Effects of variation in temperature and preservation buffer on canine fecal microbiota

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Studies involving gut microbiome analysis play an increasing role in the evaluation of health and disease in humans and animals alike. Fecal sampling methods for DNA preservation in laboratory, clinical, and field settings can greatly influence inferences of microbial composition and diversity, but are often inconsistent and under-investigated between studies. Many laboratories have utilized either temperature control or preservation buffers, but few studies have evaluated the effects of combining both methods to preserve fecal microbiota. To determine the optimal method for fecal DNA preservation, we collected fecal samples from one canine donor and stored aliquots in RNAlater, 70% ethanol, 50:50 glycerol:PBS, or without buffer at 25°C, 4°C, and -80°C. Fecal DNA was extracted, quantified, and 16S rRNA gene analysis performed on days 0, 7, 14, and 56 to evaluate changes in DNA concentration, purity, and microbial diversity and composition over time. We detected overall effects on bacterial community of storage buffer (F-value= 6.87, DF= 3, P<0.001), storage temperature (F-value=1.77, DF= 3, P=0.037), and duration of sample storage (F-value=3.68, DF= 3, P<0.001). Changes in microbial composition were observed in samples stored in -80°C without buffer, a commonly used method for fecal DNA storage, suggesting that simply freezing samples may not be optimal for bacterial analysis. Fecal preservation with 70% ethanol and RNA later very closely resembled that of fresh samples, though RNA later yielded significantly lower DNA concentrations (DF=8.57, P<0.001). Although microbial composition varies with temperature and buffer storage, 70% ethanol was the best method for preserving bacterial DNA in canine feces, yielding the highest DNA concentration and minimal changes in microbial diversity and composition. The differences observed between samples highlights the need to consider optimized post-collection methods in microbiome research.

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ABSTRACT

Studies involving gut microbiome analysis play an increasing role in the evaluation of health and disease in humans and animals alike. Fecal sampling methods for DNA preservation in laboratory, clinical, and field settings can greatly influence inferences of microbial composition and diversity, but are often inconsistent and under-investigated between studies. Many laboratories have utilized either temperature control or preservation buffers for optimization of DNA preservation, but few studies have evaluated the effects of combining both methods to preserve fecal microbiota. To determine the optimal method for fecal DNA preservation, we collected fecal samples from one canine donor and stored aliquots in RNAlater, 70% ethanol, 50:50 glycerol:PBS, or without buffer at 25°C, 4°C, and -80°C. Fecal DNA was extracted, quantified, and 16S rRNA gene analysis performed on days 0, 7, 14, and 56 to evaluate changes in DNA concentration, purity, and microbial diversity and composition over time. We detected overall effects on bacterial community of storage buffer (F-value= 6.87, DF= 3, $P<0.001$), storage temperature (F-value=1.77, DF= 3, P=0.037), and duration of sample storage (F-value=3.68, DF= 3, $P<0.001$). Changes in microbial composition were observed in samples stored in -80°C without buffer, a commonly used method for fecal DNA storage, suggesting that simply freezing samples may be suboptimal for bacterial analysis. Fecal preservation with 70% ethanol and RNAlater very closely resembled that of fresh samples, though RNAlater yielded significantly lower DNA concentrations (DF=8.57, $P<0.001$). Although microbial composition varied with temperature and buffer storage, 70% ethanol was the best method for preserving bacterial DNA in canine feces, yielding the highest DNA concentration and minimal changes in microbial diversity and composition. The differences observed between samples highlights the need to consider optimized post-collection methods in microbiome research.

INTRODUCTION

The intestinal microbiota is comprised of trillions of bacteria that contribute to nutrition, digestion, immune defense, and various disease processes (Peterson & Round, 2014; West et al., 2015). However, it is estimated that 60-70% of resident gut bacteria cannot be discerned using culture-dependent methods (Hayashi, Sakamoto, & Benno, 2002). Microbiota research involving culture-independent techniques such as DNA sequencing have gained great momentum in the past decade with recent technological advances in next-generation sequencing and bioinformatics, providing more accurate taxonomic analysis and reproducibility between studies (Wu et al., 2012). The human gut is the perhaps the most well-known and well-studied microbiome, but there is increasing interest in other biological sites in humans, domestic animals, and wildlife in an effort to study the interaction between the host and its environment (Paulino et al., 2006; Verhulst et al., 2011; Weese, 2013; Fliegerova et al., 2014; Lowrey et al., 2015). These studies have paved the way for linking distinct microbial communities within and between individuals. Recently, these efforts have greatly contributed to molecular fingerprinting and DNA identification in forensic science (Fierer et al., 2010; Tims et al., 2010). Despite the technological advances in noninvasive genetics (Beja-Pereira et al., 2009), there remains a lack of standardized methods for sample collection, bacterial preservation, and DNA extraction (Yuan et al., 2012; Kennedy et al., 2014; Gorzelak et al., 2015). Many studies that have evaluated bacterial preservation and DNA extraction showed variability in processing samples, leading to significant over- or underrepresentation of microbial populations. For example, fecal storage at room temperature decreases the relative abundance of Firmicutes and increases Bacteroidetes, whereas storage in freezing conditions introduces the opposite effect (Bahl, Bergstrom, & Licht, 2012; Choo, Leong, & Rogers, 2015). Furthermore, the use of fecal swabs in human patients led

to an overestimation of Enterobacteriaceae and Ruminococcaceae bacterial families (Tedjo et al., 2015). This highlights the need for more comprehensive evaluation of current techniques for optimal fecal DNA storage and isolation.

For many research laboratories, it is a challenge to minimize exposure of samples to the environment and minimize time between sample collection and DNA extraction (Hale et al., 2016). Preserving DNA through deactivation of nucleases, removal of cations or lowering temperature becomes crucial to inhibit enzymes that degrade DNA. Most studies have utilized freezing conditions (Carroll et al., 2012; Wu et al., 2012) or the use of preservatives such as ethanol (Murphy et al., 2002; Bressan et al., 2014; Huang et al., 2016), RNAlater (Nechvatal et al., 2008; Sorensen et al., 2016), and PBS-glycerol (McKain et al., 2013; Fliegerova et al., 2014) to preserve bacterial DNA before extraction. However, few studies have examined the combination of chemical buffers and temperature reduction to preserve DNA and optimize microbial analysis. We designed a longitudinal study to evaluate the effects of temperature (room temperature at 25°C vs. refrigeration at 4°C vs. freezing at -80°C) as well as immersion in 70% ethanol, RNAlater, and 50:50 glycerol:PBS on fecal DNA preservation over the course of a 56-day storage period.

METHODS

Stool collection and storage

One fresh stool sample from a healthy canine donor was collected at the UC Davis Veterinary Medical Teaching Hospital. The donor, a male Labrador retriever, was on a consistent commercial diet and had access to both indoors and outdoors. Fecal sampling occurred

93 immediately after defecation. The sample was then homogenized and processed (within 1 hour of
94 collection) in the laboratory. Untreated feces were placed in three common fecal preservation
95 buffers RNAlater (Ambion, Austin, TX, USA), 70% ethanol, and 50:50 Glycerol:PBS. The fecal
96 sample was homogenized, divided into ninety-six 0.25 g aliquots (4 treatment groups, 3
97 temperatures, 4 time points, in duplicate), and placed in 1.5 mL Eppendorf tubes with
98 preservation buffers. All tubes were vortexed to allow buffer penetration in each fecal sample
99 and incubated for two hours at room temperature. Each treatment group was subjected to various
100 temperature conditions: room temperature (25°C), refrigeration (4°C), and freezing (-80°C) on
101 the day of collection (day 0) and on days 7, 14, and 56 post-collection. Temperatures were
102 consistently maintained using a microbiological incubator at 25°C, a refrigerator at 4°C, and a
103 Revco freezer at -80°C. Day 0 samples were processed for DNA extraction after 2 hours of
104 temperature treatment, while other samples were stored at the indicated temperatures until DNA
105 extractions on day 7, 14, and 56.
106
107 DNA extraction
108 Genomic DNA was extracted from all samples using the 100-prep MoBio PowerSoil DNA
109 Isolation kit (MoBio, Carlsbad, CA). Fecal material was isolated from preservation buffer by
110 pelleting (centrifugation at 10,000 x g for 5 minutes, pouring off supernatant). Samples were
111 placed in bead tubes containing C1 solution and incubated at 65°C for 10 minutes, followed by 1
112 minute of bead beating with the MoBio vortex adapter. The remaining extraction protocol was
113 performed as directed by the manufacturer. DNA concentration was recorded using a QUBIT™
114 dsDNA HS Assay and the DNA purity (A260/A280 ratio) was analyzed using a Nanodrop 1000
115 spectrophotometer (ThermoFisher Scientific, Wilmington, DE).

PCR and 16S rRNA Sequencing

Bacterial diversity was characterized via amplification by a PCR enrichment of the 16S rRNA gene (V4 region) using primers 515F and 806R, modified by addition of Illumina adaptor and an in-house barcode system (Lang, Eisen, & Zivkovic, 2014) After an initial denaturation step at 94°C for 3 minutes, we ran 35 cycles of the following PCR protocol: 94°C for 45 seconds, 50°C for 60 seconds and 72°C for 90 seconds, followed by a final hold at 4°C. Prior to sequencing, the amount of input DNA per sample was normalized using a SequalPrep Normalization Plate, following the standard protocol (ThermoFisher Scientific, Wilmington, DE). Libraries were sequenced using an Illumina MiSeq system, generating 250 bp paired-end amplicon reads.

Data analysis and statistics

We used a custom script (available in a GitHub repository https://github.com/gjospin/scripts/blob/master/Demul_trim_prep.pl), to assign each pair of reads to their respective samples when parsing the raw data. This script allows for one base pair difference per barcode. The paired reads were then aligned and a consensus was computed using FLASH (Magoč & Salzberg, 2011) with maximum overlap of 120 bp and a minimum overlap of 70 bp (other parameters were left as default). The custom script automatically demultiplexes the data into fastq files, executes FLASH, and parses its results to reformat the sequences with appropriate naming conventions for Quantitative Insights into Microbial Ecology {QIIME v.1.9.1, (Caporaso et al., 2010)} in fasta format. Each sample was characterized for taxonomic composition (number and abundance) using QIIME. For presence/absence analyses, representative operational taxonomic units (OTUs) were clustered at the >97 percent identity

level and an OTU table was constructed using QIIME's pick_otus_through_otu_table.py script. In addition, we removed chimeras from the OTU table and filtered for chloroplast and mitochondrial DNA.

We compared alpha diversity (mean species diversity per treatment) using the Shannon Index as implemented in the vegan library (Solymos, Stevens, & Wagner, 2016) in R (Team RC, 2016). We compared OTU richness (number of OTUs found in each sample) and Pielou's evenness (calculated by dividing the Shannon index for diversity by the log of OTU richness). We tested for statistical significance in alpha diversity measures using the Analysis of Variance (ANOVA) to determine the effects of temperature, storage buffer, and sampling date in R. We compared beta diversity (the ratio between regional and local species diversity) using Bray-Curtis dissimilarity and we used PCOA for ordination and clustering. We then used adonis, a multivariate ANOVA based on dissimilarities to test for significant categorical differences with 1000 permutations in the picante library (Kembel et al., 2010) in R. Spearman correlation coefficients and regressions were calculated on R and GraphPad Prism Software.

RESULTS

To explore the effects of preservation buffer and temperature on the composition, abundance, and quality of bacterial DNA in fecal samples, we performed a longitudinal study over 56 days to evaluate the consequences of different storage methods in grouped samples (**Fig. 1**).

DNA Concentration and Purity

We used ANOVA to test for an effect of storage method on DNA concentration. We detected an overall effect of buffer on DNA concentration (F-value= 70.733, DF= 3, P<0.00001). In all storage methods, DNA concentration decreased over time (F-value = 7.5, DF=1, P<0.01). Significant interactions between buffer and time (F-value=3.77, DF=3, P=0.015) and temperature and time (F-value=9.8, DF=1, P=0.0027) were also detected.

By Time:

The DNA concentration of fecal samples incubated at room temperature without preservation buffer exhibited an 84% decrease in DNA concentration after Day 0 (1/Slope=-0.6812, R^2=0.9935, P=0.0002) (**Fig. 2**), where levels remained through Day 56. We used ANOVA to test for an effect of storage method on DNA quality (as measured by the ratio of A260/A280). We did not detect an overall effect of storage buffer (P=0.12) or storage temperature (P=0.66) on DNA quality. However, length of storage significantly affected DNA quality (F-value=4.365, DF=1, P=0.04) and there was a significant interaction between storage buffer and length of storage (F-value=2.88, DF=3, P=0.043). DNA quality declined slightly by Day 14 (1/Slope=-43.90, R^2=0.3135, P=0.0024) and then increased in the samples stored in 70% ethanol and no storage buffer on day 56 (1/Slope=109.4, R^2=0.4400, P=0.0014) (**Fig. S1**).

By Temperature:

Reducing the temperature used to store fecal samples reduced the amount of DNA loss after Day 0 (1/Slope=-31.73, R^2=0.09954, P=.0190). Unbuffered samples and samples in 50:50 glycerol:PBS were most affected by storage at room temperature, while samples in RNAlater and 70/% ethanol were consistent across all temperatures (**Fig. 2**). Unbuffered, refrigerated samples

exhibited a 1.5-fold higher DNA concentration at Day 7 and 2.25-fold at Day 14 compared to respective samples stored at room temperature. Refrigeration of samples stored in glycerol:PBS samples exhibited a similar trend in DNA concentration with fold-changes up to 4.9 times that of respective samples stored at room temperature, near levels found in fresh control samples. Of the three temperatures observed, freezing at -80°C yielded the highest DNA concentration over 56 days (AUC=76.291), with refrigeration close behind (AUC=72.528), and room temperature with the lowest yield (AUC=52.700). Freezing glycerol:PBS samples at days 14 and 56 led to a 706% and 811% higher DNA concentration, respectively, compared to samples stored in glycerol:PBS at room temperature. Little change was observed in DNA concentration of samples stored in RNAlater and 70% ethanol. DNA purity remained unchanged across all temperatures (1/Slope=-727.4, R^2=0.0436, P=0.0976) (**Fig. S1**).

By Buffer:

Preservation buffers were evaluated (RNAlater, 70% ethanol, and 50:50 glycerol:PBS) in comparison with unbuffered controls. DNA concentrations in unbuffered samples decreased by 75-80% over time, which were greatly improved with the addition of 70% ethanol (**Fig. 2**). 70% ethanol was the optimal method, exhibiting no significant changes in 56 days (1/Slope=-61.48, R^2=0.0365, P=0.4473). Preservation with 50:50 glycerol:PBS also improved DNA yield, but only under refrigeration or freezing conditions. Preservation with RNAlater yielded the lowest amount of DNA across all temperature and preservation buffers.

Microbial Diversity and Composition

206 Alpha Diversity measures: We performed an ANOVA to test for effects of storage buffer,
207 storage temperature, and duration of sample storage on the Shannon Diversity Index, Species
208 Richness, and Species Evenness. Storage buffer had a statistically significant effect on Shannon
209 Diversity Index values (F-value=3.07, DF=3, P=0.03). We did not detect an effect of storage
210 temperature (F-value=2.2, DF=1, P=0.14) or duration of sample storage (F-value=0.69, DF=1,
211 P=0.4) on Shannon Diversity values. Storage buffer (F-value=12.4, DF=3, P<0.00001), duration
212 of sample storage (F-value=10.8, DF=1, P=0.0016), and the interaction between storage buffer
213 and duration of sample storage (F-value=9.67, DF=3, P<0.00001) significantly affected observed
214 species richness levels. In addition, for species evenness, we found that there were significant
215 effects associated with interactions between storage buffer and storage temperature (F-
216 value=3.98, DF=3, P=0.01), storage buffer and duration of sample storage (F-value=4.9, DF=3,
217 P=0.004), and buffer, storage temperature, and duration of sample storage (F-value=3.1, DF=3,
218 P=0.03).
219 Beta Diversity measures: We used a permutational ANOVA to test for effects of storage method
220 on the degree of clustering of bacterial communities in our stored samples. We detected overall
221 effects on bacterial community of storage buffer (F-value= 6.87, DF= 3, P<0.001), storage
222 temperature (F-value=1.77, DF= 3, P=0.037), and duration of sample storage (F-value=3.68,
223 DF= 3, P<0.001).
224
225 *By Time:*
226 Complementary to the differences detected in DNA concentration, we found that the microbial
227 composition in unbuffered samples also changed over time. Analysis of weighted and
228 unweighted PCoA plots show that most Day 0 samples (in red) clustered together, indicating a

high degree of similarity among samples extracted regardless of buffer preservation at the starting time point. From Day 7 to Day 56 (in purple, blue, green), unbuffered samples did not cluster with fresh control samples, indicating a shift in microbial composition between 0 and 7 days after fecal collection (**Fig. 3**). Accordingly, taxonomic evaluation also revealed shifts in relative abundance of bacterial phyla of unbuffered samples, particularly after Day 0 at room temperature. This was exhibited by a marked increase in Firmicutes and decrease in Bacteroidetes (**Fig. 4**), or increase in Firmicutes:Bacteroidetes ratio (1/Slope=1.385, R^2=0.4687, P=0.0419) (**Fig. 5B**) compared to fresh samples.

By Temperature:

Weighted PCoA analysis of unbuffered samples indicated that shifts in microbial composition at room temperature can be minimized by lowering the temperature (**Fig. 3**). Samples clustered very closely when stored at -80°C, and segregated with increasing temperature. Samples stored at room temperature without buffer or with 50:50 glycerol:PBS past Day 7 did not cluster with the sample controls at Day 0. Samples with 50:50 glycerol:PBS clustered closer to control samples with refrigeration and even closer with freezing. However, this pattern was not observed in unbuffered samples. Unbuffered samples did not cluster closely with control samples under refrigeration and freezing conditions. Investigation of the bacterial phyla in these communities revealed a notable reduction in relative abundance of Bacteroidetes and minimal increase in Firmicutes in unbuffered samples stored at refrigeration and freezing conditions (**Fig. 4A**). Although freezing is one of the most commonly used tools for stool preservation, we observed that freezing without buffer lowers the relative abundance of Bacteroidetes (**Fig. 5A**) as previously described (Bahl, MI, Bergstrom, A, & Licht, TR, 2012) and increases the

252 Firmicutes:Bacteroidetes ratio (**Fig. 5B**) compared to fresh samples. With the addition of
253 preservation buffer, a collective analysis of all frozen samples showed that temperature reduction
254 to -80°C led to negligible changes in relative abundances of bacterial phyla compared to fresh
255 samples (**Fig. 5C**).
256
257 *By Buffer:*
258 Weighted PCoA analysis shows that across all temperatures, microbial communities in samples
259 stored in 70% ethanol and RNA clustered very closely with those in fresh control samples (**Fig.**
260 **3**). Samples stored in 50:50 glycerol:PBS clustered with control samples only under refrigeration
261 and freezing conditions, while samples without buffer did not cluster with fresh samples
262 regardless of temperature. Supporting this notion, 70% ethanol and RNAlater preserved relative
263 abundances of bacterial phyla regardless of temperature, though RNAlater storage led to slightly
264 higher levels of Bacteroidetes and lower levels of Firmicutes. Samples preserved with 50:50
265 glycerol:PBS at room temperature were different from samples without buffer, characterized by
266 a notable increase in bacterial proportions of phylum Actinobacteria, class Coriobacteriia . (**Fig.**
267 **4B**).
268
269 DNA Concentration and Microbial Composition
270 Since both temperature and buffer preservation influenced fecal DNA concentration levels, we
271 explored how DNA concentration levels might be associated with microbiota composition. A
272 correlation analysis demonstrated that there was a significant positive association between DNA
273 concentration and relative abundance of Fusobacteria and Proteobacteria (**Fig. 6**).
274

275

276 **DISCUSSION**

277 There is increasing evidence highlighting the importance of microbial DNA preservation in
278 multitude of settings, including health evaluations, research endeavors, and forensic science.
279 Characteristic signatures of microbiota have been explored as a result of the availability of next-
280 generation sequencing, extending our knowledge past culturable methods. One of the most
281 readily available resources to study microbes in humans and animals is fecal collection (Hale et
282 al., 2016). Not surprisingly, microbiota, including that of the gut, is often transient and dynamic,
283 posing a challenge for scientists to make sense of samples post-collection. Microbial DNA can
284 be degraded through environmental perturbation and subsequent hydrolysis, oxidation, and
285 methylation, supporting the need to limit spontaneous decay (Lindahl, 1993). Nevertheless, there
286 is increasing interest in studying animal, human, and environmental health by distinguishing
287 small changes in the microbiome in high resolution. This is accompanied by a need for more
288 effective storage methods that precisely and accurately capture the microbial community at a
289 given time-point. Assessing changes in fecal microbiota over time would provide insight on
290 whether changes we observe are biologically relevant and useful for outcome measures.

291

292 This approach has paved the way for developing microbiome tools in forensic science (Kim, M,
293 Zorraquino & Tagkopoulos, 2015) Microorganisms are ubiquitous and collectively form a
294 potentially unique signature, providing at least in theory the means to locate people in space and
295 time. Next-generation sequencing provides the possibility that microbial information could
296 eventually be used to locate and identify individuals as well as their patterns of decomposition
297 (Fornaciari, 2017) A combination of machine learning techniques and microbiome data may

298 provide unprecedented scientific data for the criminal justice system (Metcalf et al., 2017). It is
299 evident that investigation of optimal methods for DNA preservation will have important impacts
300 on microbiome studies in field, clinic, and laboratory settings.
301
302 A variety of studies have examined preservation methods to minimize post-sampling alterations
303 in fecal microbial DNA. Such experiments have evaluated fixation conditions with 95% ethanol,
304 70% ethanol, FTA card, OMNI gene Gut, RNAlater, glycerol, refrigeration, and freezing.
305 However, there is little consensus on the optimal buffer and temperature condition for microbial
306 preservation (Nechvatal et al., 2008; Cardona et al., 2013; Kolodziej et al., 2013; McKain et al.,
307 2013; Fliegerova et al., 2014; Hale et al., 2015; Song et al., 2016; Hale et al., 2016; Metzler-
308 Zebeli et al., 2016). Furthermore, a limited number of studies have evaluated the combination of
309 these chemical buffers with temperature reduction to optimize DNA preservation. Our study
310 examined the influence of three commonly used preservation buffers on the microbial integrity
311 of canine feces after eight weeks of storage at room temperature (25°C), refrigeration (4°C), and
312 freezing (-80°C). Since we were interested in measuring change over time in identical fecal
313 samples, we utilized one canine donor and homogenized the stool sample prior to tube allocation.
314 We speculate that there may be inter-individual differences in storage because different dogs
315 may have different bacterial compositions and some fecal bacterial groups may be more prone to
316 temperature or buffer alterations. We report that fecal DNA concentration and microbial
317 composition changes over time, and that the common practice of preservation by freezing may
318 not be adequate in maintaining bacterial DNA. In fact, correlations between fecal DNA
319 concentration and bacterial composition indicate that DNA loss is associated with reductions in
320 Fusobacteria and Proteobacteria. Results showed clear differences between preservation methods

and analysis of DNA concentration, purity, and microbial diversity and composition, highlighting the need to consider post-collection dynamics in microbiome research. Although there were minor differences, total DNA recovery and fecal composition of samples stored at 4°C were like that of samples stored at -80°C over 56 days, suggesting that deep freezing conditions may not be necessary to maintain stability of microbiota in fecal samples. Our findings are consistent with a study previously showing that refrigeration at 4°C is adequate in preserving human fecal microbiota (Choo, Leong, & Rogers, 2015) for subsequent analysis.

To evaluate methods that are most cost-effective and accessible to all studies, we were especially interested in the efficacy of ethanol preservation. Previous studies have utilized 70% and 95% ethanol, each with varying results in DNA yield and microbial community stability (Hale et al., 2015; Song et al., 2016) Efficacy of ethanol preservation may be dependent on concentration due to species-species differences in stool consistency. Based on our canine stool sample, we decided to use 70% ethanol for fecal preservation. We found that it yielded the highest amount of DNA and most closely resembled that of fresh samples within 56 days compared to other buffer solutions. Studies with contrasting results report that perhaps the penetration of 70% ethanol in fecal samples may play a role in inadequate preservation, but we circumvented this by including a homogenization step. Additional experiments are warranted to examine 70% and 95% ethanol using a homogenizing protocol and various stool consistencies. Another historically supported and commonly used DNA stabilization buffer is RNAlater (Schnecker et al., 2012). Studies have recently shown that this method yields low levels of DNA yield (Hale et al., 2015; Song et al., 2016) due to DNA degradation. We hypothesize that residual RNAlater remaining in fecal samples may interfere with the cell lysis and protein digestion in DNA extraction, inhibiting

optimal DNA isolation. However, a thorough analysis of the microbial composition over time showed that RNAlater was effective and consistent across all three temperatures, comparable to that of 70% ethanol. This consistency suggests that temperature control may not be as critical to DNA yield and microbial composition with the use of preservation buffers such as RNAlater and 70% ethanol. Further investigation of DNA concentration was performed using both a QUBIT™ dsDNA HS Assay and a Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE). We noted that Nanodrop-obtained values were consistently higher than that of QUBIT, and markedly higher in RNAlater samples (**Figure S2**). Lastly, fecal DNA is also commonly preserved by subjecting samples to freezing conditions without the use of DNA stabilization buffers. In this study, we showed that despite temperature reduction, microbial DNA changes over time without buffer. Changes in relative abundances of bacterial phyla observed in unbuffered samples were eliminated with the addition of preservation buffer. For example, the use of 50:50 glycerol:PBS in refrigeration and freezing temperatures improved both DNA composition and concentration compared to unbuffered controls at room temperature. Storage of fecal DNA in 70% ethanol was the optimal preservation method regardless of temperature, highlighting its utility in circumstances devoid of temperature control. We conclude that while preservation buffer and temperature have an additive effect, the contributions to microbial preservation are largely buffer-specific.

It is clear that post-sampling conditions may impact results and interpretations of microbial DNA. Microbial DNA outcomes are widely used in medicine and healthcare, such as the Firmicutes:Bacteroidetes ratio, a comparison of butyrate to propionate/acetate-producing bacteria (Bahl, Bergstrom, & Licht, 2012). This ratio, along with levels of Proteobacteria and

Actinobacteria, is used as a potential indicator of gastrointestinal health and immune balance (Honneffer et al., 2014) While there is no direct comparison to disease state in this study, we showed that alterations in fecal DNA composition can markedly shift this ratio, particularly with inadequate temperature reduction or preservation buffer. More studies investigating fecal DNA preservation and stability in chronic diseases are warranted. Preservation buffers may enrich for certain bacteria such as Coriobacteriia, a group whose known members are Gram-positive, nonsporulating, non-motile, facultative anaerobes common to the animal and human gut microbiota population, which we observed in samples with 50:50 glycerol:PBS. Coriobacteriia have been associated with lipid and xenobiotic metabolism (Cho et al., 2016), as well as severity of various human diseases such as periodontitis, halitosis, blood bacteremia, and ulcerative colitis (Saunders et al., 2009; Gupta et al., 2013)

Stabilizers such as glycerol can provide a supplemental carbon source (Murarka et al., 2008) for selective bacterial enrichment at room temperature. Our data suggests that caution should be taken in sample handling and use of adequate storage buffers to accurately and consistently analyze fecal microbial DNA.

CONCLUSIONS

A 56-day longitudinal study of canine fecal microbiota was conducted to evaluate storage conditions with RNAlater, 70% ethanol, 50:50 glycerol:PBS, and no buffer at -80°C, 4°C, and 25°C. Fecal samples exhibited DNA degradation and altered composition and diversity regardless of temperature reduction, suggesting that gold standard methods of immediate freezing at -80°C may not be optimal for fecal preservation. In fact, minimal differences were seen between samples stored at 4°C and those at -80°C. While temperature reduction and storage

buffer have an additive effect, effective preservation was largely driven by buffer, most notably in samples stored in 70% ethanol. Fecal preservation with 70% ethanol yielded DNA concentrations and microbial composition closest to that of fresh samples at all temperatures, highlighting the potential of its utility in field, laboratory, and clinical settings. This study underlines the need for more comprehensive evaluation of fecal DNA storage methods for accurate downstream microbial analysis.

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REFERENCES

Bahl, MI, Bergstrom, A, & Licht, TR (2012). Freezing fecal samples prior to DNA extraction affects the *Firmicutes* to *Bacteroidetes* ratio determined by downstream quantitative PCR analysis. *FEMS Microbiology Letters*, 329: 193-197. doi:10.1111/j.1574-6968.2012.02523.x

Beja-Pereira, A, Oliveira, R, Alves, PC, Schwartz, MK, & Luikart, G (2009). Advancing ecological understandings through technological transformations in noninvasive genetics. *Molecular Ecology Resources,* 9: 1279-1301. doi:10.1111/j.1755-0998.2009.02699.x

412 Bressan, EA, Rossi, ML, Gerald, LT, & Figueira, A (2014). Extraction of high-quality DNA
413 from ethanol-preserved tropical plant tissues. *BMC Research Notes,* 7: 268.
414 doi:10.1186/1756-0500-7-268
415 Caporaso, JG, Kuczynski, J, Stombaugh, J, Bittinger, K, Bushman, FD, Costello, EK, Fierer, N,
416 Pena, AG, Goodrich, JK, Gordon, JI, Huttley, GA, Kelley ST, Knights, D, Koenig, JE,
417 Ley, RE, Lozupone, CA, McDonald, D, Muegge, BD, Pirrung, M, Reeder, J, Sevinsky,
418 JR, Turnbaugh, PJ, Walters, WA, Widmann, J, Yatsunenko, T, Zaneveld, J, & Knight, R
419 (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature*
420 *Methods*. 7: 335-336. doi:10.1038/nmeth.f.303
421 Cardona, S, Eck, A, Cassellas, M, Gallart, M, Alastrue, C, Dore, J, Azpiroz, F, Roca, J, Guarner,
422 F, & Manichanh, C (2012). Storage conditions of intestinal microbiota matter in
423 metagenomics analysis. *BMC Microbiology*. 12: 158. doi:10.1186/1471-2180-12-158
424 Carroll, IM, Ringel-Kulka, T, Siddle, JP, Klaenhammer, TR, & Ringel, Y (2012).
425 Characterization of the fecal microbiota using high-throughput sequencing reveals a
426 stable microbial community during storage. *PloS one,* 7: e46953-e46953.
427 doi:10.1371/journal.pone.0046953
428 Cho GS, Ritzmann F, Eckstein M, Huch, M, Briviba, K, Behsnilian, D, Neve, H, & Franz, CM
429 (2016). Quantification of *Slackia* and *Eggerthella* spp. in Human Feces and Adhesion of
430 Representatives Strains to Caco-2 Cells. *Frontiers in Microbiology*. 7: 658.
431 doi:10.3389/fmicb.2016.00658
432 Choo, JM, Leon, LEX, & Rogers, GB (2015). Sample storage conditions significantly influence
433 faecal microbiome profiles. *Scientific Reports.* 5: 16350. doi:10.1038/srep16350

Fierer, N, Lauber, CL, Zhou, N, McDonald, D, Costello, EK, & Knight, R (2010). Forensic identification using skin bacterial communities. *Proceedings of the National Academy of Sciences of the USA,* 107: 6477-6481. doi:10.1073/pnas.1000162107

Fliegerova, K, Tapio, I, Bonin, A, Mrazek, J, Callegari, ML, Bani, P, Bayat, A, Vilkki, J, Kopecny, J, Shingfield, KJ, Boyer, F, Coissac, E, Taberlet, P, & Wallace, RJ (2014). Effect of DNA extraction and sample preservation method on rumen bacterial population. *Anaerobe,* 29: 80-84. doi:10.1016/j.anaerobe.2013.09.015

Fornaciari, A (2017) Environmental microbial forensics and archaeology of past pandemics. *Microbiology Spectrum*, 5. doi:10.1128/microbiolspec.EMF-0011-2016

Gorzelak, MA, Gill, SK, Tasnim, N, Ahmadi-Vand, Z, Jay, M, & Gibson, DL (2015). Methods for Improving Human Gut Microbiome Data by Reducing Variability through Sample Processing and Storage of Stool. *PloS one*, 10: e0134802. doi:10.1371/journal.pone.0134802

Gupta, RS, Chen, WJ, Adeolu, M, & Chai, Y (2013). Molecular signatures for the class Coriobacteriia and its different clades; proposal for division of the class Coriobacteriia into the emended order Coriobacteriales, containing the emended family Coriobacteriaceae and Atopobiaceae fam. nov., and Eggerthellales ord. nov., containing the family Eggerthellaceae fam. nov. *International Journal of Systematic and Evolutionary Microbiology,* 63: 3379-3397. doi:10.1099/ijs.0.048371-0

Hale, VL, Tan, VL, Knight, R, Amato, KR (2015). Effect of preservation method on spider monkey (Ateles geoffroyi) fecal microbiota over 8 weeks. *Journal of Microbiological Methods.* 113: 16-26. doi:10.1016/j.mimet.2015.03.021

456 Hale, VL, Tan, CL, Niu, K, Yang, Y, Cui, D, Zhao, H, Knight, R, & Amato, KR (2016). Effects
457 of field conditions on fecal microbiota. *Journal of Microbiological Methods,* 130: 180-
458 188. doi:10.1016/j.mimet.2016.09.017
459 Hayashi, H, Sakamoto, M, & Benno, Y (2002). Phylogenetic analysis of the human gut
460 microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods.
461 *Microbiology and Immunology,* 46: 535-548. doi:10.1111/j.1348-0421.2002.tb02731.x
462 Honnefer, JB, Minamoto, Y, & Suchodolski, JS (2014). Microbiota alterations in acute and
463 chronic gastrointestinal inflammation of cats and dogs. *World Journal of*
464 *Gastroenterology.* 20: 16489-97. doi:10.3748/wjg.v20.i44.16489
465 Huang, M, Sun, XJ, Zhou, Y, & Wang, XM (2016). Comparison of methods to preserve Rheum
466 palmatum (Polygonaceae) for efficient DNA extraction and PCR amplification. *Genetics*
467 *and Molecular Research,* 15: gmr.15038019. doi:10.4238/gmr.15038019
468 Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, Blomberg SP, &
469 Webb CO (2010). Picante: R tools for integrating phylogenies and ecology.
470 *Bioinformatics.* 26:1463-1464. doi:10.1093/bioinformatics/btq166
471 Kolodziej, K, Nikolov, I, Schulz, HK, Theissinger, K, & Schulz, R (2013). Evaluation of fecal
472 storage and DNA extraction methods in wild boat (Sus scrofa). *Biochemical Genetics*. 51:
473 406-412. doi:10.1007/s10528-013-9573-1
474 Kennedy, NA, Walker, AW, Berry, SH, Duncan, SH, Farquarson, FM, Louis, P, & Thoomson,
475 JM (2014). The impact of different DNA extraction kits and laboratories upon the
476 assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PloS*
477 *one, 9*(2), e88982. doi:10.1371/journal.pone.0088982

478 Kim, M, Zorraquino, V, & Tagkopoulous, I (2015). Microbial forensics: predicting phenotypic
479 characteristics and environmental conditions from large-scale gene expression profiles.
480 *Plos Computational Biology*. 11: e1004127. doi:10.1371/journal.pcbi.1004127
481 Lang, JM, Eisen, JA, Zivkovic, AM (2014). The microbes we eat: abundance and taxonomy of
482 microbes consumed in a day's worth of meals for three diet types. *PeerJ.* 2: e659.
483 doi:10.7717/peerj.659
484 Lindahl, T (1993). Instability and decay of the primary structure of DNA. *Nature*. 362: 709-715.
485 doi:10.1038/362709a0
486 Lowrey, L, Woodhams, DC, Tacchi, L, & Salinas, I (2015). Topographical Mapping of the
487 Rainbow Trout (Oncorhynchus mykiss) Microbiome Reveals a Diverse Bacterial
488 Community with Antifungal Properties in the Skin. *Applied and Environmental*
489 *Microbiology*, 81: 6915-6925. doi:10.1128/aem.01826-15
490 Magoč, T & Salzberg, SL (2011). FLASH: fast length adjustment of short reads to improve
491 genome assemblies. *Bioinformatics*. 1: 2957-63. doi:10.1093/bioinformatics/btr507
492 Markowitz, V, Hugenholtz, P, Kyrpides, NC, Klenk, HP, & Han, C (2009). Complete genome
493 sequence of Eggerthella lenta type strain (IPP VPI 0255). *Standards in Genomic*
494 *Sciences.* 1: 174–82. doi:10.4056/sigs.33592
495 Metcalf, JL, Xu, ZZ, Bouslimani, A, Dorrestein, P, Carter, DO, & Knight, R (2017). Microbiome
496 tools for forensic science. *Trends in Biotechnology*. 35: 814-823.
497 doi:10.1016/j.tibtech.2017.03.006
498 Metzler-Zebeli, BU, Lawlor, PG, Magowan, E, & Zebeli, Q (2016). Effect of freezing conditions
499 on fecal bacterial composition in pigs. *Animals.* 6: E18. doi:10.33990/ani6030018

McKain, N, Genc, B, Snelling, TJ, & Wallace, RJ (2013). Differential recovery of bacterial and archaeal 16S rRNA genes from ruminal digesta in response to glycerol as cryoprotectant. *Journal of Microbiological Methods,* 95: 381-383. doi:10.1016/j.mimet.2013.10.009

Murarka, A, Dharmadi, Y, Yazdani, SS, & Gonzalez R (2008). Fermentative utilization of glycerol by Escherichia coli and its implications for the production of fuels and chemicals. *Applied and Environmental Microbiology*. 74: 1125-1135. doi:10.1128/AEM.02192-07

Murphy, MA, Waits, LP, Kendall, KC, Wasser, SK, Higbee, JA, & Bogden, R (2002). An evaluation of long-term preservation methods for brown bear (Ursus arctos) faecal DNA samples. *Conservation Genetics,* 3: 435-440. doi:10.1023/a:1020503330767

Nechvatal, JM. Ram, JL, Basson, MD, Namprachan, P, Niec, SR, Badsha, KZ, Matherly, LH, Majumdar, APN, & Kato, I (2008). Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. *Journal of Microbiological Methods,* 72: 124-132. doi:10.1016/j.mimet.2007.11.007

Paulino, LC, Tseng, CH, Strober, BE, & Blaser, MJ (2006). Molecular analysis of fungal microbiota in samples from healthy human skin and psoriatic lesions. *Journal of Clinical Microbiology,* 44: 2933-2941. doi:10.1128/JCM.00785-06

Petersen, C,& Round, JL (2014). Defining dysbiosis and its influence on host immunity and disease. *Cell Microbiology,* 16: 1024-1033. doi:10.1111/cmi.12308

Saunders E, Pukall R, Abt, B, Lapidus, A, Glavina Del Rio, T, Copeland, A, Tice, H, Cheng, JF, Lucas, S, Chen, F, Nolan, M, Bruce, D, Goodwin, L, Pitluck, S, Ivanova, N, Mavromatis, K, Ovchinnikova, G, Pati, A, Chen, A, Palaniappan, K, Land, M, Hauser, L, Chang, YJ,

Jeffries, CD, Chain, P, Meincke, L, Sims, D, Brettin, T, Detter, JC, Goker, M, Bristow, J, Eisen, JA,

Schnecker, J, Wild, B, Fuchsleuger, L, & Richter, A (2012). A field method to store samples from temperature mountain grassland soils for analysis of phospholipid fatty acids. *Soil Biology and* Biochemistry, 51: 81-83. doi: 10.1016/j.soilbio.2012.02.029

Solymos P, Stevens MHH, & Wagner H. 2016. vegan: Community Ecology Package. R package version 2.3-5 https://cran.r-project.org/package=vegan

Song, SJ, Amir, A, Metcalf, JL, Amato, KR, Xu, ZZ, Humphrey, G, & Knight, R (2016). *Applied and Environmental Science*. 1: e00021-16. doi:10.1128/mSystems.0021-16

Sorensen, A, Rahman, E, Canela, C, Gangitano, D, & Hughes-Stamm, S (2016). Preservation and rapid purification of DNA from decomposing human tissue samples. *Forensic Science International: Genetics,* 25: 182-190. doi:10.1016/j.fsigen.2016.05.013

Team RC. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/

Tedjo, DI, Jonkers, DMAE, Masclee, AA, van Best, N, Pierik, MJ, & Penders, J (2015) Effect of sampling and storage on fecal microbiota composition in healthy and diseased subjects. *PloS one, 10: e0126685. doi:10.1371/journal.pone.0126685*

Tims, S, van Wamel, W, Endtz, H P, van Belkum, A, & Kayser, M (2010). Microbial DNA fingerprinting of human fingerprints: dynamic colonization of fingertip microflora challenges human host inferences for forensic purposes. *International Journal of Legal Medicine,* 124: 477-481. doi:10.1007/s00414-009-0352-9

Verhulst, NO, Qiu, YT, Beijleveld, H, Maliepaard, C, Knights, D, Schulz, S, Berg-Lyons, D, Lauber, CL, Verduijn, W, Haasnoot, GW, Mumm, R, Bouwmeester, FHJ, Claas, Dicke,

M, A. van Loon, JJ, Takken, W, Knight, R, & Smallegange, RC (2011). Composition of human skin microbiota affects attractiveness to malaria mosquitoes. *PloS one,* 6: e28991. doi:10.1371/journal.pone.0028991

Weese, JS (2013). The canine and feline skin microbiome in health and disease. *Veterinary Dermatology,* 24: 137-145.e131. doi:10.1111/j.1365-3164.2012.01076.x

West, CE, Renz, H, Jenmalm, MC, Kozyrskyj, AL, Allen, KJ, Vuillermin, P, & Prescott, SL (2015). The gut microbiota and inflammatory noncommunicable diseases: Associations and potentials for gut microbiota therapies. *Journal of Allergy and Clinical Immunology*, 135: 3-13. doi:10.1016/j.jaci.2014.11.012

Wu, GD, Lewis, JD, Hoffmann, C, Chen, YY, Knight, R, Bittinger, K, Hwang, J, Chen, J, Berkowsky, R, Nessel, L, Li, H, & Bushman, FD (2010). Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. *BMC Microbiology,* 10: 206. doi:10.1186/1471-2180-10-206

Yuan, S, Cohen, DB, Ravel, J, Abdo, Z, & Forney, LJ (2012). Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PloS one,* 7: e33865. doi:10.1371/journal.pone.0033865

Figure 1(on next page)

Chronological flowchart of fecal DNA preservation experiment

Fecal samples from one canine donor were collected, aliquoted, and treated with no buffer (P), RNA later (R), 70% ethanol (E), and 50:50 Glycerol:PBS (G). After 2 hours of incubation at 25°C, Day 0 samples were immediately processed for DNA extraction, while other samples were stored at the indicated temperatures (room temp: 25°C, refrigeration: 4°C, freezing: -80°C) until extractions on days 7, 14, and 56.

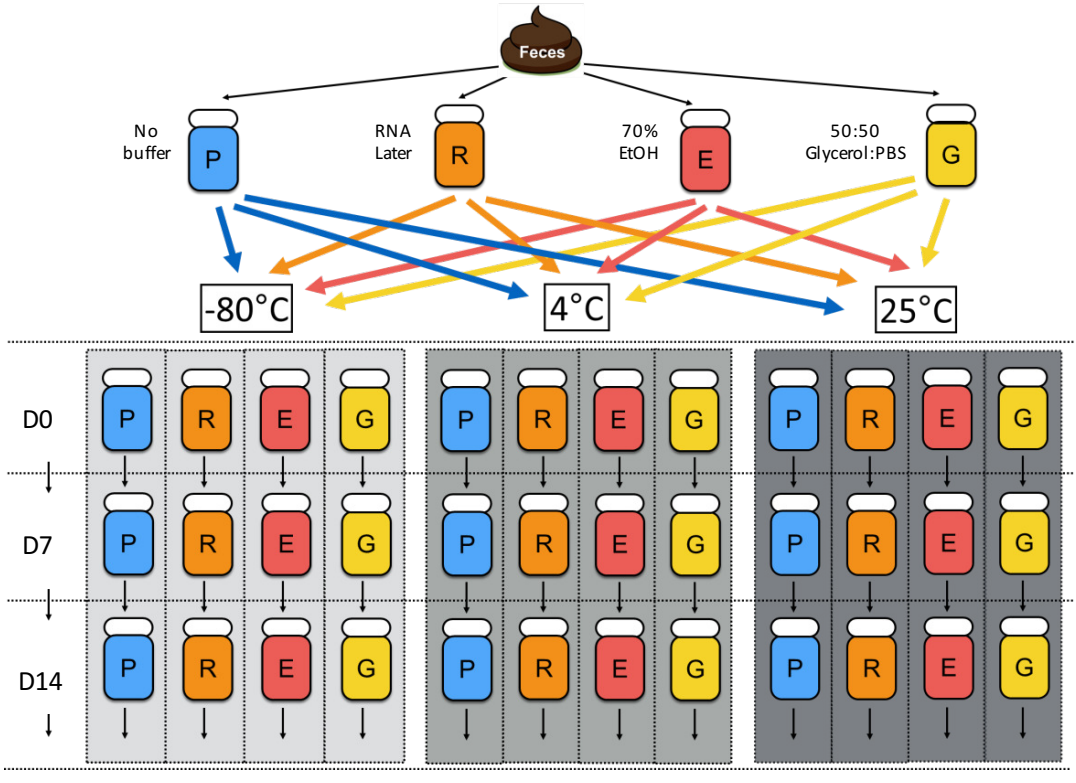

Feces
No buffer
P
RNA Later
R
70% EtOH
E
50:50 Glycerol:PBS
G
-80°C
4°C
25°C
D0
D7
D14
D56

Figure 2(on next page)

Average DNA concentration (µg/mL) by preservation method from Day 0 to 56 at 25°C, 4°C, and -80°C

DNA concentrations of fecal samples were obtained with QUBIT dsDNA HS Assay after DNA extraction. In all storage methods, DNA concentration decreased over time (F-value = 7.5, DF=1, P<0.01). Concentrations significantly varied by buffer, with the highest in samples preserved with 70% ethanol and lowest in samples with RNA later (DF=8.57, P<0.001).

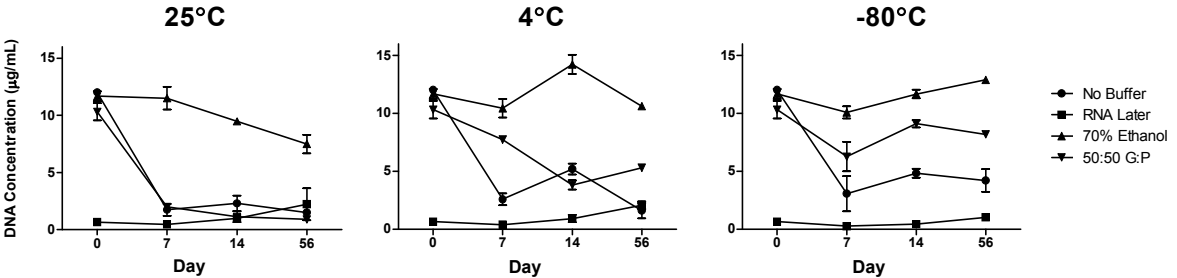

25°C
4°C
-80°C
DNA Concentration (µg/mL)
15
10
5
0
0
7
14
56
Day
No Buffer
RNA Later
70% Ethanol
50:50 G:P

Figure 3(on next page)

Principal coordinate analysis (PCoA) of weighted and unweighted UniFrac distances of microbial communities by temperature

Weighted UniFrac distances between communities (the evolutionary relatedness of taxa) and weighted UniFrac distances (the relative abundance of bacterial phyla) reveal that all samples clustered regardless of temperature on Day 0. Samples preserved without buffer and in 50:50 glycerol:PBS diverged after Day 0, whereas those in RNA later and 70% ethanol clustered with fresh control samples regardless of temperature.

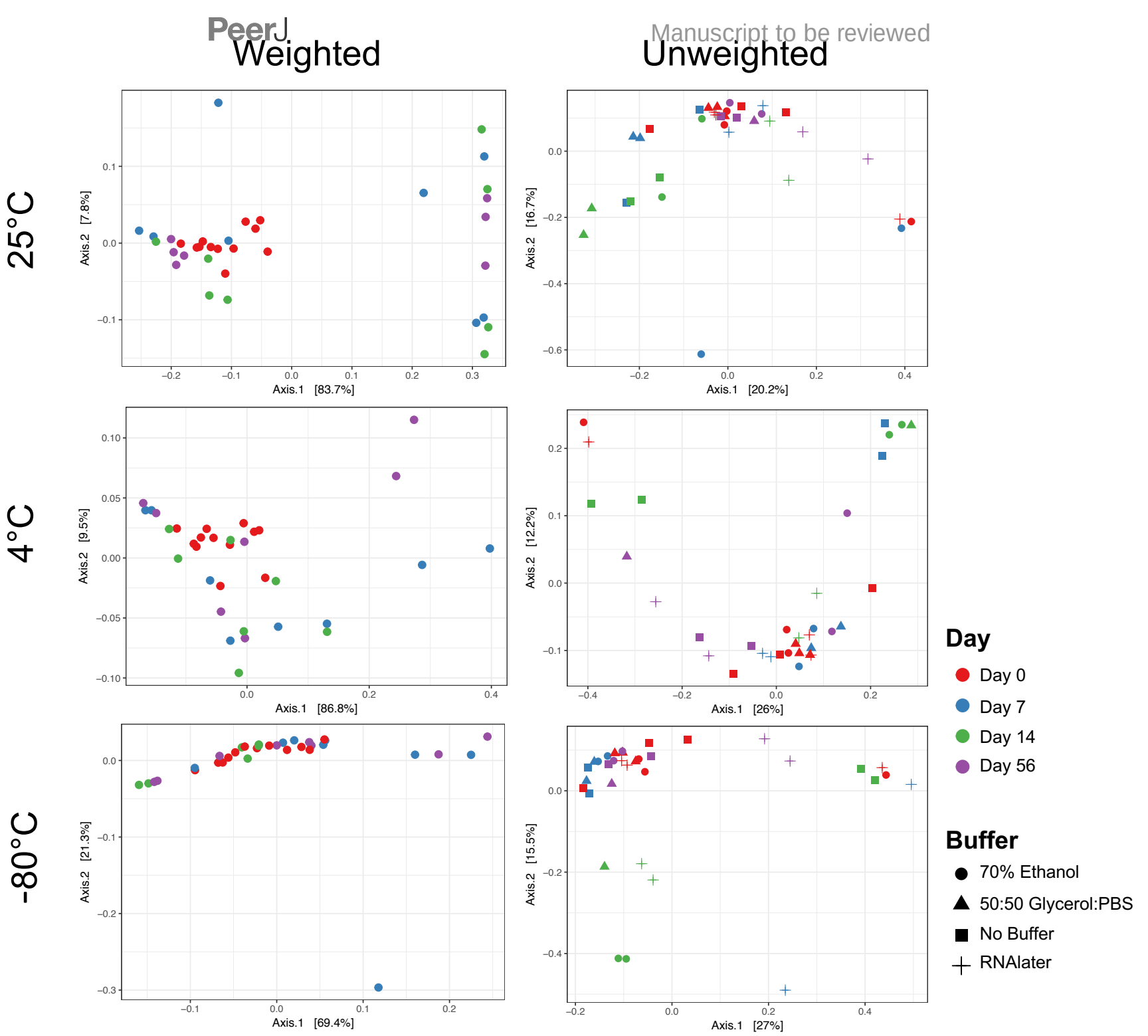
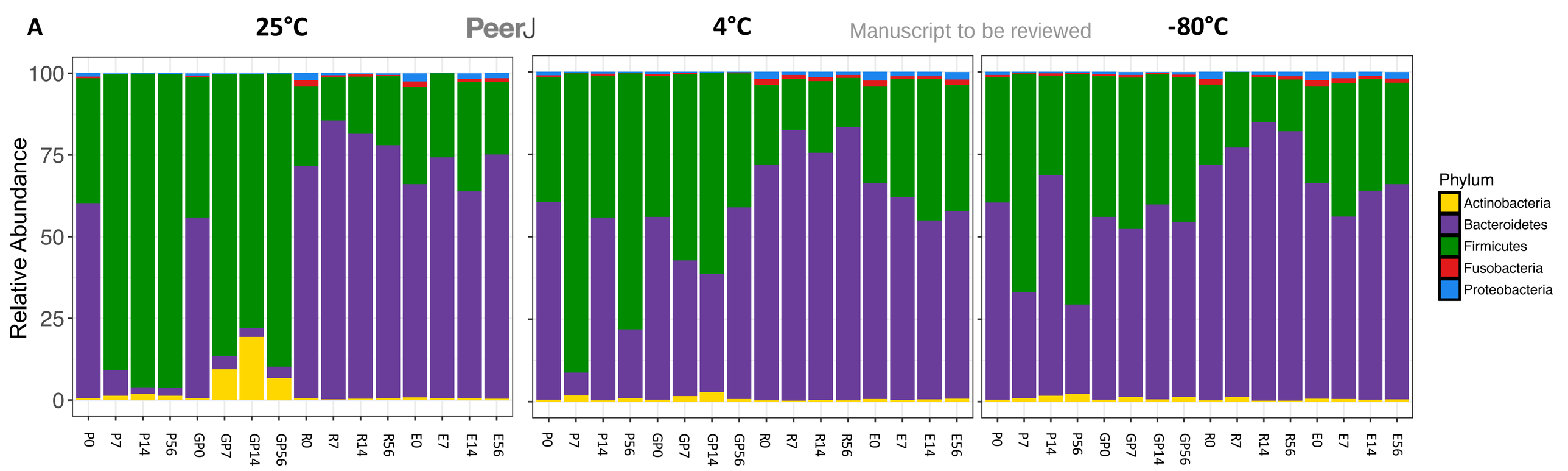

Weighted
Unweighted
25°C
4°C
-80°C
Axis.1 [83.7%]
Axis.2 [7.8%]
Axis.1 [20.2%]
Axis.2 [16.7%]
Axis.1 [86.8%]
Axis.2 [9.5%]
Axis.1 [26%]
Axis.2 [12.2%]
Axis.1 [69.4%]
Axis.2 [21.3%]
Axis.1 [27%]
Axis.2 [15.5%]
Day
Day 0
Day 7
Day 14
Day 56
Buffer
70% Ethanol
50:50 Glycerol:PBS
No Buffer
RNAlater

Figure 4(on next page)

Relative abundances of (A) bacterial phyla at 25°C, 4°C, -80°C and (B) bacterial class at 25°C

A) Across all temperatures, fresh control samples (X0) exhibited near identical relative abundances of the top five bacterial phyla. At room temperature after day 0, samples without buffer and samples immersed in 50:50 Glycerol:PBS showed significant relative increases in Phylum Firmicutes and decreases in Phylum Bacteroidetes. Refrigeration or freezing reduced changes in relative abundances of bacterial phyla in plain and glycerol:PBS samples over time. Samples preserved with RNA Later and 70% ethanol exhibited microbial compositions similar with that of fresh control samples. B) The notable increase in Phylum Actinobacteria in samples with 50:50 glycerol:PBS was further investigated by taxonomic class, and found to be attributed to an increase in Coriobacteriia.


A
25°C
4°C
-80°C
Relative Abundance
100
75
50
25
0
P0
P7
P14
P56
GP0
GP7
GP14
GP56
R0
R7
R14
R56
E0
E7
E14
E56
Phylum
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria

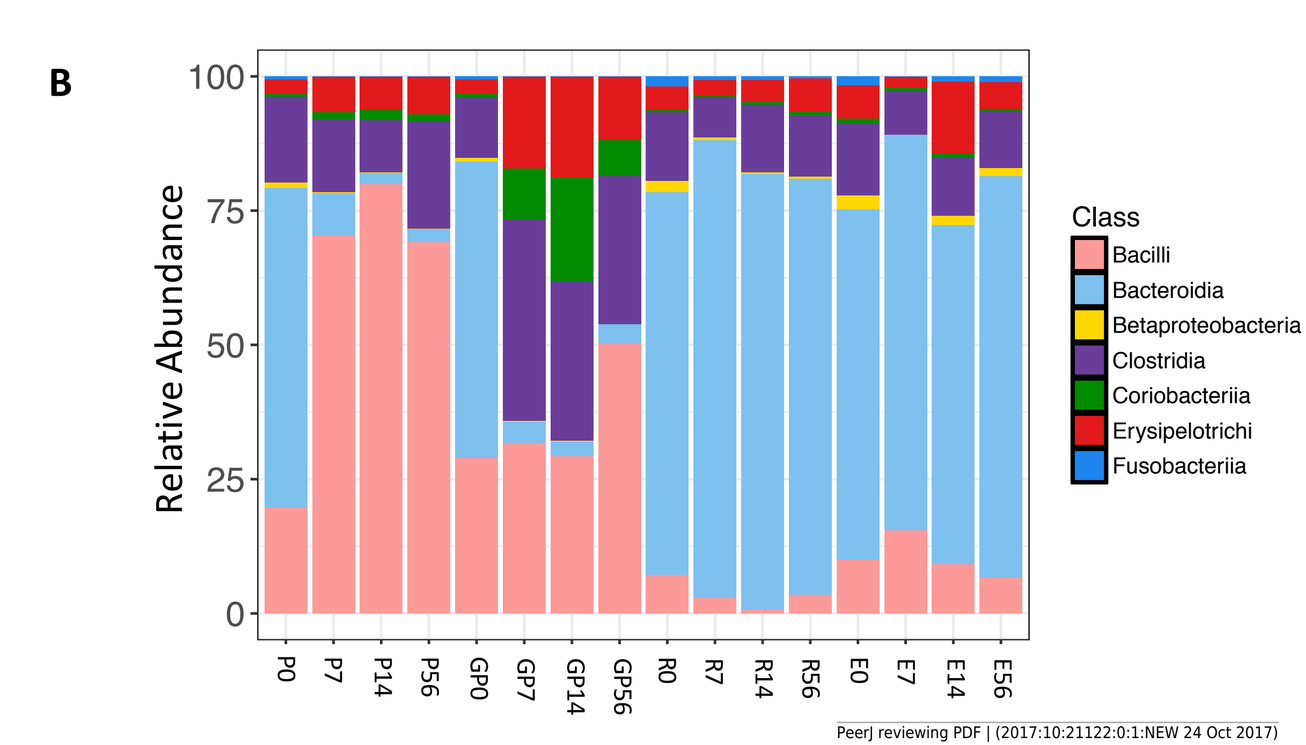

B
Relative Abundance
100
75
50
25
0
P0
P7
P14
P56
GP0
GP7
GP14
GP56
R0
R7
R14
R56
E0
E7
E14
E56
Class
Bacilli
Bacteroidia
Betaproteobacteria
Clostridia
Coriobacteriia
Erysipelotrichi
Fusobacteriia

Figure 5(on next page)

Effects of buffer and temperature on relative abundance of bacterial phyla over 56 days

Relative abundances of phyla **A)** Bacteroidetes and **B)** Firmicutes:Bacteroidetes ratio show maintenance of bacterial composition under freezing conditions and preservation with RNA later or 70% ethanol. C) Mean fold-change (frozen/fresh) of bacterial phyla in all buffered and unbuffered sample.

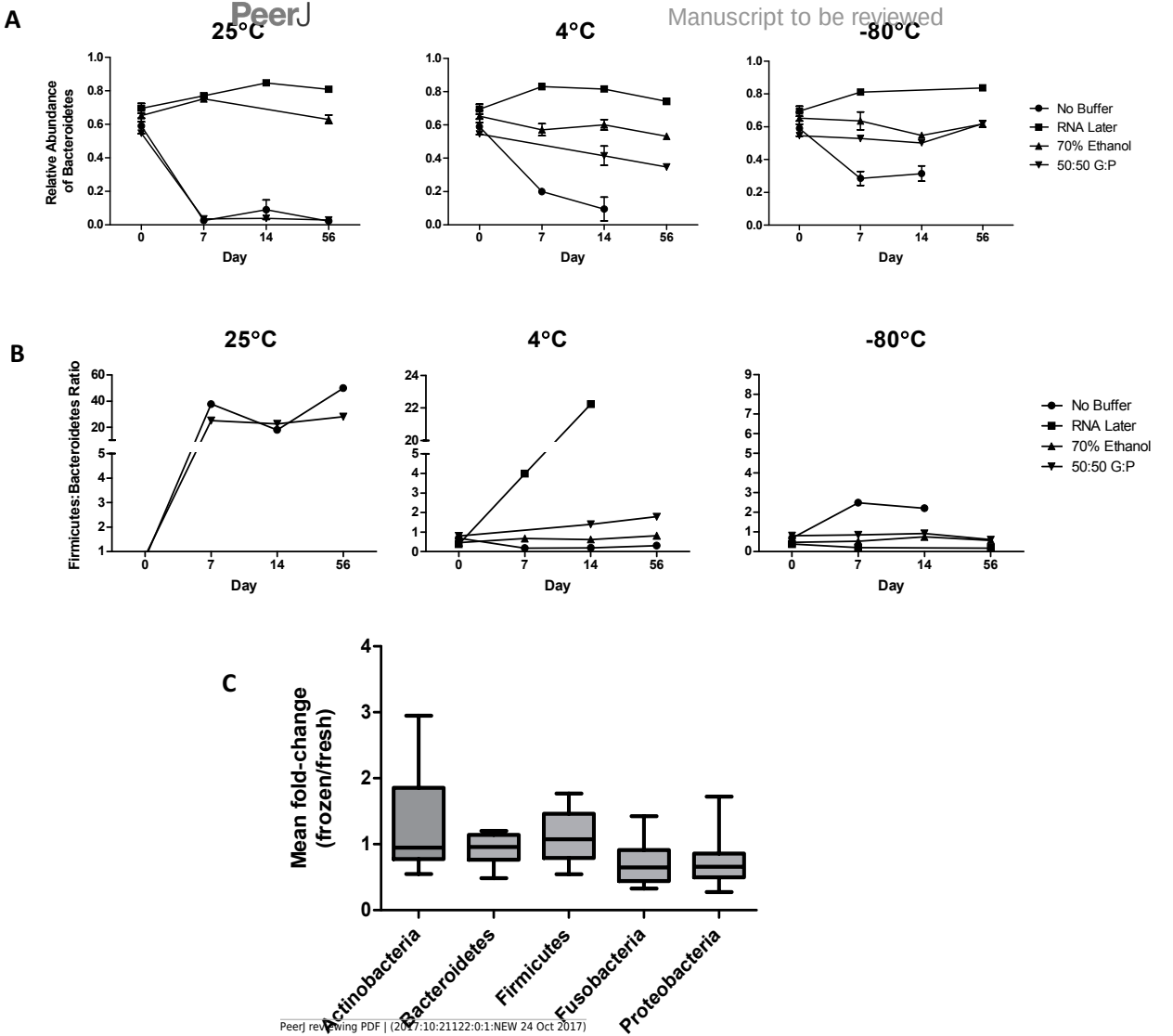
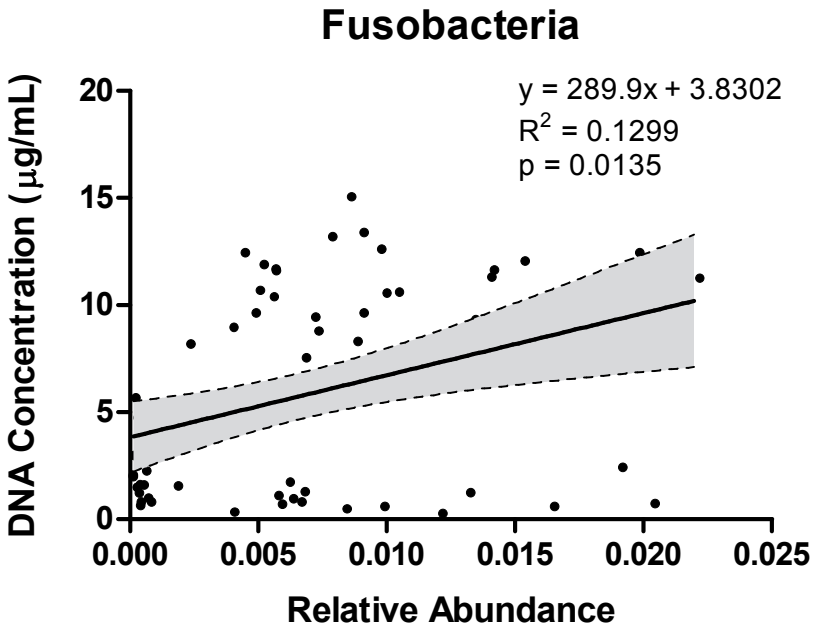
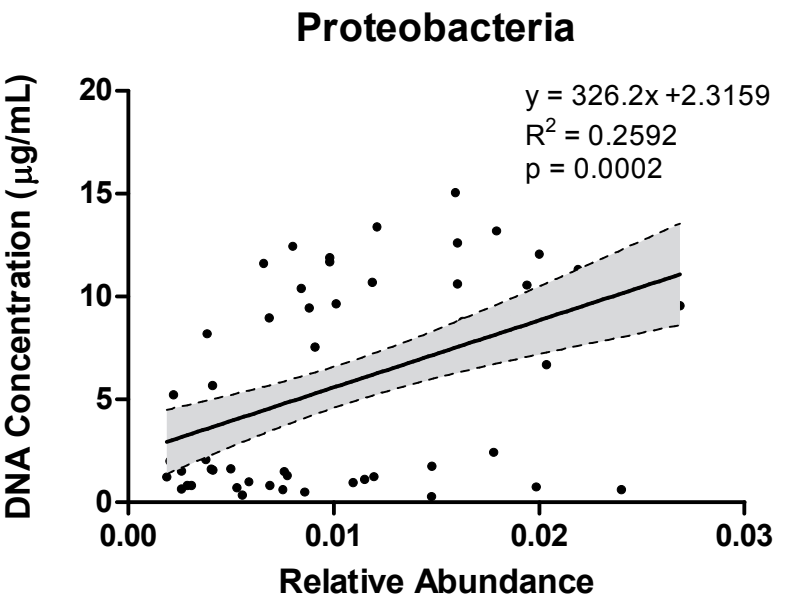

A
25°C
4°C
-80°C
Relative Abundance of Bacteroidetes
Day
No Buffer
RNA Later
70% Ethanol
50:50 G:P
B
25°C
4°C
-80°C
Firmicutes:Bacteroidetes Ratio
Day
No Buffer
RNA Later
70% Ethanol
50:50 G:P
C
Mean fold-change (frozen/fresh)
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria

Figure 6(on next page)

Correlation between DNA concentration and relative abundance of bacterial phyla

Shown as a linear regression, Spearman's correlation coefficient analysis of all samples in the study revealed a significant positive correlation between DNA concentration and phylum Fusobacteria and Proteobacteria.


Fusobacteria
y = 289.9x + 3.8302
R2 = 0.1299
p = 0.0135
DNA Concentration (µg/mL)
Relative Abundance
0
5
10
15
20
0.000
0.005
0.010
0.015
0.020
0.025


Proteobacteria
y = 326.2x +2.3159
R2 = 0.2592
p = 0.0002
DNA Concentration (µg/mL)
Relative Abundance
0
5
10
15
20
0.00
0.01
0.02
0.03