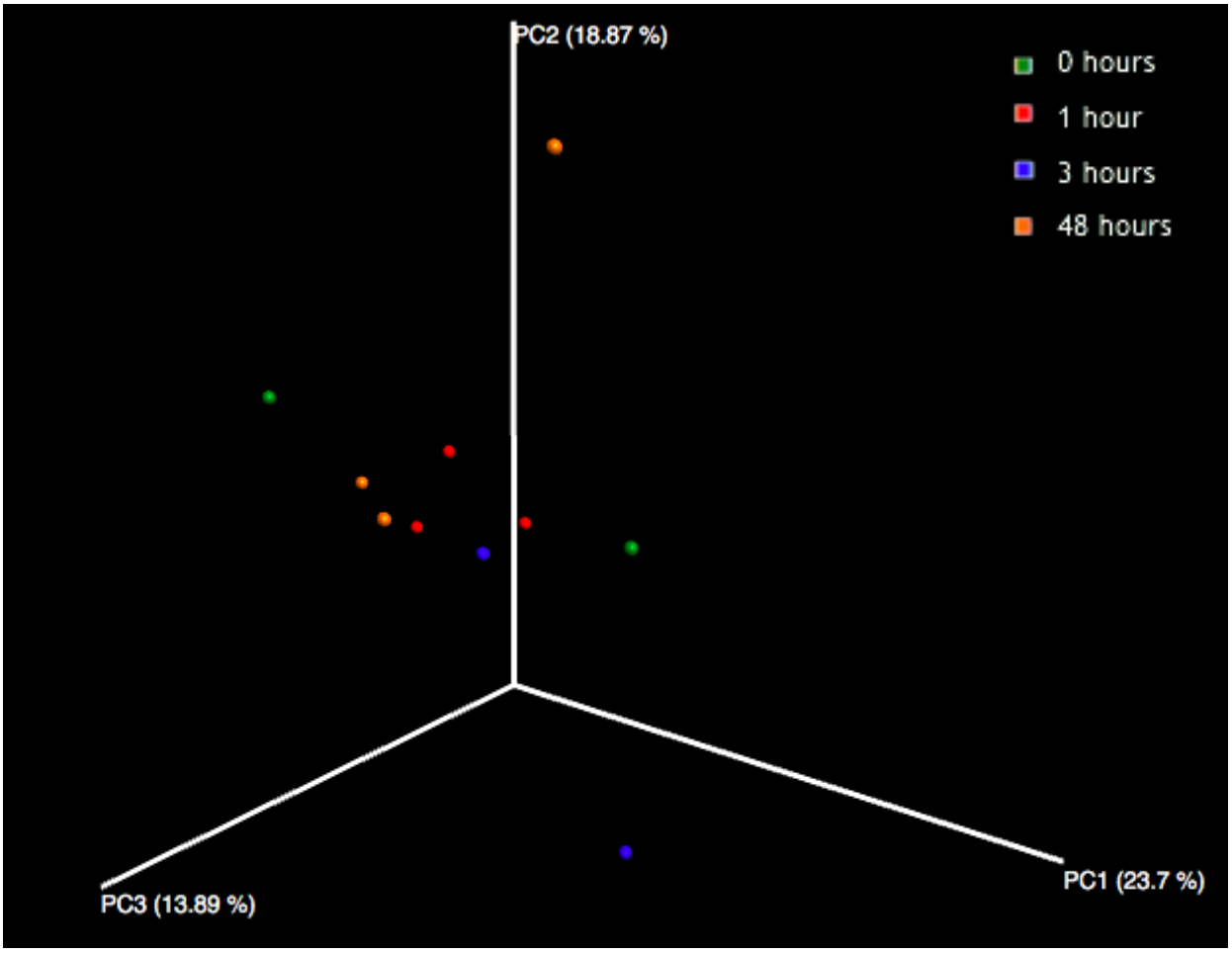


Figure 6: Unweighted PCoA plots of *Canis familiaris* stool samples (3D overview).

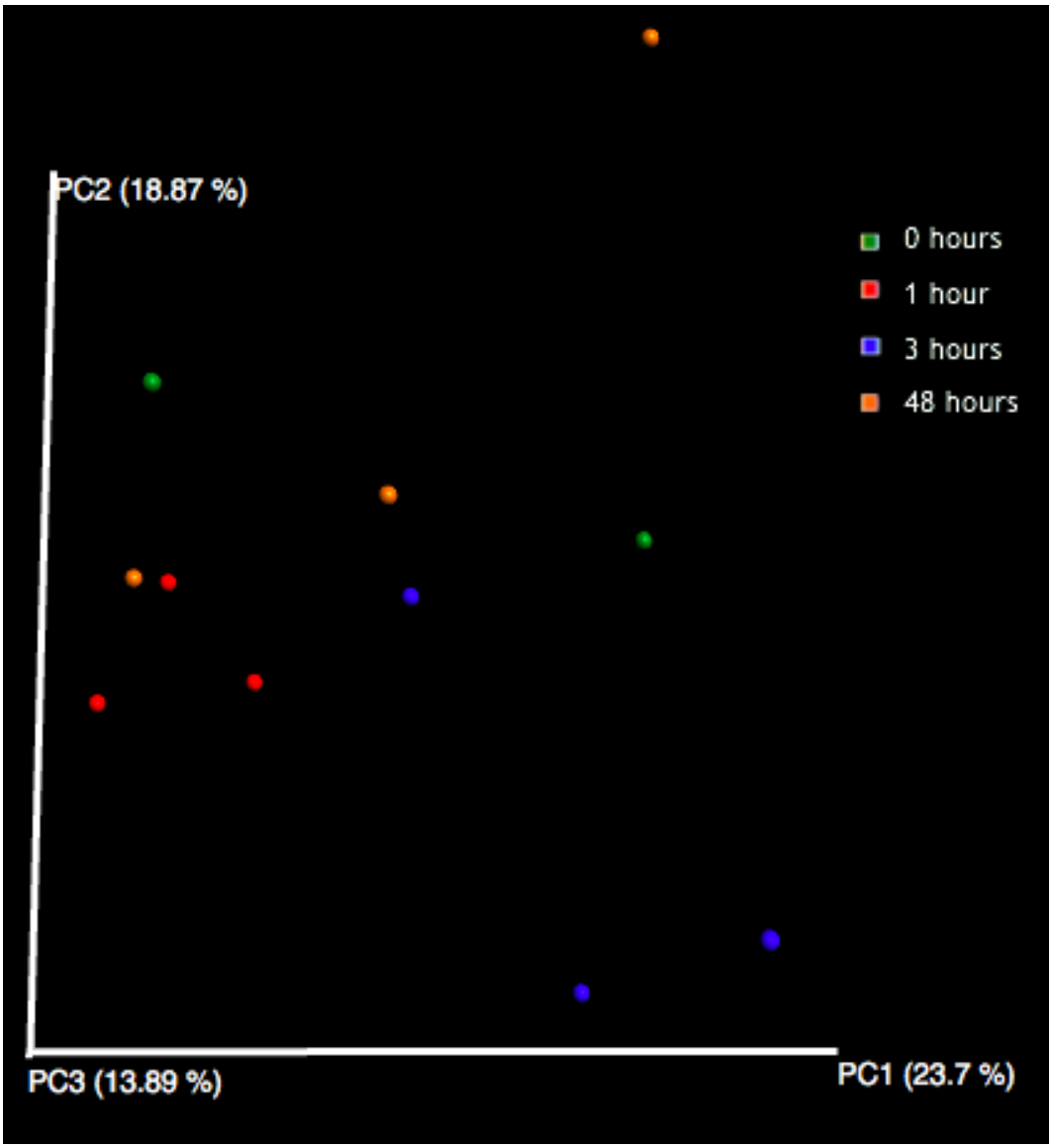


Figure 7: Unweighted PCoA plots of *Canis familiaris* stool samples (PC1 vs PC2).

Figure 6 and figure 7 best represent the causes of variation within the dataset which is visually shown with these PCoA plots. The percentages on each principal coordinate axis shows the dissimilarity between the data. The percentage on each axis is the amount of variability that is summarized on that axis alone. Therefore, figure 6 represents about 50% of the variation observed in the data given the sum of the axis. Figure 7 best represents the grouping of similar sample times with the cluster of the 1 hour and 3 hours group, which helps verify similarities within groups.

Discussion

The *Canis familiaris* stool samples were exposed to room air at room temperature at various times prior to stool processing and DNA extraction. The procedure was performed in order to determine stool microbiome changes as time progressed to best simulate lab backlogs and shipping samples. It was hypothesized that more opportunistic bacteria would increase in their relative community abundance while bacteria that were not well adjusted to room temperature and room air would not thrive. The most significant findings from these data were aptly summarized in figure 3 with the drastic increase in relative community abundance in the Clostridiales order within the Firmicutes phyla paired with the drastic decrease of the Bacteroidales order within the Bacteroidetes phyla between the 3 hr and 48 hr treatment groups.

The families within the Clostridiales order that increased the most were the Clostridiaceae, Lachnospiraceae, and Erysipelotrichaceae families. These families were found to be positively correlated in diets high in meat and crude protein (Bermingham et al., 2017). For the Bacteriodales order, the Bacteroidaceae and Prevotellaceae families significantly decreased and were found to be positively correlated with the presence of carbohydrates within the *Canis familiaris* diet and negatively correlated with a high presence of crude protein within their diet (Bermingham et al., 2017). These metabolic findings would suggest that the carbohydrates were the prioritized source of energy initially until resources were depleted, which could have led to the loss in Bacteroidetes while the Clostridiales order would be able to persist with the digestion of proteins present within the stool sample.

An alternative theory may also be that the Clostridiales order could have utilized their endospore formation ability (Setlow 2018) to persist in the room temperature environment better

than the non-endospore forming bacteria present. Regardless, these findings could be concerning given that *Clostridium difficile* is classified under the Clostridiales order. There was no evidence of Clostridia present within the taxonomic data acquired, which is common for about 95% of the asymptomatic *Canis familiaris* population (Wetterwick et al., 2013).

Conclusion

These findings support the hypothesis that there would be a change over time in bacterial composition with a shift in bacteria that would be more well suited for the room air environment with limited supplies in the stool sample. These findings were evident with the drastic increase in the relative community abundance of the Clostridiales order which contains bacteria that can develop endospores and metabolize proteins well (Setlow et al., 2018). This order also contains *Clostridium difficile,* which is concerning because it could mean that the conditions used could enrich for *Clostridium difficile*, which would accelerate its growth and presence within the stool sample.

to better study the ramifications of these findings, it would be best to obtain more time intervals between 3 hours and 48 hours to determine the inflection point of these massive microbiome changes (i.e., 6 hours, 9 hours, 12 hours, 24 hours, etc.,). In addition, obtaining human stool samples, especially from *Clostridium difficile* carriers, would prove beneficial in determining how pathogens of concern would change over time. Finally, performing DNA extraction in a way that exclusively tests for endospore DNA would assist in determining the cause of the increase in the Firmicutes phyla because a large presence of endospores would determine that the bacteria are persisting better than the others, while no notable change would suggest that the bacteria are outperforming the other species through their metabolic processes.

Even with these current findings, it was apparent that changes did occur to the microbiome as time progressed, such as the small decrease in Proteobacteria and Fusobacteria. In order to obtain a sample size that best represents the stool microbiome, immediate processing of the stool sample would be the best practice. This could be done through various means. For example, there could be consumer-end preservation where the stool donor could place preservation fluids into the sample, such as what is currently done with commercial at-home genomic tests. Alternatively, physical stool banking locations could be constructed, which would eliminate concerns of shipping samples as well as ensure that there are minimal backlogs by having the facility be open through appointments only.

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