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Effects of preservation method on canine (*Canis lupus familiaris*) 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 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 bacterial 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 bacterial 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 closely resembled that of fresh samples, though RNAlater yielded significantly lower DNA concentrations (DF=8.57, P<0.001). Although bacterial 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 bacterial diversity and composition. The differences observed between samples highlight the need to consider optimized post-collection methods in microbiome research.

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23

24 **ABSTRACT**

25 Studies involving gut microbiome analysis play an increasing role in the evaluation of health and
26 disease in humans and animals alike. Fecal sampling methods for DNA preservation in
27 laboratory, clinical, and field settings can greatly influence inferences of microbial composition
28 and diversity, but are often inconsistent and under-investigated between studies. Many
29 laboratories have utilized either temperature control or preservation buffers for optimization of
30 DNA preservation, but few studies have evaluated the effects of combining both methods to
31 preserve fecal microbiota. To determine the optimal method for fecal DNA preservation, we
32 collected fecal samples from one canine donor and stored aliquots in RNAlater, 70% ethanol,
33 50:50 glycerol:PBS, or without buffer at 25°C, 4°C, and -80°C. Fecal DNA was extracted,
34 quantified, and 16S rRNA gene analysis performed on days 0, 7, 14, and 56 to evaluate changes
35 in DNA concentration, purity, and bacterial diversity and composition over time. We detected
36 overall effects on bacterial community of storage buffer (F-value= 6.87, DF= 3, P<0.001),
37 storage temperature (F-value=1.77, DF= 3, P=0.037), and duration of sample storage (F-
38 value=3.68, DF= 3, P<0.001). Changes in bacterial composition were observed in samples stored
39 in -80°C without buffer, a commonly used method for fecal DNA storage, suggesting that simply
40 freezing samples may be suboptimal for bacterial analysis. Fecal preservation with 70% ethanol
41 and RNAlater closely resembled that of fresh samples, though RNAlater yielded significantly
42 lower DNA concentrations (DF=8.57, P<0.001). Although bacterial composition varied with
43 temperature and buffer storage, 70% ethanol was the best method for preserving bacterial DNA
44 in canine feces, yielding the highest DNA concentration and minimal changes in bacterial
45 diversity and composition. The differences observed between samples highlight the need to
46 consider optimized post-collection methods in microbiome research.

47 INTRODUCTION

48 The intestinal microbiota is comprised of trillions of bacteria that contribute to nutrition,
49 digestion, immune defense, and various disease processes (Peterson & Round, 2014; West et al.,
50 2015). However, it is estimated that 60-70% of resident gut bacteria cannot be discerned using
51 culture-dependent methods (Hayashi, Sakamoto, & Benno, 2002). Microbiota research
52 involving culture-independent techniques such as DNA sequencing have made headway in the
53 past decade with recent technological advances in next-generation sequencing and
54 bioinformatics, providing more accurate taxonomic analysis and reproducibility between studies
55 (Wu et al., 2012). The human gut is the perhaps the most well-known and well-studied
56 microbiome, but there is increasing interest in other biological sites in humans, domestic
57 animals, and wildlife in an effort to study the interaction between the host and its environment
58 (Paulino et al., 2006; Verhulst et al., 2011; Weese, 2013; Fliegerova et al., 2014; Lowrey et al.,
59 2015). These studies have paved the way for linking distinct bacterial communities within and
60 between individuals. Recently, these efforts have greatly contributed to molecular fingerprinting
61 and DNA identification in forensic science (Fierer et al., 2010; Tims et al., 2010). Despite the
62 technological advances in noninvasive genetics (Beja-Pereira et al., 2009), there remains a lack
63 of standardized methods for sample collection, bacterial preservation, and DNA extraction (Yuan
64 et al., 2012; Kennedy et al., 2014; Gorzelak et al., 2015). Many studies that have evaluated
65 bacterial preservation and DNA extraction showed variability in processing samples, leading to
66 significant over- or underrepresentation of bacterial populations. For example, fecal storage at
67 room temperature decreases the relative abundance of Firmicutes and increases Bacteroidetes,
68 whereas storage in freezing conditions introduces the opposite effect (Bahl, Bergstrom, & Licht,
69 2012; Choo, Leong, & Rogers, 2015). Furthermore, the use of fecal swabs in human patients led

70 to an overestimation of Enterobacteriaceae and Ruminococcaceae bacterial families (Tedjo et al.,
71 2015). This highlights the need for more comprehensive evaluation of current techniques for
72 optimal fecal DNA storage and isolation.

73

74 For many research laboratories, it is a challenge to minimize exposure of samples to the
75 environment and minimize time between sample collection and DNA extraction (Hale et al.,
76 2016). Preserving DNA through deactivation of nucleases, removal of cations or lowering
77 temperature becomes crucial to inhibit enzymes that degrade DNA. Most studies have utilized
78 freezing conditions (Carroll et al., 2012; Wu et al., 2012) or the use of preservatives such as
79 ethanol (Murphy et al., 2002; Bressan et al., 2014; Huang et al., 2016), RNAlater (Nechvatal et
80 al., 2008; Sorensen et al., 2016), and glycerol:PBS (McKain et al., 2013; Fliegerova et al., 2014)
81 to preserve bacterial DNA before extraction. However, few studies have examined the
82 combination of chemical buffers and temperature reduction to preserve DNA and optimize
83 bacterial analysis. We designed a longitudinal study to evaluate the effects of temperature (room
84 temperature at 25°C, refrigeration at 4°C, freezing at -80°C) as well as immersion in 70%
85 ethanol, RNAlater, and 50:50 glycerol:PBS on fecal DNA preservation over the course of a 56-
86 day storage period.

87

88 **METHODS**

89 Stool collection and storage

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

93 for its entire life by a veterinarian and had not been exposed to oral antibiotics 36 months prior to
94 fecal collection. Fecal sampling occurred immediately after defecation. The sample was then
95 homogenized and processed (within 1 hour of collection) in the laboratory. Untreated feces were
96 placed in three common fecal preservation buffers: RNAlater (Ambion, Austin, TX, USA), 70%
97 ethanol, or 50:50 glycerol:PBS. The fecal sample was homogenized, divided into ninety-six
98 0.25g aliquots (4 treatment groups, 3 temperatures, 4 time points, in duplicate), and placed in 1.5
99 mL Eppendorf tubes with preservation buffers. All tubes were vortexed to allow buffer
100 penetration in each fecal sample and incubated for two hours at room temperature. Each
101 treatment group was subjected to various temperature conditions: room temperature (25°C),
102 refrigeration (4°C), and freezing (-80°C) on the day of collection (day 0) and on days 7, 14, and
103 56 post-collection. Temperatures were consistently maintained using a microbiological incubator
104 at 25°C, a refrigerator at 4°C, and a Revco freezer at -80°C. Day 0 samples were processed for
105 DNA extraction after 2 hours of temperature treatment, while other samples were stored at the
106 indicated temperatures until DNA extractions on day 7, 14, and 56.

107

108 DNA extraction

109 Genomic DNA was extracted from all samples using the 100-prep MoBio PowerSoil DNA
110 Isolation kit (MoBio, Carlsbad, CA). Fecal material was isolated from preservation buffer by
111 pelleting (centrifugation at 10,000 x g for 5 minutes, pouring off supernatant). Samples were
112 placed in bead tubes containing C1 solution and incubated at 65°C for 10 minutes, followed by 1
113 minute of bead beating with the MoBio vortex adapter. The remaining extraction protocol was
114 performed as directed by the manufacturer. DNA concentration was recorded using a QUBIT™

115 dsDNA HS Assay and the DNA purity (A260/A280 ratio) was analyzed using a Nanodrop 1000
116 spectrophotometer (ThermoFisher Scientific, Wilmington, DE).

117

118 PCR and 16S rRNA Sequencing

119 Bacterial diversity was characterized via amplification by a PCR enrichment of the 16S rRNA
120 gene (V4 region) using primers 515F and 806R, modified by addition of Illumina adaptor and an
121 in-house barcode system (Lang, Eisen, & Zivkovic, 2014). After an initial denaturation step at
122 94°C for 3 minutes, we ran 35 cycles of the following PCR protocol: 94°C for 45 seconds, 50°C
123 for 60 seconds and 72°C for 90 seconds, followed by a final hold at 4°C. Prior to sequencing, the
124 amount of input DNA per sample was normalized using a SequalPrep Normalization Plate,
125 following the standard protocol (ThermoFisher Scientific, Wilmington, DE). Libraries were
126 sequenced using an Illumina MiSeq system, generating 250 bp paired-end amplicon reads.

127

128 Data analysis and statistics

129 We used a custom script (available in a GitHub repository
130 https://github.com/gjospin/scripts/blob/master/Demul_trim_prep.pl), to assign each pair of reads
131 to their respective samples when parsing the raw data. This script allows for one base pair
132 difference per barcode. The paired reads were then aligned and a consensus was computed using
133 FLASH (Magoč & Salzberg, 2011) with maximum overlap of 120 bp and a minimum overlap of
134 70 bp (other parameters were left as default). The custom script automatically demultiplexes the
135 data into fastq files, executes FLASH, and parses its results to reformat the sequences with
136 appropriate naming conventions for Quantitative Insights into Microbial Ecology {QIIME
137 v.1.9.1, (Caporaso et al., 2010)} in fasta format. Each sample was characterized for taxonomic

138 composition (number and abundance) using QIIME. For presence/absence analyses,
139 representative operational taxonomic units (OTUs) were clustered at the >97 percent identity
140 level and an OTU table was constructed using QIIME's pick_otus_through_otu_table.py script.
141 In addition, we removed chimeras from the OTU table and filtered for chloroplast and
142 mitochondrial DNA. The resulting table was rarefied at 4000 reads and filtered to remove low
143 abundance OTUs. Raw sequencing data files have been uploaded to NCBI Bioproject
144 (Accession: PRJNA414515) and Figshare at the following link:

145 https://figshare.com/articles/Canine_fecal_samples/5510422.

146

147 We compared alpha diversity (mean species diversity per treatment) using the Shannon Index as
148 implemented in the vegan library (Solymos, Stevens, & Wagner, 2016) in R (Team RC, 2016).
149 We compared OTU richness (number of OTUs found in each sample) and Pielou's evenness
150 (calculated by dividing the Shannon index for diversity by the log of OTU richness). We tested
151 for statistical significance in alpha diversity measures using the Analysis of Variance (ANOVA)
152 with post-hoc Tukey HSD to determine the effects of temperature, storage buffer, and duration of
153 storage in R. We compared beta diversity (the ratio between regional and local species diversity)
154 using Bray-Curtis dissimilarity and weighted Unifrac, and we used PCoA for ordination and
155 clustering. We then used adonis, a multivariate ANOVA based on dissimilarities to test for
156 significant categorical differences with 1000 permutations in the picante library (Kembel et al.,
157 2010) in R. OTU frequencies across buffer, temperature, and time were compared using QIIME
158 script group_significance.py. Spearman correlation coefficients and regressions were calculated
159 on R and GraphPad Prism Software.

160

161 **RESULTS**

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

165

166 DNA Concentration and Purity

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

172

173 *By Time:*

174 The DNA concentration of fecal samples incubated at room temperature without preservation
175 buffer exhibited an 84% decrease in DNA concentration after Day 0 (1/Slope=-0.6812,
176 $R^2=0.9935$, $P=0.0002$) (**Fig. 2A**), where levels remained through Day 56. We used ANOVA to
177 test for an effect of storage method on DNA quality (as measured by the ratio of A260/A280).
178 We did not detect an overall effect of storage buffer ($P=0.12$) or storage temperature ($P=0.66$) on
179 DNA quality. However, length of storage significantly affected DNA quality (F-value=4.365,
180 $DF=1$, $P=0.04$) and there was a significant interaction between storage buffer and length of
181 storage (F-value=2.88, $DF=3$, $P=0.043$). DNA quality declined slightly by Day 14 (1/Slope=-
182 43.90, $R^2=0.3135$, $P=0.0024$) and then increased in the samples stored in 70% ethanol and no
183 storage buffer on day 56 (1/Slope=109.4, $R^2=0.4400$, $P=0.0014$) (**Fig. S1**).

184

185 *By Temperature:*

186 Reducing the temperature used to store fecal samples reduced the amount of DNA loss after Day
187 0 (1/Slope=-31.73, $R^2=0.09954$, $P=.0190$). Unbuffered samples and samples in 50:50
188 glycerol:PBS were most affected by storage at room temperature, while samples in RNAlater and
189 70% ethanol were consistent across all temperatures (**Fig. 2A-C**). Unbuffered, refrigerated
190 samples exhibited a 1.5-fold higher DNA concentration at Day 7 and 2.25-fold at Day 14
191 compared to respective samples stored at room temperature. Refrigeration of samples stored in
192 glycerol:PBS samples exhibited a similar trend in DNA concentration with fold-changes up to
193 4.9 times that of respective samples stored at room temperature, near levels found in fresh
194 control samples. Of the three temperatures observed, freezing at -80°C yielded the highest DNA
195 concentration over 56 days ($\text{AUC}=76.291$), with refrigeration close behind ($\text{AUC}=72.528$), and
196 room temperature with the lowest yield ($\text{AUC}=52.700$). Freezing glycerol:PBS samples at days
197 14 and 56 led to a 706% and 811% higher DNA concentration, respectively, compared to
198 samples stored in glycerol:PBS at room temperature. Little change was observed in DNA
199 concentration of samples stored in RNAlater and 70% ethanol. DNA purity remained unchanged
200 across all temperatures (1/Slope=-727.4, $R^2=0.0436$, $P=0.0976$) (**Fig. S1**).

201

202 *By Buffer:*

203 Preservation buffers were evaluated (RNAlater, 70% ethanol, and 50:50 glycerol:PBS) in
204 comparison with unbuffered controls. DNA concentrations in unbuffered samples decreased by
205 75-80% over time, which were greatly improved with the addition of 70% ethanol regardless of
206 temperature (**Fig. 2A-C**). 70% ethanol was the optimal method, exhibiting no significant changes

207 in 56 days ($1/\text{Slope}=-61.48$, $R^2=0.0365$, $P=0.4473$). Preservation with 50:50 glycerol:PBS also
208 improved DNA yield, but only under refrigeration or freezing conditions. Preservation with
209 RNAlater yielded the lowest amount of DNA across all temperature and preservation buffers.

210

211 Bacterial Diversity and Composition

212 Alpha Diversity measures: We performed an ANOVA to test for effects of storage buffer,
213 storage temperature, and duration of sample storage on the Shannon Diversity Index, Species
214 Richness, and Species Evenness. Storage buffer had a statistically significant effect on Shannon
215 Diversity Index values ($F\text{-value}=3.07$, $DF=3$, $P=0.03$) (**Fig. S2**). Preservation with glycerol:PBS
216 had the highest Shannon Diversity values compared to 70% ethanol ($P=0.0167$), RNAlater
217 ($P<0.0001$), and no buffer ($P=0.0004$). Samples stored with ethanol and without buffer did not
218 differ significantly ($P=0.5178$), while samples stored in RNAlater had the lowest Shannon
219 Diversity values (**Table S1**). We did not detect an effect of storage temperature ($F\text{-value}=2.2$,
220 $DF=1$, $P=0.14$) or duration of sample storage ($F\text{-value}=0.69$, $DF=1$, $P=0.4$) on Shannon Diversity
221 values. Additionally, storage buffer ($F\text{-value}=12.4$, $DF=3$, $P<0.00001$), duration of sample
222 storage ($F\text{-value}=10.8$, $DF=1$, $P=0.0016$), the interaction between storage buffer and temperature
223 ($F\text{-value}=3.443$, $DF=3$, $P=0.0218$), and the interaction between storage buffer and duration of
224 sample storage ($F\text{-value}=9.67$, $DF=3$, $P<0.00001$) significantly affected observed species
225 richness levels (**Fig. S3**). Effect of storage buffer on species richness levels were similar to that
226 of Shannon Diversity, with the highest in glycerol:PBS and lowest in RNA later. Samples
227 exhibited a reduction in species richness levels at 7 days compared to fresh samples ($P=0.0724$),
228 and a subsequent increase at Day 14 ($P=0.0018$) and Day 56 ($P<0.0001$) compared to Day 7.
229 Effects of the interaction between buffer and duration of storage on species richness were also

230 noted (**Table S2**). Lastly, we found no significant effects of buffer, duration of storage, nor
231 temperature on species evenness (**Fig. S4**). Significant effects were associated with interactions
232 between storage buffer and storage temperature (F-value=3.98, DF=3, P=0.01), storage buffer
233 and duration of sample storage (F-value=4.9, DF=3, P=0.004), and buffer, storage temperature,
234 and duration of sample storage (F-value=3.1, DF=3, P=0.03) (**Table S3**).

235 Beta Diversity measures: We used a permutational ANOVA to test for effects of storage method
236 on the degree of clustering of bacterial communities in our stored samples. We detected overall
237 effects on bacterial community of storage buffer (F-value= 6.87, DF= 3, P<0.001), storage
238 temperature (F-value=1.77, DF= 3, P=0.037), and duration of sample storage (F-value=3.68,
239 DF= 3, P<0.001). Significance testing on the Bray Curtis dissimilarities using a two-sided
240 Student's two-sample t-test indicated that samples stored in 70% ethanol and samples stored in
241 glycerol:PBS had lower dissimilarities from fresh samples when compared to samples stored in
242 RNALater and samples stored without a storage buffer (**Fig. S5, Table S4**).

243

244 *By Time:*

245 Complementary to the differences detected in DNA concentration, we found that the bacterial
246 composition in unbuffered samples also changed over time. Visualization of weighted (**Fig. 3A-**
247 **C**) and unweighted (**Fig. 3D-F**) PCoA plots showed that while most Day 0 samples (in red)
248 clustered together, some differences were observed between buffers, likely due to immediate
249 shifts in bacterial composition after contact with preservation buffer (**Fig. S5**). From Day 7 to
250 Day 56, unbuffered samples did not cluster with fresh control samples, indicating a shift in
251 bacterial composition between 0 and 7 days after fecal collection (**Fig. 3A-C**). Accordingly,
252 taxonomic evaluation also revealed shifts in relative abundance of bacteria in unbuffered

253 samples, particularly after Day 0 at room temperature. This was exhibited by a marked increase
254 in *Streptococcus*, decrease in *Prevotella* (**Fig. 4A**), and increase in overall
255 Firmicutes:Bacteroidetes ratio (1/Slope=1.385, $R^2=0.4687$, $P=0.0419$) (**Fig. 5A**) compared to
256 fresh samples. The increase in Firmicutes:Bacteroidetes ratio in unbuffered samples over time
257 was attenuated by temperature reduction (**Fig. 5B, C**). Overall, alterations in OTU frequencies
258 were observed in genera *Collinsella* ($P=0.0020$), *Enterococcus* ($P=0.0048$), *Prevotella*
259 ($P=0.0052$), *Megamonas* ($P=0.0129$), and *Streptococcus* ($P=0.0413$) over 56 days.

260

261 *By Temperature:*

262 Weighted PCoA analysis of unbuffered samples indicated that shifts in bacterial composition at
263 room temperature can be minimized by lowering the temperature (**Fig. 3A-C**). Samples clustered
264 very closely when stored at -80°C (**Fig. 3C**), and segregated with increasing temperature (**Fig.**
265 **3A, B**). Samples stored at room temperature without buffer or with 50:50 glycerol:PBS past Day
266 7 did not cluster with the sample controls at Day 0 (**Fig. 3A**). Analysis of Bray-Curtis
267 dissimilarity revealed that samples with 50:50 glycerol:PBS clustered closer to control samples
268 with refrigeration and freezing (**Fig. S5**). However, this pattern was not observed in unbuffered
269 samples. Unbuffered samples did not cluster closely with control samples under refrigeration and
270 freezing conditions. Investigation of the bacterial phyla in these communities revealed a notable
271 reduction in relative abundance of *Prevotella* and increase in *Streptococcus* in unbuffered
272 samples stored at refrigeration (**Fig. 4B**) and freezing conditions (**Fig. 4C**). Although freezing is
273 one of the most commonly used tools for stool preservation, we observed that samples frozen
274 without buffer exhibited a lower relative abundance of Bacteroidetes (**Fig. 5F**) as previously
275 described (Bahl, MI, Bergstrom, A, & Licht, TR, 2012) and an increase in

276 Firmicutes:Bacteroidetes ratio (**Fig. 5C**) compared to samples stored with buffer. Although these
277 alterations were observed in frozen samples without buffer, overall changes in Bacteroidetes and
278 Bacteroidetes:Firmicutes ratios were attenuated by freezing when compared to storage at room
279 temperature (**Fig. 5A, D**) or refrigeration (**Fig. 5B, E**). Across all buffers, storage at various
280 temperatures introduced changes in OTU frequencies of genera *Prevotella* (P=0.0006),
281 *Collinsella* (P=0.0068), *Streptococcus* (P=0.0094), *Megamonas* (P=0.0094), *Bacteroidetes*
282 (P=0.0094), and *Catenibacterium* (P=0.0156).

283

284 *By Buffer:*

285 Weighted PCoA analysis shows that across all temperatures, bacterial communities in samples
286 stored in 70% ethanol clustered relatively closely with those in fresh control samples (**Fig. 3A-**
287 **C**). Samples without buffer did not cluster with fresh samples over time despite temperature
288 reduction. This was consistent with taxonomic evaluation of bacterial genera, as indicated by a
289 large increase in *Streptococcus* and reduction in *Prevotella* (**Fig. 4A-C**). Samples stored in 50:50
290 glycerol:PBS clustered with control samples only under refrigeration and freezing conditions,
291 which was consistent with taxonomic analysis at the OTU level. At room temperature, storage in
292 glycerol:PBS introduced an increase in genus *Collinsella*. Storage with both RNAlater and 70%
293 ethanol rapidly introduced permanent changes in bacterial composition after a 2-hour incubation
294 period on Day 0. RNAlater preservation was consistent across all temperatures, but led to a
295 reduction in relative abundance of *Streptococcus* and *Megamonas*, and increase in *Prevotella*
296 species. Storage in 70% ethanol also preserved fecal microbiota composition at all temperatures,
297 with slight increases in *Catenibacterium* and *Bacteroidetes*. Overall, variation in storage buffer
298 introduced significant changes in OTU frequencies of genera *Streptococcus* (P<0.0001),

299 *Megamonas* ($P < 0.0001$), *Collinsella* ($P < 0.0001$), *Catenibacterium* ($P < 0.0001$). Although
300 statistically insignificant, alterations in genera *Prevotella* ($P = 0.0563$) and *Bacteroides*
301 ($P = 0.0758$) were also noted across storage buffers.

302

303 **DISCUSSION**

304 There is increasing evidence highlighting the importance of bacterial DNA preservation in
305 multitude of settings, including health evaluations, research endeavors, and forensic science.
306 Characteristic signatures of microbiota have been explored as a result of the availability of next-
307 generation sequencing, extending our knowledge beyond culturable methods. One of the most
308 readily available resources to study microbes in humans and animals is fecal collection (Hale et
309 al., 2016). Not surprisingly, microbiota, including that of the gut, is often transient and dynamic,
310 posing a challenge for scientists to make sense of samples post-collection. Bacterial DNA can be
311 degraded through environmental perturbation and subsequent hydrolysis, oxidation, and
312 methylation, supporting the need to limit spontaneous decay (Lindahl, 1993). Nevertheless, there
313 remains considerable value in distinguishing changes in the microbiome in high resolution for
314 improving animal, human, and environmental health. This is accompanied by a need for more
315 effective storage methods that precisely and accurately capture the bacterial community at a
316 given time-point. Additionally, studying longitudinal changes in microbiota has paved the way
317 for developing microbiome tools to study unique signatures and providing, at least in theory, the
318 means to locate people in space and time in forensic science (Kim, M, Zorraquino &
319 Tagkopoulos, 2015; Fornaciari, 2017). A subset of our study investigates longitudinal changes in
320 fecal microbiota at various temperatures, which provides insight on how various bacterial
321 signatures could be maintained or altered over time. Studies tracking how certain microbes

322 change over time could be utilized to interpret chronological events in the past. While this study
323 focuses on optimal preservation of DNA for future analysis, information gathered at various time
324 points is valuable to extrapolate longitudinal data in a forensic context. It is evident that
325 investigation of optimal methods for DNA preservation will have important impacts on
326 microbiome studies in field, clinic, and laboratory settings. Assessing changes in preserved fecal
327 microbiota over time provides insight on whether changes we observe are biologically relevant
328 and useful for outcome measures.

329

330 A variety of studies have examined preservation methods to minimize post-sampling alterations
331 in fecal bacterial DNA. Such experiments have evaluated fixation conditions with 95% ethanol,
332 70% ethanol, FTA card, OMNI gene Gut, RNAlater, glycerol, refrigeration, and freezing.
333 However, there is little consensus on the optimal buffer and temperature condition for bacterial
334 preservation (Nechvatal et al., 2008; Cardona et al., 2013; Kolodziej et al., 2013; McKain et al.,
335 2013; Fliegerova et al., 2014; Hale et al., 2015; Song et al., 2016; Hale et al., 2016; Metzler-
336 Zebeli et al., 2016). Furthermore, a limited number of studies have evaluated the combination of
337 these chemical buffers with temperature reduction to optimize DNA preservation. Our study
338 examined the influence of three commonly used preservation buffers on the bacterial integrity of
339 canine feces after eight weeks of storage at room temperature (25°C), refrigeration (4°C), and
340 freezing (-80°C). Since we were interested in measuring change over time in identical fecal
341 samples, we utilized one canine donor and homogenized the stool sample prior to tube allocation.
342 While one donor was used for this study, we speculate that there may be inter-individual
343 differences in storage because different dogs may have different bacterial compositions and some
344 fecal bacterial groups may be more prone to temperature or buffer alterations. Therefore, more

345 studies involving additional animals and species are needed to make definitive conclusions about
346 bacterial changes with preservation methods. We report that fecal DNA concentration and
347 by freezing may not be adequate in maintaining bacterial DNA. Overall, total DNA recovery and
348 fecal composition of samples stored at 4°C were similar to that of samples stored at -80°C over
349 56 days, both of which were vastly different from samples stored at 25°C. Minor differences
350 between storage at 4°C and -80°C were observed in samples without buffer, which exhibited
351 higher abundances of *Streptococcus* and lower abundances of *Prevotella* at 4°C compared to -
352 80°C. Storage in buffers such as 70% ethanol, RNA later, and 50:50 glycerol:PBS greatly
353 reduced the changes observed between samples stored at 4°C and -80°C. This suggests that deep
354 freezing may not be needed when samples are stored with a preservation buffer, a particularly
355 useful tool in field conditions or settings without a laboratory-grade freezer.

356

357 To evaluate methods that are most cost-effective and accessible to all studies, we were especially
358 interested in the efficacy of ethanol preservation. Previous studies have utilized 70% and 95%
359 ethanol, each with varying results in DNA yield and microbial community stability (Hale et al.,
360 2015; Song et al., 2016). Efficacy of ethanol preservation may be dependent on concentration
361 due to species-species differences in stool consistency. Based on the lack of moisture in our
362 canine stool sample, we decided to use 70% ethanol for fecal preservation. We found that it
363 yielded the highest amount of DNA and most closely resembled that of fresh samples within 56
364 days compared to other buffer solutions. Song, et al. reported that 70% was inadequate for DNA
365 preservation, but sample preparation methods did not include a homogenization step after the
366 fecal sample is immersed in ethanol. The penetration of 70% ethanol in fecal samples, along with
367 stool consistency, may play a role in DNA preservation, which we attempted to circumvent by

368 including a homogenization step. Additional experiments are warranted to examine 70% and
369 95% ethanol using a homogenizing protocol and various stool consistencies. We also evaluated
370 DNA preservation by RNAlater, a historically-supported and commonly used DNA stabilization
371 buffer (Schnecker et al., 2012). Consistent with our findings, studies have recently shown that
372 this method yields very low amounts of DNA (Hale et al., 2015; Song et al., 2016) due to
373 degradation. We hypothesize that residual RNAlater remaining in fecal samples may interfere
374 with the cell lysis and protein digestion in DNA extraction, inhibiting optimal DNA isolation.
375 Investigation of DNA concentration using both a QUBIT™ dsDNA HS Assay and a Nanodrop
376 1000 spectrophotometer revealed that Nanodrop-obtained values were markedly higher in
377 RNAlater samples than that of QUBIT (**Fig. S6**). Due to the methodological variability of DNA
378 concentration in samples with RNAlater, deeper analyses of bacterial composition was
379 performed. Our data showed that both RNAlater and 70% ethanol were effective and consistent
380 across all temperatures, and that DNA yields did not reflect bacterial composition. While
381 preservation buffers are a useful tool, it is important to note that storage in both RNAlater and
382 70% ethanol rapidly introduced changes in bacterial composition, even at Day 0. Compared to
383 fresh, unbuffered samples, ethanol preservation slightly increased the relative abundance of
384 *Catenibacterium* and *Bacteroidetes* while RNAlater preservation greatly increased abundances
385 of *Prevotella* and decreased *Streptococcus* species. These alterations did not change over time.
386
387 It is clear that post-sampling conditions may impact results and interpretations of bacterial DNA.
388 Bacterial DNA outcomes are widely used in medicine and healthcare, such as the
389 Firmicutes:Bacteroidetes ratio, a comparison of butyrate to propionate/acetate-producing bacteria
390 (Bahl, Bergstrom, & Licht, 2012). This ratio, along with levels of Proteobacteria and

391 Actinobacteria, is used as a potential indicator of gastrointestinal health and immune balance
392 (Honneffer et al., 2014). While there is no direct comparison to disease state in this study, we
393 showed that alterations in fecal DNA composition can markedly shift this ratio, particularly with
394 inadequate temperature reduction or preservation buffer. More studies investigating fecal DNA
395 preservation and stability in chronic diseases are warranted. Preservation buffers such as glycerol
396 at room temperature may promote the selective growth of bacteria such as *Collinsella*, which has
397 been linked to production of inflammatory cytokine IL-17A and disease states such as
398 rheumatoid arthritis (Chen et al., 2016). Use of carbon-containing buffers like glycerol may
399 provide energy sources for certain bacterial groups, creating disease preservation biases that
400 could be falsely interpreted as a clinical concern (Murarka et al., 2008). Therefore, careful
401 selection of preservation buffers is advised when measuring specific biological outcomes. In this
402 study, we also showed that despite temperature reduction, bacterial DNA changes over time
403 without buffer, highlighting the need to consider post-collection dynamics in microbiome
404 research. Changes in bacterial composition observed in unbuffered samples were eliminated
405 with the addition of preservation buffers such as 70% ethanol, RNA later, and 50:50
406 glycerol:PBS. For example, the use of 50:50 glycerol:PBS in refrigeration and freezing
407 temperatures improved both DNA composition and concentration compared to unbuffered
408 controls at room temperature. While temperature reduction preserved DNA to an extent, storage
409 buffer had the greatest impact on DNA preservation. Storage of fecal DNA in 70% ethanol was
410 the optimal preservation method across all temperatures, highlighting its utility in settings
411 without access to temperature control. Our data suggests that caution should be taken in sample
412 handling and use of adequate storage buffers to accurately and consistently analyze fecal
413 microbial DNA.

414

415 **CONCLUSIONS**

416 A 56-day longitudinal study of fecal microbiota from one canine donor was conducted to
417 evaluate storage conditions with RNAlater, 70% ethanol, 50:50 glycerol:PBS, and no buffer at -
418 80°C, 4°C, and 25°C. We report that temperature, time, and buffer significantly changed the
419 composition of fecal microbiota, which was comprised predominantly of genera *Streptococcus*,
420 *Prevotella*, *Collinsella*, and *Megamonas*. Samples stored without buffer exhibited DNA
421 degradation and altered composition and diversity despite temperature reduction, suggesting that
422 gold standard methods of immediate freezing at -80°C may not be optimal for fecal preservation.
423 The efficacy of DNA preservation was largely driven by storage buffer, which produced an
424 additive effect in glycerol:PBS when combined with temperature reduction. Most notably, fecal
425 preservation with 70% ethanol yielded DNA concentrations and bacterial composition closest to
426 that of fresh samples at all temperatures, highlighting the potential of its utility in field,
427 laboratory, and clinical settings without access to a laboratory freezer. Because this study utilizes
428 samples from only one canine donor, research involving more animals and species are warranted
429 to better evaluate the efficacy of fecal microbiota preservation. In conclusion, this study
430 underlines the need for more comprehensive evaluation of fecal DNA storage methods for
431 accurate downstream microbial analysis.

432

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439

440 REFERENCES

- 441 Bahl, MI, Bergstrom, A, & Licht, TR (2012). Freezing fecal samples prior to DNA extraction
442 affects the *Firmicutes* to *Bacteroidetes* ratio determined by downstream quantitative PCR
443 analysis. *FEMS Microbiology Letters*, 329: 193-197. doi:10.1111/j.1574-
444 6968.2012.02523.x
- 445 Beja-Pereira, A, Oliveira, R, Alves, PC, Schwartz, MK, & Luikart, G (2009). Advancing
446 ecological understandings through technological transformations in noninvasive genetics.
447 *Molecular Ecology Resources*, 9: 1279-1301. doi:10.1111/j.1755-0998.2009.02699.x
- 448 Bressan, EA, Rossi, ML, Gerald, LT, & Figueira, A (2014). Extraction of high-quality DNA
449 from ethanol-preserved tropical plant tissues. *BMC Research Notes*, 7: 268.
450 doi:10.1186/1756-0500-7-268
- 451 Caporaso, JG, Kuczynski, J, Stombaugh, J, Bittinger, K, Bushman, FD, Costello, EK, Fierer, N,
452 Pena, AG, Goodrich, JK, Gordon, JI, Huttley, GA, Kelley ST, Knights, D, Koenig, JE,
453 Ley, RE, Lozupone, CA, McDonald, D, Muegge, BD, Pirrung, M, Reeder, J, Sevinsky,
454 JR, Turnbaugh, PJ, Walters, WA, Widmann, J, Yatsunenko, T, Zaneveld, J, & Knight, R
455 (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature*
456 *Methods*. 7: 335-336. doi:10.1038/nmeth.f.303
- 457 Cardona, S, Eck, A, Cassellas, M, Gallart, M, Alastrue, C, Dore, J, Azpiroz, F, Roca, J, Guarner,
458 F, & Manichanh, C (2012). Storage conditions of intestinal microbiota matter in
459 metagenomics analysis. *BMC Microbiology*. 12: 158. doi:10.1186/1471-2180-12-158
- 460 Carroll, IM, Ringel-Kulka, T, Siddle, JP, Klaenhammer, TR, & Ringel, Y (2012).
461 Characterization of the fecal microbiota using high-throughput sequencing reveals a

462 stable microbial community during storage. *PloS one*, 7: e46953-e46953.
463 doi:10.1371/journal.pone.0046953

464 Chen, J, Wright, K, Davis, JM, Jeraldo, P, Marietta, EV, Murray, J, Nelson, H, Matteson, EL, &
465 Taneja, V (2016). An expansion of rare lineage intestinal microbes characterizes
466 rheumatoid arthritis. *Genome Medicine*. 8:43. doi:10.1186/s13073-016-0299-7

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

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

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

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

480 Gupta, RS, Chen, WJ, Adeolu, M, & Chai, Y (2013). Molecular signatures for the class
481 Coriobacteriia and its different clades; proposal for division of the class Coriobacteriia
482 into the emended order Coriobacteriales, containing the emended family
483 Coriobacteriaceae and Atopobiaceae fam. nov., and Eggerthellales ord. nov., containing

- 484 the family Eggerthellaceae fam. nov. *International Journal of Systematic and*
485 *Evolutionary Microbiology*, 63: 3379-3397. doi:10.1099/ijs.0.048371-0
- 486 Hale, VL, Tan, VL, Knight, R, Amato, KR (2015). Effect of preservation method on spider
487 monkey (*Ateles geoffroyi*) fecal microbiota over 8 weeks. *Journal of Microbiological*
488 *Methods*. 113: 16-26. doi:10.1016/j.mimet.2015.03.021
- 489 Hale, VL, Tan, CL, Niu, K, Yang, Y, Cui, D, Zhao, H, Knight, R, & Amato, KR (2016). Effects
490 of field conditions on fecal microbiota. *Journal of Microbiological Methods*, 130: 180-
491 188. doi:10.1016/j.mimet.2016.09.017
- 492 Hayashi, H, Sakamoto, M, & Benno, Y (2002). Phylogenetic analysis of the human gut
493 microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods.
494 *Microbiology and Immunology*, 46: 535-548. doi:10.1111/j.1348-0421.2002.tb02731.x
- 495 Honnefer, JB, Minamoto, Y, & Suchodolski, JS (2014). Microbiota alterations in acute and
496 chronic gastrointestinal inflammation of cats and dogs. *World Journal of*
497 *Gastroenterology*. 20: 16489-97. doi:10.3748/wjg.v20.i44.16489
- 498 Huang, M, Sun, XJ, Zhou, Y, & Wang, XM (2016). Comparison of methods to preserve *Rheum*
499 *palmatum* (Polygonaceae) for efficient DNA extraction and PCR amplification. *Genetics*
500 *and Molecular Research*, 15: gmr.15038019. doi:10.4238/gmr.15038019
- 501 Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, Blomberg SP, &
502 Webb CO (2010). Picante: R tools for integrating phylogenies and ecology.
503 *Bioinformatics*. 26:1463-1464. doi:10.1093/bioinformatics/btq166
- 504 Kolodziej, K, Nikolov, I, Schulz, HK, Theissinger, K, & Schulz, R (2013). Evaluation of fecal
505 storage and DNA extraction methods in wild boar (*Sus scrofa*). *Biochemical Genetics*. 51:
506 406-412. doi:10.1007/s10528-013-9573-1

- 507 Kennedy, NA, Walker, AW, Berry, SH, Duncan, SH, Farquarson, FM, Louis, P, & Thoomson,
508 JM (2014). The impact of different DNA extraction kits and laboratories upon the
509 assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PloS*
510 *one*, 9(2), e88982. doi:10.1371/journal.pone.0088982
- 511 Kim, M, Zorraquino, V, & Tagkopoulous, I (2015). Microbial forensics: predicting phenotypic
512 characteristics and environmental conditions from large-scale gene expression profiles.
513 *Plos Computational Biology*. 11: e1004127. doi:10.1371/journal.pcbi.1004127
- 514 Lang, JM, Eisen, JA, Zivkovic, AM (2014). The microbes we eat: abundance and taxonomy of
515 microbes consumed in a day's worth of meals for three diet types. *PeerJ*. 2: e659.
516 doi:10.7717/peerj.659
- 517 Lindahl, T (1993). Instability and decay of the primary structure of DNA. *Nature*. 362: 709-715.
518 doi:10.1038/362709a0
- 519 Lowrey, L, Woodhams, DC, Tacchi, L, & Salinas, I (2015). Topographical Mapping of the
520 Rainbow Trout (*Oncorhynchus mykiss*) Microbiome Reveals a Diverse Bacterial
521 Community with Antifungal Properties in the Skin. *Applied and Environmental*
522 *Microbiology*, 81: 6915-6925. doi:10.1128/aem.01826-15
- 523 Magoč, T & Salzberg, SL (2011). FLASH: fast length adjustment of short reads to improve
524 genome assemblies. *Bioinformatics*. 1: 2957-63. doi:10.1093/bioinformatics/btr507
- 525 Markowitz, V, Hugenholtz, P, Kyrpides, NC, Klenk, HP, & Han, C (2009). Complete genome
526 sequence of *Eggerthella lenta* type strain (IPP VPI 0255). *Standards in Genomic*
527 *Sciences*. 1: 174–82. doi:10.4056/sigs.33592
- 528 Metzler-Zebeli, BU, Lawlor, PG, Magowan, E, & Zebeli, Q (2016). Effect of freezing conditions
529 on fecal bacterial composition in pigs. *Animals*. 6: E18. doi:10.33990/ani6030018

- 530 McKain, N, Genc, B, Snelling, TJ, & Wallace, RJ (2013). Differential recovery of bacterial and
531 archaeal 16S rRNA genes from ruminal digesta in response to glycerol as cryoprotectant.
532 *Journal of Microbiological Methods*, 95: 381-383. doi:10.1016/j.mimet.2013.10.009
- 533 Murarka, A, Dharmadi, Y, Yazdani, SS, & Gonzalez R (2008). Fermentative utilization of
534 glycerol by *Escherichia coli* and its implications for the production of fuels and
535 chemicals. *Applied and Environmental Microbiology*. 74: 1125-1135.
536 doi:10.1128/AEM.02192-07
- 537 Murphy, MA, Waits, LP, Kendall, KC, Wasser, SK, Higbee, JA, & Bogden, R (2002). An
538 evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA
539 samples. *Conservation Genetics*, 3: 435-440. doi:10.1023/a:1020503330767
- 540 Nechvatal, JM, Ram, JL, Basson, MD, Namprachan, P, Niec, SR, Badsha, KZ, Matherly, LH,
541 Majumdar, APN, & Kato, I (2008). Fecal collection, ambient preservation, and DNA
542 extraction for PCR amplification of bacterial and human markers from human feces.
543 *Journal of Microbiological Methods*, 72: 124-132. doi:10.1016/j.mimet.2007.11.007
- 544 Paulino, LC, Tseng, CH, Strober, BE, & Blaser, MJ (2006). Molecular analysis of fungal
545 microbiota in samples from healthy human skin and psoriatic lesions. *Journal of Clinical*
546 *Microbiology*, 44: 2933-2941. doi:10.1128/JCM.00785-06
- 547 Petersen, C, & Round, JL (2014). Defining dysbiosis and its influence on host immunity and
548 disease. *Cell Microbiology*, 16: 1024-1033. doi:10.1111/cmi.12308
- 549 Saunders E, Pukall R, Abt, B, Lapidus, A, Glavina Del Rio, T, Copeland, A, Tice, H, Cheng, JF,
550 Lucas, S, Chen, F, Nolan, M, Bruce, D, Goodwin, L, Pitluck, S, Ivanova, N, Mavromatis,
551 K, Ovchinnikova, G, Pati, A, Chen, A, Palaniappan, K, Land, M, Hauser, L, Chang, YJ,

552 Jeffries, CD, Chain, P, Meincke, L, Sims, D, Brettin, T, Detter, JC, Goker, M, Bristow, J,
553 Eisen, JA,
554 Schnecker, J, Wild, B, Fuchsleuger, L, & Richter, A (2012). A field method to store samples
555 from temperature mountain grassland soils for analysis of phospholipid fatty acids. *Soil*
556 *Biology and Biochemistry*, 51: 81-83. doi: 10.1016/j.soilbio.2012.02.029
557 Solymos P, Stevens MHH, & Wagner H. 2016. vegan: Community Ecology Package. R package
558 version 2.3-5 <https://cran.r-project.org/package=vegan>
559 Song, SJ, Amir, A, Metcalf, JL, Amato, KR, Xu, ZZ, Humphrey, G, & Knight, R (2016).
560 Preservation methods differ in fecal microbiome stability, affecting suitability for field
561 studies. *Applied and Environmental Science*. 1: e00021-16. doi:10.1128/mSystems.0021-
562 16
563 Sorensen, A, Rahman, E, Canela, C, Gangitano, D, & Hughes-Stamm, S (2016). Preservation
564 and rapid purification of DNA from decomposing human tissue samples. *Forensic*
565 *Science International: Genetics*, 25: 182-190. doi:10.1016/j.fsigen.2016.05.013
566 Team RC. 2016. R: A language and environment for statistical computing. R Foundation for
567 Statistical Computing, Vienna, Austria. <https://www.r-project.org/>
568 Tedjo, DI, Jonkers, DMAE, Masclee, AA, van Best, N, Pierik, MJ, & Penders, J (2015) Effect of
569 sampling and storage on fecal microbiota composition in healthy and diseased subjects.
570 *PloS one*, 10: e0126685. doi:10.1371/journal.pone.0126685
571 Tims, S, van Wamel, W, Endtz, H P, van Belkum, A, & Kayser, M (2010). Microbial DNA
572 fingerprinting of human fingerprints: dynamic colonization of fingertip microflora
573 challenges human host inferences for forensic purposes. *International Journal of Legal*
574 *Medicine*, 124: 477-481. doi:10.1007/s00414-009-0352-9

- 575 Verhulst, NO, Qiu, YT, Beijleveld, H, Maliepaard, C, Knights, D, Schulz, S, Berg-Lyons, D,
576 Lauber, CL, Verduijn, W, Haasnoot, GW, Mumm, R, Bouwmeester, FHJ, Claas, Dicke,
577 M, A. van Loon, JJ, Takken, W, Knight, R, & Smallegange, RC (2011). Composition of
578 human skin microbiota affects attractiveness to malaria mosquitoes. *PloS one*, 6: e28991.
579 doi:10.1371/journal.pone.0028991
- 580 Weese, JS (2013). The canine and feline skin microbiome in health and disease. *Veterinary*
581 *Dermatology*, 24: 137-145.e131. doi:10.1111/j.1365-3164.2012.01076.x
- 582 West, CE, Renz, H, Jenmalm, MC, Kozyrskyj, AL, Allen, KJ, Vuillermin, P, & Prescott, SL
583 (2015). The gut microbiota and inflammatory noncommunicable diseases: Associations
584 and potentials for gut microbiota therapies. *Journal of Allergy and Clinical Immunology*,
585 135: 3-13. doi:10.1016/j.jaci.2014.11.012
- 586 Wu, GD, Lewis, JD, Hoffmann, C, Chen, YY, Knight, R, Bittinger, K, Hwang, J, Chen, J,
587 Berkowsky, R, Nessel, L, Li, H, & Bushman, FD (2010). Sampling and pyrosequencing
588 methods for characterizing bacterial communities in the human gut using 16S sequence
589 tags. *BMC Microbiology*, 10: 206. doi:10.1186/1471-2180-10-206
- 590 Yuan, S, Cohen, DB, Ravel, J, Abdo, Z, & Forney, LJ (2012). Evaluation of methods for the
591 extraction and purification of DNA from the human microbiome. *PloS one*, 7: e33865.
592 doi:10.1371/journal.pone.0033865
- 593

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.

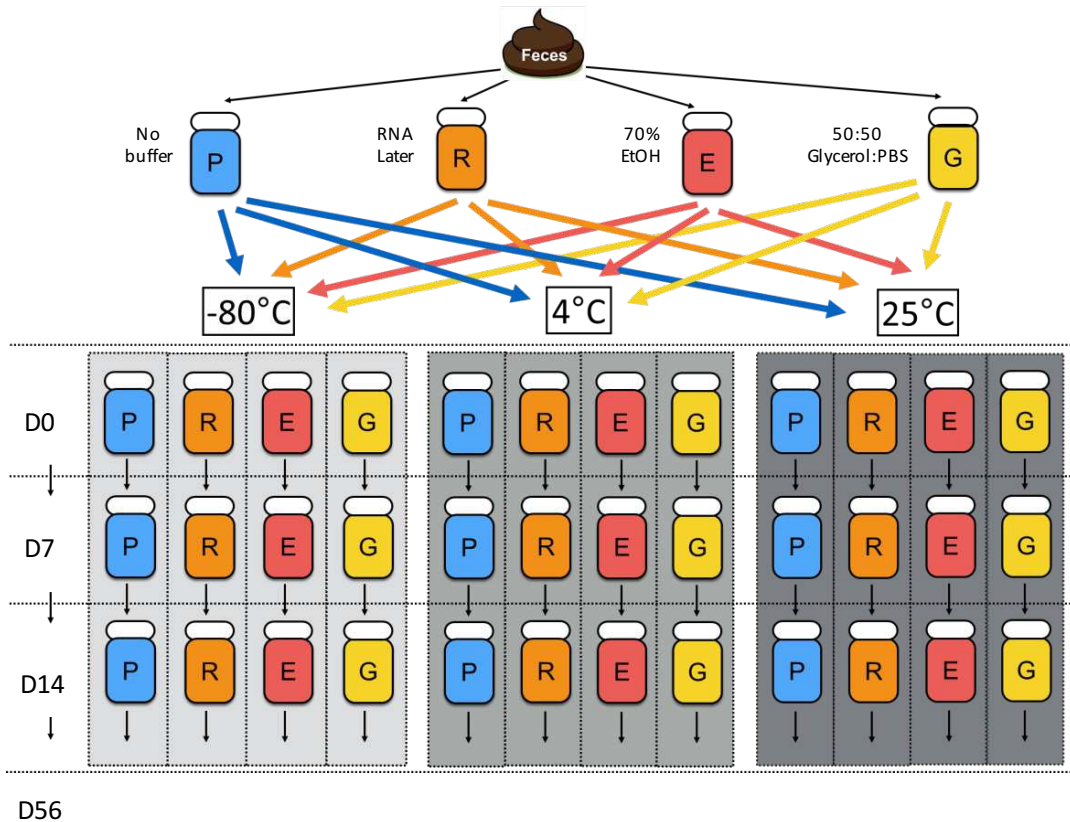
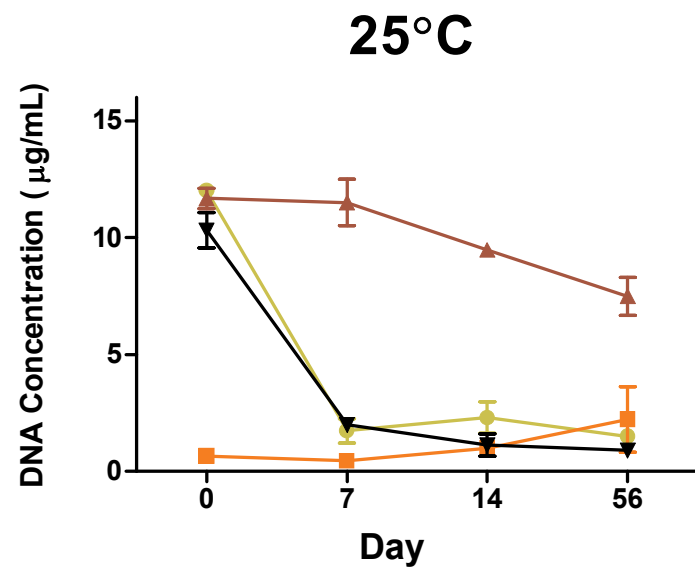


Figure 2(on next page)

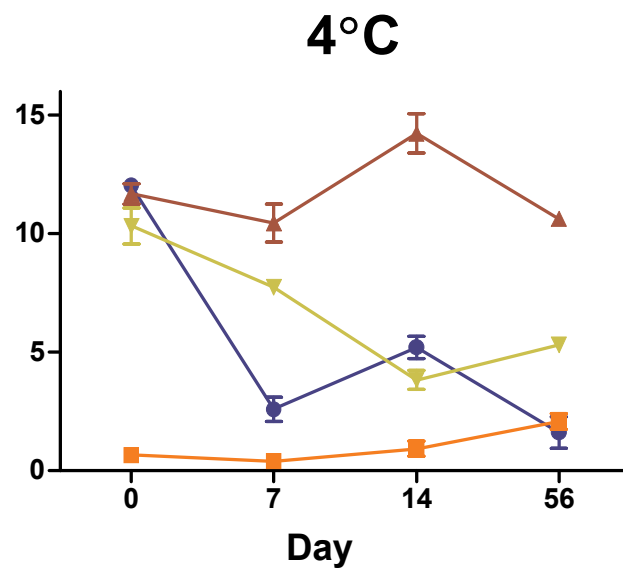
Average DNA concentration ($\mu\text{g}/\text{mL}$) \pm standard error by preservation method over 56 days.

DNA concentrations of samples stored at (A) 25°C, (B) 4°C, and (C) -80°C. Symbols represent average DNA concentrations by buffer and error bars represent standard error of arithmetic means.

A



B



C

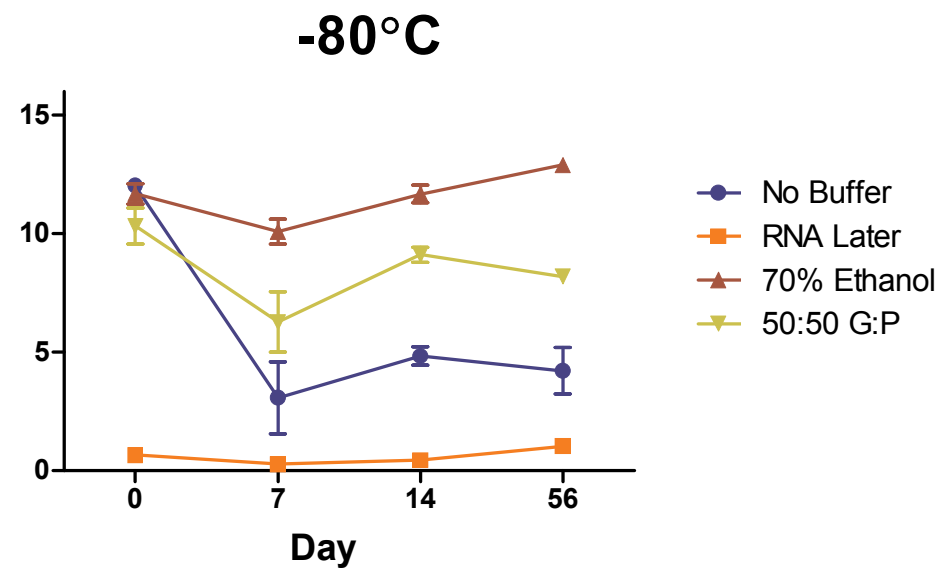


Figure 3(on next page)

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

Weighted Unifrac distances (left) measures relatedness between communities and relative abundance of bacterial phyla at (A) 25°C, (B) 4°C, and (C) -80°C. Unweighted UniFrac distances (right) represent only distances between communities and the evolutionary relatedness of taxa at (D) 25°C, (E) 4°C, and (F) -80°C. Symbols denote method of buffer preservation, while colors represent time over 56 days.

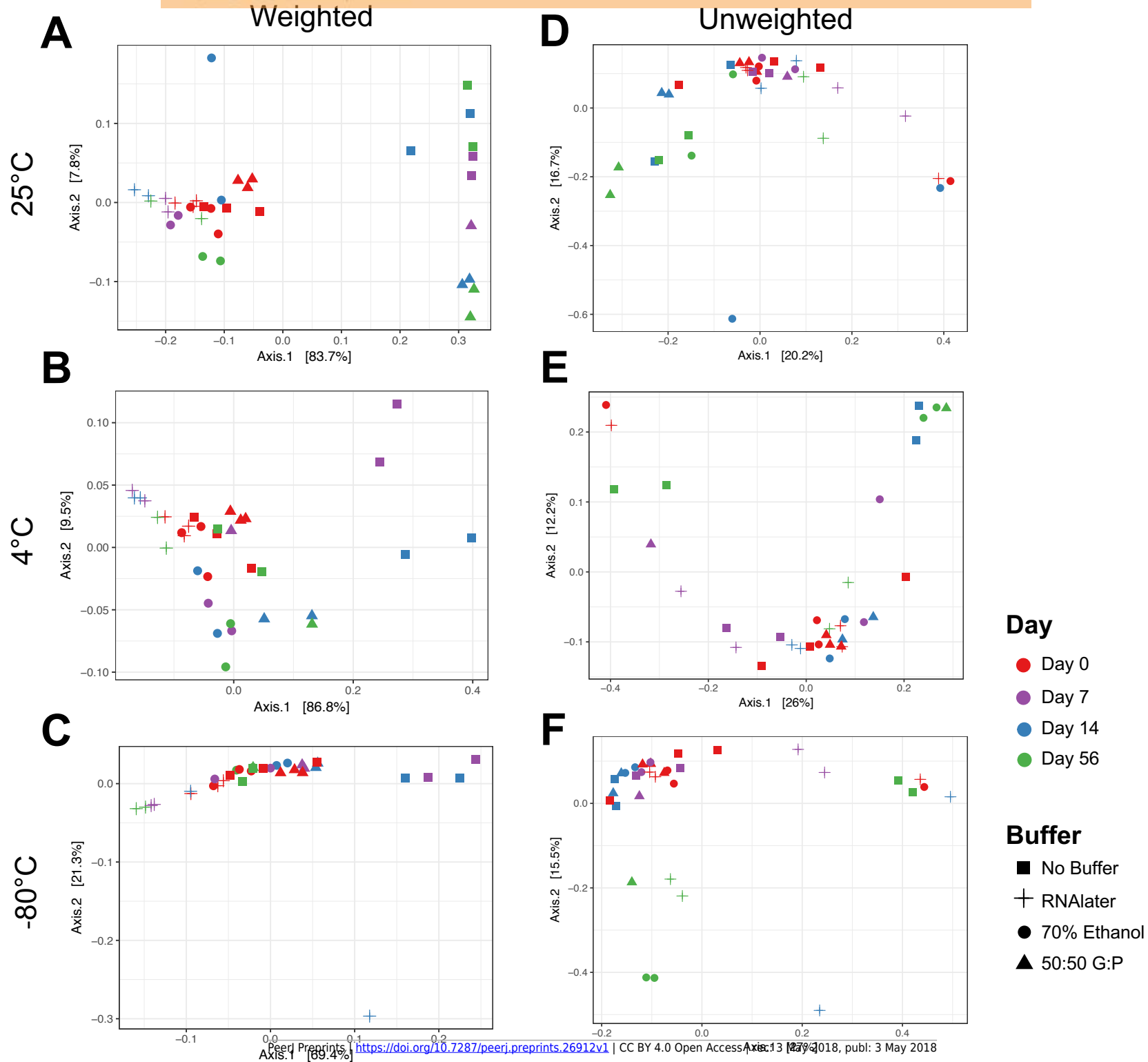
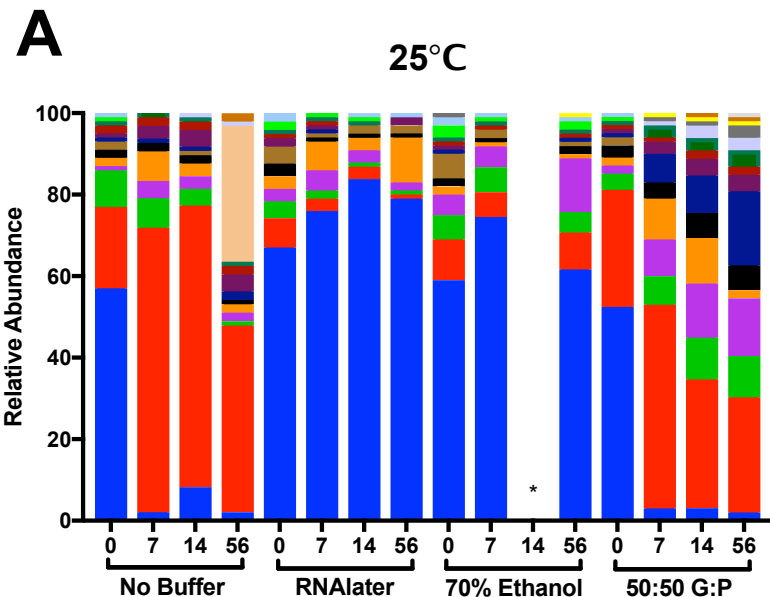


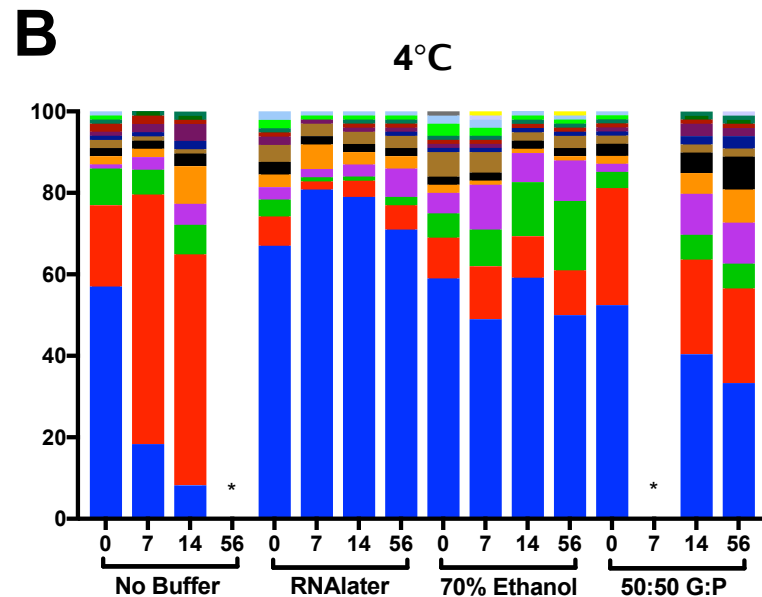
Figure 4(on next page)

Relative abundances of 24 highest taxonomic classifications of bacteria over 56 days.

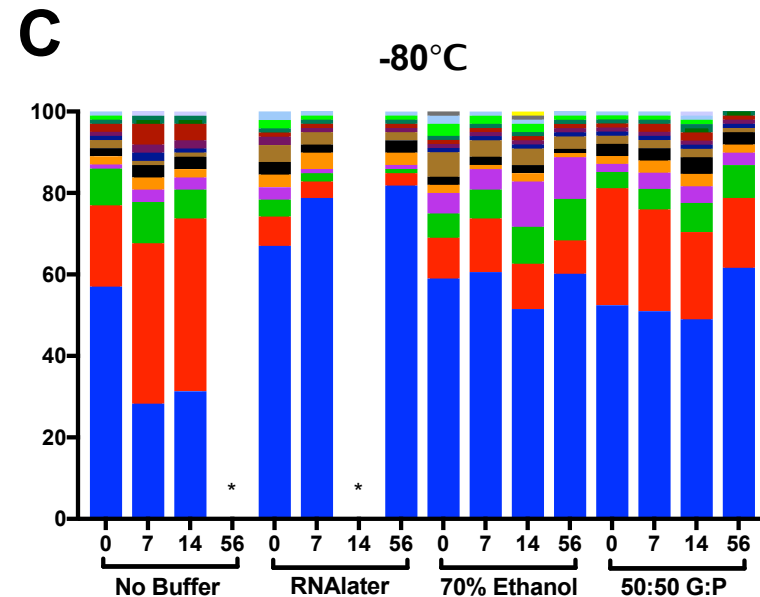
Each column represents the average of samples collected on Day 0, 7, 14, and 56 at (A) 25°C, (B) 4°C, and (C) -80°C. Canine fecal microbiota was largely dominated by genera *Prevotella* and *Streptococcus*. Comparison of Day 0 samples in different preservation buffers revealed rapid changes in abundance of *Prevotella* and *Streptococcus* with 70% ethanol and RNAlater. *Time points were excluded from samples that did not pass sequencing quality or low abundance OTU filtering.



f__Enterobacteriaceae
o__Clostridiales_other
g__Dorea
f__Lachnospiraceae_other
g__Helicobacter
g__Turicibacter
f__Clostridiaceae_g__
o__Clostridiales_f_g__



f__Ruminococcaceae_g__
f__Peptostreptococcaceae_g__
g__Fusobacterium
g__Sutterella
g__Enterococcus
f__Lachnospiraceae_g__
g__Clostridium
g__[Eubacterium]



g__Collinsella
g__Bacteroides
g__[Ruminococcus]
g__Blautia
g__Catenibacterium
g__Megamonas
g__Streptococcus
g__Prevotella

Figure 5(on next page)

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

Symbols represent relative abundances \pm standard error of the Firmicutes:Bacteroidetes ratio in samples stored at (A) 25°C, (B) 4°C, and (C) -80°C. Relative abundances of phyla Bacteroidetes in various storage buffers are shown after storage at (D) 25°C, (E) 4°C, and (C) -80°C. Preservation of bacterial composition at the phyla level was optimal under freezing conditions and storage with RNA later or 70% ethanol.

