Journal of Microbiological Methods 113 (2015) 16–26

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Effect of preservation method on spider monkey (*Ateles geoffroyi*) fecal microbiota over 8 weeks

Vanessa L. Hale [a,*], Chia L. Tan [b], Rob Knight [c,d], Katherine R. Amato [e]

[a] Department of Biological Sciences, Purdue University, West Lafayette, IN 47906, USA
[b] San Diego Zoo Institute for Conservation Research, Escondido, CA 92027, USA
[c] Department of Pediatrics, University of California San Diego, La Jolla, CA 92093, USA
[d] Department of Computer Science & Engineering, University of California San Diego, La Jolla, California 92093, USA
[e] Department of Anthropology, University of Colorado, Boulder, CO 80309, USA

ARTICLE INFO

Article history:
Received 26 November 2014
Received in revised form 24 March 2015
Accepted 24 March 2015
Available online 27 March 2015

Keywords:
Gut microbiota
Primate
Fecal preservation method
Fecal microbial community
Ateles geoffroyi

ABSTRACT

Studies of the gut microbiome have become increasingly common with recent technological advances. Gut microbes play an important role in human and animal health, and gut microbiome analysis holds great potential for evaluating health in wildlife, as microbiota can be assessed from non-invasively collected fecal samples. However, many common fecal preservation protocols (e.g. freezing at −80 °C) are not suitable for field conditions, or have not been tested for long-term (greater than 2 weeks) storage. In this study, we collected fresh fecal samples from captive spider monkeys (*Ateles geoffroyi*) at the Columbian Park Zoo (Lafayette, IN, USA). The samples were pooled, homogenized, and preserved for up to 8 weeks prior to DNA extraction and sequencing. Preservation methods included: freezing at −20 °C, freezing at −80 °C, immersion in 100% ethanol, application to FTA cards, and immersion in RNAlater. At 0 (fresh), 1, 2, 4, and 8 weeks from fecal collection, DNA was extracted and microbial DNA was amplified and sequenced. DNA concentration, purity, microbial diversity, and microbial composition were compared across all methods and time points. DNA concentration and purity did not correlate with microbial diversity or composition. Microbial composition of frozen and ethanol samples were most similar to fresh samples. FTA card and RNAlater-preserved samples had the least similar microbial composition and abundance compared to fresh samples. Microbial composition and diversity were relatively stable over time within each preservation method. Based on these results, if freezers are not available, we recommend preserving fecal samples in ethanol (for up to 8 weeks) prior to microbial extraction and analysis.

1. Introduction

The gastrointestinal (GI) tract is home to trillions of microbes that play an important role in shaping diet and digestion (Backhed et al., 2004; Ley et al., 2008; Martin et al., 2007; Turnbaugh et al., 2006), host immunity (Cho and Blaser, 2012; Chung et al., 2012; Hooper et al., 2012; Littman and Pamer, 2011), and disease processes (Petersen and Round, 2014; Round and Mazmanian, 2009; Sekirov et al., 2010). Recent advances in next-generation sequencing and bioinformatics have allowed us to analyze and compare entire gut microbial communities efficiently and effectively. As a result, the number of gut microbial studies published over the last 15 years has grown dramatically (Sekirov et al., 2010). Studies of the gut microbiome have also begun expanding to wildlife (Amato et al., 2013; Nelson et al., 2013; Uenishi et al., 2007; Villers et al., 2008; Xenoulis et al., 2010). These studies hold great potential for evaluating health in wildlife, as microbiota can be assessed from non-invasively collected fecal samples. However, there is limited information available on long-term (greater than 2 weeks) fecal microbial preservation methods under field conditions (Frantzen et al., 1998; Vlčková et al., 2012). Preserving fecal samples via freezing is commonly considered the 'gold-standard' for microbial analysis (Rochelle et al., 1994; Wu et al., 2010, but see Bahl et al., 2012), and most protocols focus on short term (less than 2 weeks) human or animal fecal preservation in highly controlled conditions including laboratories or hospitals with electricity and −20 °C or −80 °C freezers (Carroll et al., 2012; Dominianni et al., 2014; Lauber et al., 2010; Nechvatal et al., 2008; Ott et al., 2004; Roesch et al., 2009; Wu et al., 2010). Evaluation of methods for long-term storage under field conditions (i.e. without electricity/freezers) is necessary to understand if or how fecal microbial communities are affected by preservation method and time.

Feces are already used for many different types of wildlife studies including monitoring reproductive status (Dehnhard et al., 2008; Stoops

Abbreviations: GI, gastrointestinal; OTU, Operational Taxonomic Unit; QIIME, Quantitative Insights Into Microbial Ecology; FTA, Fast Technology for Analysis of nucleic acids; PCoA, Principal coordinate analysis; FDR, False Discovery Rate.

* Corresponding author at: Mayo Clinic, 200 1st St. SW, Harwick-3, Rochester, MN 55905, USA.
E-mail addresses: hale.vanessa@mayo.edu (V.L. Hale), ctan@sandiegozoo.org (C.L. Tan), robknight@ucsd.edu (R. Knight), Katherine.Amato@colorado.edu (K.R. Amato).

http://dx.doi.org/10.1016/j.mimet.2015.03.021
0167-7012/

et al., 1999), physiological stress (Cavigelli, 1999; Shutt et al., 2012), parasite load (Muller-Graf et al., 1999; Rietmann and Walzer, 2014), and genetic relatedness of populations (Adams et al., 2003; Mowry et al., 2011). Each of these types of studies have differing requirements in terms of fecal preservation, and much research has been devoted to optimizing fecal preservation in wildlife (for fecal steroid analysis (Khan et al., 2002; Shutt et al., 2012); for microsatellite amplification (Bubb et al., 2011; Murphy et al., 2002; Vallet et al., 2008); for parasite detection (Nielsen et al., 2010; Rietmann and Walzer, 2014)). Gut microbial studies have only recently begun in wildlife, and field-friendly microbial preservation methods still need to be validated, particularly with host species and dietary ecology in mind. For example, feces from herbivorous or folivorous host species (e.g. Barbary macaques, lowland gorillas) may contain high concentrations of secondary compounds that inhibit DNA extraction or PCR success (Vallet et al., 2008). Feces from animals that practice geophagy (consumption of soil directly or incidentally as a part of their diet) may contain large quantities of soil microbes (Delsuc et al., 2013). Preservation and analysis of such samples require thought regarding the transience or biological relevance of soil microbes within the gut.

To guide future fecal collection and preservation protocols for gut microbial studies in wildlife, particularly in herbivorous primates we assessed the effect of different preservation methods on the fecal microbiome of *Ateles geoffroyi*, the spider monkey, at the Columbian Park Zoo (Lafayette, IN, USA). Our study compared 5 methods of fecal preservation: freezing at −20 °C, freezing at −80 °C, immersion in 100% ethanol, application to FTA cards, and immersion in RNAlater.

These methods were selected because they are relatively common fecal preservation techniques with varying advantages and disadvantages. Freezing, one of the most common preservation methods, inhibits microbial growth, limits opportunities for contamination, and effectively preserves DNA over time (Rochelle et al., 1994; Wu et al., 2010). However, few studies have examined differences between freezing at −20 °C and −80 °C. Additionally, freezing is often not a viable method for field studies. Chemical means of fecal preservation such as ethanol, RNAlater or FTA cards are more "field friendly" methods of preservation. Like freezing, ethanol is recognized as another common and effective fecal DNA preservation method (Murphy et al., 2002), but restrictions apply to ethanol transport due to its status as a 'hazardous chemical.' RNAlater is a nonhazardous liquid that preserves both RNA and DNA (Nechvatal et al., 2008). While RNAlater faces fewer transport restrictions than ethanol, both RNAlater and ethanol pose another challenge; carrying large quantities of liquid into remote locations can be logistically difficult. FTA cards are the easiest to transport and most convenient to use in the field, but convenience comes at a price: FTA cards and RNAlater are the most expensive preservation methods.

We hypothesized that freezing would be the most effective method for preserving microbial DNA. Specifically, we predicted that frozen (at −20 °C and −80 °C) samples would be the most stable in terms of DNA concentration, purity, and microbial composition over 8 weeks. Previous studies have found that freezing results in greater DNA concentrations with higher purity values compared to RNAlater, ethanol, and FTA card preservation (Nechvatal et al., 2008; Vlčková et al., 2012). We also predicted that the microbial communities of frozen samples would most closely resemble the microbial communities of fresh, never-preserved, immediately-extracted fecal samples.

2. Methods

Spider monkeys are herbivorous primates native to South and Central America (González-Zamora et al., 2009) that consume leaves and fruits (González-Zamora et al., 2009). The captive diet of these monkeys consists of fresh fruits and vegetables along with primate pellets (Mazuri leaf eater biscuits, Richmond, IN, USA). In terms of nutritional value, the captive and wild diets are similar.

2.1. Fecal collection and processing

Fecal samples were collected in September 2013 from a group of spider monkeys (n = 6) at the Columbian Park Zoo (Lafayette, IN, USA). The group was composed of 1 adult male and 5 adult females. Two of the monkeys were related (father/daughter); the other 4 adult females were unrelated. The monkeys were all housed in the same enclosure, and none of the monkeys had been treated with antibiotics during the 12 months prior to sample collection. All fecal samples were fresh (<1 hour old) and were immediately transported on ice to Purdue University (West Lafayette, IN, USA), a 3 mile trip that takes approximately 15 min. Upon arrival at Purdue, all samples were pooled and homogenized.

Five common fecal storage methods were tested: freezing at −20 °C, freezing at −80 °C, immersion in 100% ethanol, application to FTA cards (Whatman Inc., Florham Park, NJ, USA), and immersion in RNAlater (Ambion, Austin, TX, USA). Immediately after pooling and homogenization of the feces, feces were divided into 0.25 g aliquots. A total of 42 aliquots were prepared: 8 aliquots for each of the 5 storage methods, and 2 aliquots for immediate extraction. For samples subjected to freezing or immersion in ethanol or RNAlater, fecal aliquots were placed in 1.5 ml Eppendorf tubes. RNAlater and ethanol were then added to the appropriate tubes, fully immersing each fecal sample. RNAlater and ethanol samples were stored at room temperature throughout the study (8 weeks). For FTA card storage, the 0.25 g aliquots of feces were applied to FTA card sample circles using sterile cotton swabs (Dynarex, Orangeburg, NY, USA). FTA cards were allowed to air dry on a laboratory bench for 12–24 h. Then, cards were stored at room temperature in individual Ziploc bags with MiniPax desiccant packets (Multisorb technologies Inc., Buffalo, NY, USA). Desiccant packets were replaced periodically. The 2 fecal aliquots prepared for immediate extraction were not subjected to any type of preservation method and are henceforth denoted as "week 0" samples. DNA from these "week 0" samples was extracted within 3 h of fecal collection. The rest of the aliquots were extracted in duplicate at 1, 2, 4, and 8 weeks from the day of fecal collection.

DNA extraction, amplification, and library preparation were performed according to Earth Microbiome Project (EMP) protocols ((Gilbert et al., 2010); web page: http://www.earthmicrobiome.org/emp-standard-protocols/) with one modification. Prior to DNA extraction, a 2 mm Harris Uni-Core biopsy punch (TedPella, Redding, CA, USA) was used to make 20 punches in each FTA card sample circle. These 20 punches per sample were used in lieu of whole feces during DNA extraction. One important caveat: FTA card samples started the DNA extraction process with less fecal matter than all other methods. In all preservation methods, 0.25 g of feces was used for DNA extraction. For FTA cards, 20 punches from a sample circle equated to approximately 0.008 g of feces used in each FTA card extraction. If DNA concentrations were calculated in terms of ng/μl/g feces, then FTA cards would yield the highest DNA concentrations relative to all other preservation methods. However, because DNA concentrations are most commonly assessed and compared using nanograms per microliter (ng/μl), this is how we chose to analyze and display our results.

At weeks 0, 2, 4, and 8, DNA extraction was performed using a 50-prep PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). At week 1, DNA extraction was performed using a 96-well plate PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). The only difference between the 50-prep kit and the 96-well plate kit is the amount of supernatant transferred after the "C3" step. In the 96-well kit, 650 μl of supernatant is transferred to the spin filter; in the 50-prep kit, 750 μl of supernatant is transferred (M.T. Carlson, MoBio, personal communication). A NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE) was used to quantify DNA

concentration and purity (A260/A280 ratio) after extraction. Amplification targeted the ~300 bp variable region 4 (V4) of the 16S rRNA gene using bacterial primers 515F and 806R (Caporaso et al., 2012). Golay barcodes and Illumina adaptors were included in the primer construct for each sample. Amplicons were pooled for sequencing and cleaned using a MoBio UltraClean PCR Clean-Up Kit (MoBio Laboratories, Carlsbad, CA, USA). Samples were paired-end sequenced on an Illumina MiSeq. Sequencing was performed by the BioFrontiers Institute Next-Generation Genomics Facility at the University of Colorado Boulder (Boulder, CO, USA) (Gilbert et al., 2010). Sample processing, sequencing and core amplicon data analysis were performed as part of the Earth Microbiome Project (www.earthmicrobiome.org), and all amplicon and metadata have been made public through the data portal (www.microbio.me/emp and http://www.microbio.me/qiime/).

2.2. Microbial taxonomic assignment

We used default parameters in QIIME (Quantitative Insights Into Microbial Ecology — software version 1.8.0) to de-multiplex, quality-filter, and cluster 16S rRNA amplicon sequences (Caporaso et al., 2010b). A total of 1,306,754 reads (mean: 31,113; standard deviation: 10,442) were obtained after filtering. A threshold of 97% sequence identity was used to cluster sequences into operational taxonomic units (OTUs). In other words, microbial DNA sequences that were at least 97% similar were grouped as a single OTU. We then used QIIME's implementation of UCLUST to perform open-reference OTU picking, using the Greengenes reference data set (version 13_8, release date August 2013 (McDonald et al., 2011)) (http://greengenes.secondgenome.com). Using PyNAST, representative sequences for OTUs were then aligned to the Greengenes reference alignment (Caporaso et al., 2010a). RDP classifier was used to assign de novo OTUs to taxonomies (Wang et al., 2007) with a minimum of 80% confidence. All samples were rarified at 14,120 reads. Two samples fell below this cutoff and were removed from the analyses: 1 sample preserved in ethanol for 8 weeks (total reads: 2), and 1 sample preserved at −80 °C for 2 weeks (total reads: 20).

2.3. Statistical analyses

DNA concentration was compared across preservation method and week using a repeated measures mixed model in SAS (version 9.3, released in July 2011) with LSMEANS and post-hoc pairwise comparisons (LSMEANS/diff). The model included preservation method and week as fixed factors. DNA purity (A260/A280 ratio) was compared across preservation method and week using a Friedman's test with post-hoc comparisons in RStudio (version 0.98.501, 2013) (R: (RCoreTeam, 2013); R package "coin": (Hothorn et al., 2006, 2008b); R package "multcomp": (Hothorn et al., 2008a); post-hoc tests: (Hollander and Wolfe, 1999); R code: (Galili, 2010)). Friedman's test is non-parametric repeated measures analysis of variance (Galili, 2010; UCLA, 2014). An ideal A260/280 ratio for pure nucleic acid ranges from 1.8 to 2.0. Ratios lower than this indicate contamination (excess protein or inhibitory compounds or residual reagent), or low DNA concentrations (ThermoScientific, 2011).

We calculated Shannon and Simpson diversity indices for each sample within QIIME. Both indices yielded similar patterns, so we only report Shannon index results here. Shannon diversity values were compared with LSMEANS and post-hoc pairwise comparisons (LSMEANS/diff) in SAS (version 9.3, released in July 2011). A repeated measures mixed model – including preservation method and week – was used to compare Shannon indices.

We compared OTU frequencies between preservation methods using a Kruskal–Wallis test and FDR corrected p-values (QIIME script: group_significance.py). Supervised learning analyses were also performed in QIIME to determine if preservation method or week affected microbial composition (OTUs) (Breiman, 2001; Knights et al., 2011). This analysis uses 80% of the data as a training set, and 20% of the data as a test set. 1000 decision trees were generated based on microbial composition (OTUs) and preservation methods. Results from this analysis produced feature importance scores, an error ratio and a confusion matrix. We also ran a PERMANOVA in RStudio (version 0.98.501, 2013) to assess the role of preservation method and week on microbial composition (R: (RCoreTeam, 2013); R package "vegan": (Oksanen et al., 2013); R package "qiimer": (Bittinger, 2014)).

UniFrac distance matrices were used to assess beta diversity (Lozupone and Knight, 2005). UniFrac calculates distances between microbial communities based on their microbial composition and takes phylogenetic similarity among microbes into account. Principal coordinate analysis (PCoA) was used to visualize patterns in UniFrac distances. Samples with more similar communities – in terms of microbial composition and abundance – cluster together. A weighted PCoA accounts for microbial composition and abundance. An unweighted PCoA only accounts for microbial composition but not abundance. UniFrac distances were compared within and between preservation methods (QIIME script: make_distance_boxplots.py) using two-sample t-tests between all pairs of boxplots.

3. Results

3.1. DNA concentration and purity

DNA concentrations varied significantly by preservation method and by week (method: $F_{4,21} = 34.61$; $p < 0.0001$; week: $F_{3,21} = 30.76$; $p < 0.0001$; Fig. 1). The interaction of method and week was also significant (method * week: $F_{12,21} = 8.55$; $p < 0.0001$). DNA concentrations in week 1 were significantly lower than DNA concentrations measured at all other weeks (week 2: $t_{21} = 8.35$, $p < 0.0001$; week 4: $t_{21} = 6.69$, $p < 0.001$; week 8: $t_{21} = 8.08$, $p < 0.001$). There was insufficient power to compute post-hoc comparisons between week 0 and week 1 samples. No other significant differences were detected in DNA concentrations between weeks (all $t < 1.66$, all $p > 0.11$). When we removed week 1 data from the analysis, "week" no longer had a significant effect on DNA concentration ($F_{2,16} = 1.74$; $p = 0.2$).

FTA cards yielded significantly lower DNA concentrations (ng/μl) than all other methods (freezing at −20 °C: $t_{21} = 8.82$, $p < 0.0001$; freezing at −80 °C: $t_{21} = 9.87$, $p < 0.001$; ethanol: $t_{21} = 3.78$, $p = 0.001$; RNAlater: $t_{21} = 2.89$, $p = 0.009$). (Note: As described in the Methods section, FTA card samples started the DNA extraction process with approximately 0.008 g of feces while all other preservation methods started the DNA extraction process with 0.25 g of feces.) Freezing at −20 °C or −80 °C yielded significantly greater DNA concentrations than ethanol or RNAlater-preserved samples (freezing at −20 °C versus ethanol: $t_{21} = 6.09$, $p < 0.0001$; freezing at −20 °C versus RNAlater: $t_{21} = 5.94$, $p < 0.0001$; freezing at −80 °C versus ethanol: $t_{21} = 5.05$, $p < 0.0001$; freezing at −80 °C versus RNAlater: $t_{21} = 6.98$, $p < 0.0001$).

Within a preservation method, other significant differences were observed besides differences noted in week 1. Freezing at −20 °C resulted in significantly higher DNA concentrations in week 8 compared to weeks 2 and 4 (week 2: $t_{21} = 3.11$, $p = 0.005$; week 4: $t_{21} = 2.21$, $p = 0.04$). Freezing at −80 °C also resulted in significantly higher DNA concentrations in week 8 compared to week 4 ($t_{21} = 2.29$, $p = 0.03$). RNAlater-preserved samples had significantly greater DNA concentrations in week 2 compared to weeks 4 and 8 (week 4: $t_{21} = 2.33$, $p = 0.03$; week 8: $t_{21} = 2.83$, $p = 0.01$). The only samples that did not differ significantly in DNA concentration from fresh samples were week 2 samples frozen at −80 °C, week 8 samples frozen at −20 °C, or week 8 samples frozen at −80 °C (week 2 freezing at −80 °C: $t_{21} = 1.56$, $p = 0.13$; week 8 freezing at −20 °C: $t_{21} = 1.05$, $p = 0.3$; week 8 freezing at −80 °C: $t_{21} = 1.25$, $p = 0.22$). Ethanol-preserved samples, and FTA card samples did not differ significantly in DNA concentration between weeks 2, 4, and 8 (all $t < 1.25$, all $p > 0.24$).

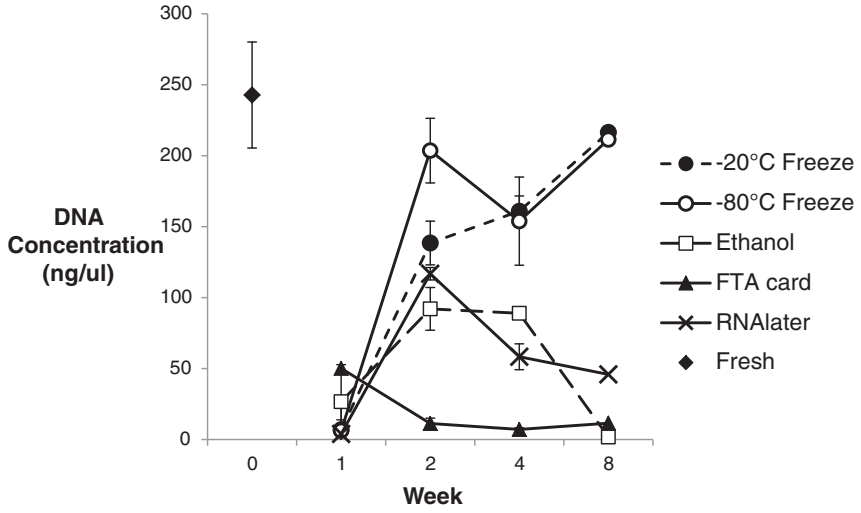

Fig. 1. DNA concentration (ng/μl) by preservation method and week. Error bars represent standard error around the arithmetic means. Week 1 DNA concentrations were significantly lower than all other weeks. FTA card concentrations were significantly lower than all other preservation methods. Freezing at −20 °C or −80 °C resulted in significantly higher DNA concentrations than preservation by ethanol or RNAlater.

DNA purity also differed by preservation method but not by week (A260/A280 ratio — preservation method: maxT = 3.96; p = 0.0008; week: maxT = 1.86; p = 0.25; Fig. 2; A260/A230 ratio — see Supplementary materials; Fig. S1). FTA card samples had significantly lower A260/A280 ratios than samples preserved by freezing at −80 °C (p = 0.0007) and marginally lower A260/A280 ratios than samples preserved by freezing at −20 °C (p = 0.052). There were no other significant differences between preservation methods (all p > 0.19). All preservation methods, except FTA cards had average A260/A280 ratios within the normal range (1.8–2.0). FTA cards had an average A260/A280 ratio of 1.5.

3.2. Microbial species diversity

Shannon diversity indices differed significantly across preservation methods ($F_{4,31} = 33.53$; $p < 0.0001$; Fig. 3) and weeks ($F_{3,31} = 16.60$; $p < 0.0001$; Fig. 4). The interaction between preservation method and week was not significant and was removed from the model ($F_{12,19} = 1.10$; $p = 0.42$). Post-hoc pairwise comparisons indicated that FTA card preservation resulted in significantly greater microbial diversity than all other preservation methods (−20 °C freezing: $t_{31} = 8.13$, $p \leq 0.0001$; −80 °C freezing: $t_{31} = 8.71$, $p \leq 0.0001$; RNAlater: $t_{31} = 6.62$, $p \leq 0.0001$; ethanol: $t_{31} = 10.51$, $p \leq 0.0001$;). Additionally, RNAlater samples had greater microbial diversity than samples preserved in ethanol ($t_{31} = 4.13$, $p = 0.0003$) or by freezing at −80 °C ($t_{31} = 2.33$, $p = 0.03$). Also, ethanol preservation resulted in greater fecal microbial diversity than freezing at −20 °C ($t_{31} = 2.68$, $p = 0.01$). As there were only 2 week 0 samples, there was insufficient power to compute post-hoc comparisons between fresh and preserved samples or week 0 and later samples. When microbial diversity was compared across the rest of the weeks (weeks 1, 2, 4 and 8), post-hoc comparisons identified week 1 microbial diversity as significantly greater than all other weeks (week 2: $t_{31} = 5.67$, $p < 0.0001$; week 4: $t_{31} = 6.18$, $p < 0.0001$ week 8: $t_{31} = 5.06$, $p < 0.0001$).

3.3. Microbial composition

Microbial composition as characterized following storage under different conditions differed across preservation methods at all taxonomic levels (phyla: Fig. 5; genera: see Supplementary material, Fig. S2). Notably, FTA cards preserved a higher relative abundance of OTUs within the *Firmicutes* phylum and RNAlater preserved a lower relative abundance

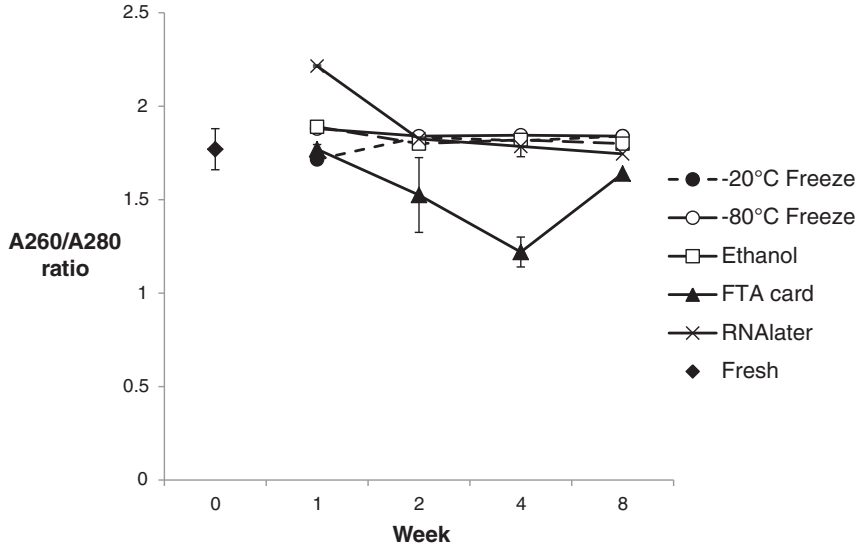

Fig. 2. A260/A280 ratio (DNA purity) by preservation method and week. Error bars represent standard error around arithmetic means.

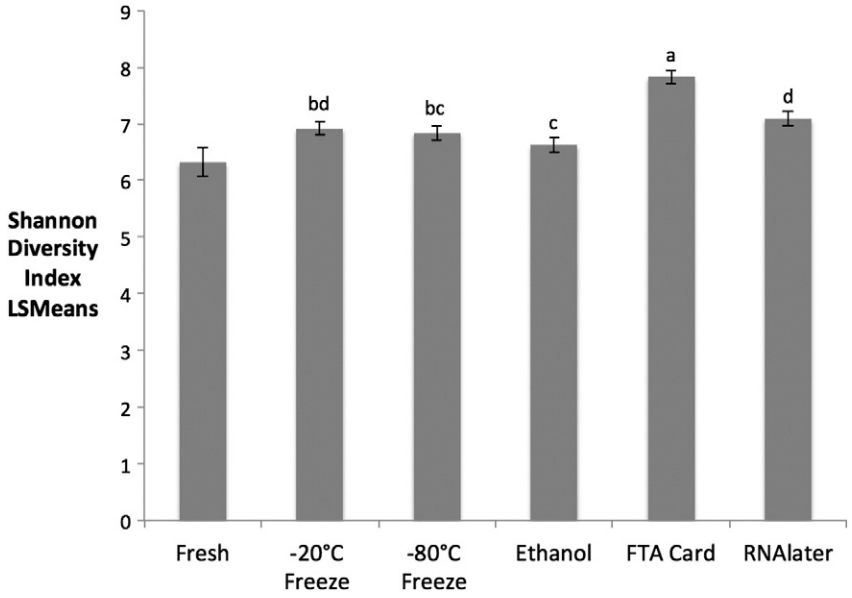

Fig. 3. Shannon diversity index values by preservation method. Methods marked with the same letter do not differ significantly in post-hoc pairwise comparisons. Error bars represent standard errors.

of OTUs within the phylum *Bacteroidetes*. These results were further supported through the group significance tests at both the phylum and genus levels. At the phylum level, relative abundance of 11 microbes differed significantly between preservation methods (all FDR corrected p-values < 0.05; see Supplementary material, Table S1). In total, 199 OTUs differed significantly between preservation methods (all FDR corrected p-values <0.05; see Supplementary material, Table S2). OTUs in the phyla *Firmicutes* (122 OTUs) and *Bacteroidetes* (18 OTUs) made up the majority of these significant OTUs. Most of the significant OTUs were designated as such due to very high or very low FTA card or RNAlater OTU frequencies. FTA cards had the highest mean OTU frequencies in 121 of the significant OTUs (including 102 OTUs in the phylum *Firmicutes*), and the lowest mean OTU frequencies in 27 of the significant OTUs. RNAlater had the highest mean OTU frequencies in 33 of the significant OTUs and the lowest mean OTU frequencies in 38 of the significant OTUs. There were 9 OTUs in the genera *Prevotella* (phylum *Bacteroidetes*) among the 199 significant OTUs. RNAlater had the lowest mean OTU frequencies in 8 of these 9 *Prevotella* OTUs. RNAlater also had the lowest OTU frequencies in 15 of the 42 OTUs in the family *Ruminococcaceae*. FTA cards had the lowest mean OTU frequencies in 3 of the 6 significant *Proteobacteria* OTUs and 4 of the 5 significant *Cyanobacteria* OTUs. In contrast, ethanol had the highest mean OTU frequencies in all 6 *Cyanobacteria* OTUs. Of the 199 significant OTUs, 140 also ranked in the top 200 based on feature importance scores from supervised learning analyses.

In the supervised learning analysis based on preservation method, the reported error ratio (error of random guessing over the sum of the error in the test sets) was 4.08. Error ratios greater than 2 indicate that groups are significantly different. Based on this analysis, preservation method significantly influences the characterization of fecal microbial community composition and abundance. The confusion matrix assigns samples to groups based on microbial composition. RNAlater, FTA card, and ethanol samples were all correctly assigned (Table 1a). Frozen samples were misassigned either to other frozen samples (e.g.

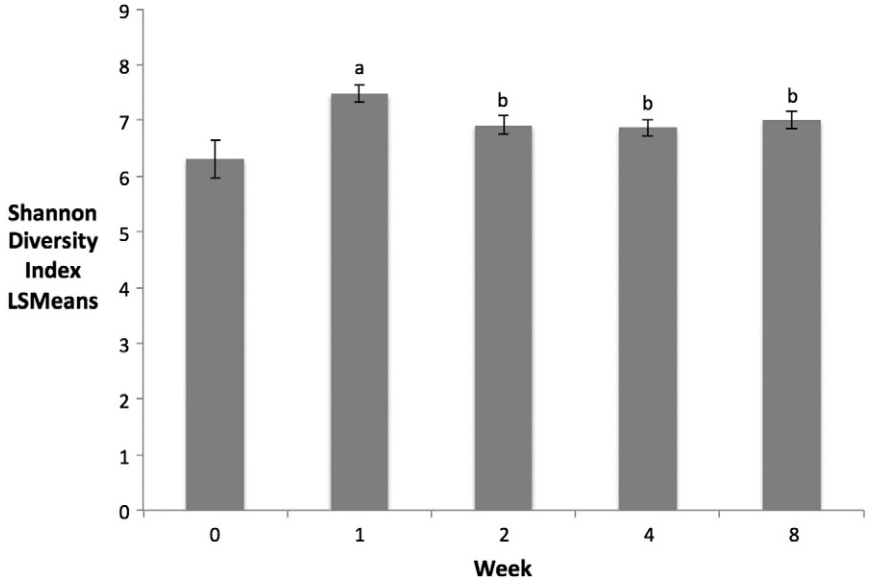

Fig. 4. Shannon diversity index values by week. Weeks marked with the same letter do not differ significantly in post-hoc pairwise comparisons. Error bars represent standard errors.

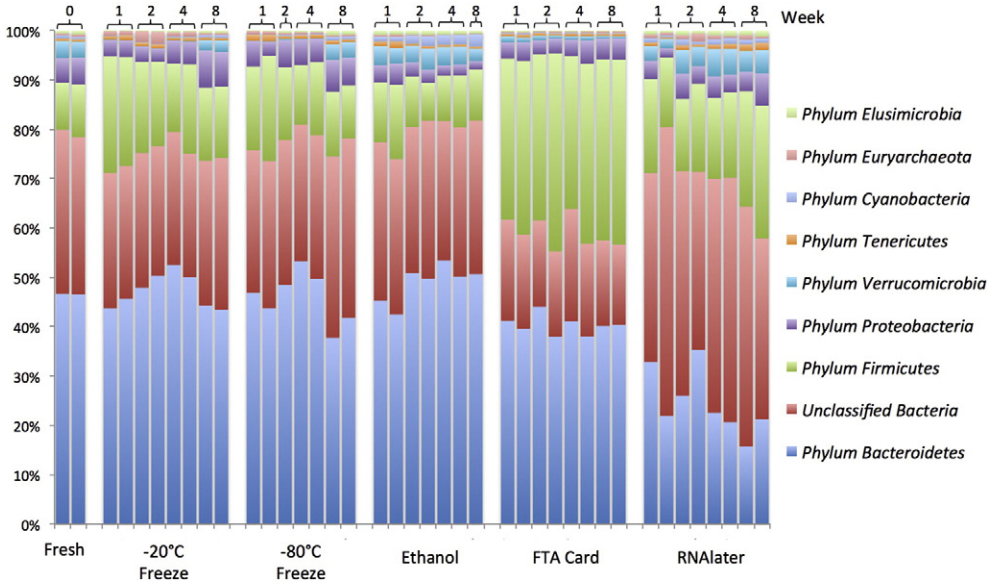

Fig. 5. Relative abundances of bacterial phyla by preservation method. Each column represents the microbial community from a single sample. Only phyla that comprised greater than 0.5% relative abundance (on average) were included below. Phylum *Bacteroidetes* (blue) and Phylum *Firmicutes* (green) made up the majority of the phyla in each sample. FTA cards preserved a significantly greater relative abundance of *Firmicutes* than any other preservation method.

−20 °C samples misassigned as −80 °C samples) or to ethanol samples. Fresh samples were all misassigned to ethanol or −80 °C samples. A supervised learning analysis was also run based on the week from fecal collection. The error ratio was 1.15 indicating no significant differences in microbial composition over time.

Greater than 50% of the samples were misassigned in all weeks except week 1 (Table 1b). This indicates that the characterization of microbial communities under different storage conditions was similar over all weeks except in week 1.

PERMANOVA results in R supported the supervised learning results: preservation method but not week resulted in significant differences in microbial composition (preservation method pseudo-$F = 21.26$; $p = 0.001$; week pseudo $F = 0.74$; $p = 0.56$). The interaction between preservation method and week was also not significant (pseudo-$F = 2.0$; $p = 0.06$).

Principal coordinate analysis (PCoA) based on unweighted and weighted UniFrac distances indicated that samples clustered by preservation method (Figs. 6, 7). Fresh samples clustered with ethanol-preserved and frozen samples. In both the weighted and unweighted PCoA, samples preserved by FTA cards clustered apart from all other samples. In the weighted analysis, RNAlater samples also clustered apart from other samples and apart from FTA cards.

PCoA analysis performed on weighted UniFrac distances with samples separated by week (Fig. 8) demonstrated that within a preservation method, microbial communities remained relatively stable over the 8 weeks.

Distance boxplot analysis compared weighted UniFrac distances within and between preservation methods (Fig. 9). The distance within the following preservation methods, freezing at −20 °C, freezing at −80 °C, and immersion in ethanol, was not significantly different than the distance between these preservation methods and fresh samples respectively (all $t < 2.3$; all Bonferroni-corrected $p > 0.05$). The distance within FTA card and RNAlater-preserved samples was significantly less than the distance between fresh samples and FTA or RNAlater-preserved samples respectively (all $t > 4.0$; all $p < 0.05$).

Results from all analyses are summarized in Table 2.

4. Discussion

Our results demonstrate clear differences in DNA concentration and purity as well as microbial species diversity and composition among fecal preservation methods. Overall, our results supported our hypothesis that freezing was the most effective and stable method for preserving microbial DNA in terms of DNA concentration over time. However,

Table 1
Confusion matrix produced by supervised learning analysis. Rows indicate true assignment of each sample. Columns indicate predicted assignment of each sample based on OUT composition. The "class error" column lists rate of misassignment for samples within a) preservation method or b) week.

	True\predicted	− 20 °C freeze	− 80 °C freeze	Ethanol	Fresh	FTA	RNAlater	Class error
A)	− 20 °C freeze	6	2	0	0	0	0	0.25
	− 80 °C freeze	3	3	1	0	0	0	0.57
	Ethanol	0	0	7	0	0	0	0
	Fresh	0	1	1	0	0	0	1
	FTA	0	0	0	0	8	0	0
	RNAlater	0	0	0	0	0	8	0

	True\predicted	Week 0	Week 1	Week 2	Week 4	Week 8	Class error
B)	Week 0	0	0	0	1	1	1
	Week 1	0	9	0	1	0	0.10
	Week 2	0	1	0	6	2	1
	Week 4	0	0	3	4	3	0.60
	Week 8	0	0	2	6	1	0.89

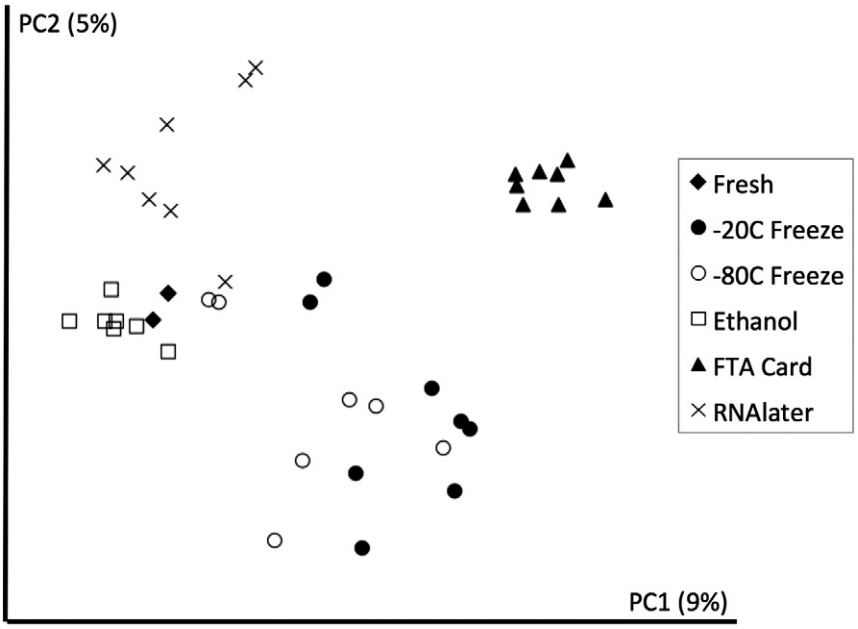

Fig. 6. Principal coordinate analysis on unweighted UniFrac distances. Unweighted UniFrac metrics account for microbial species richness but not evenness.

freezing, ethanol, and RNAlater performed similarly in terns of DNA purity over time. Also, both freezing and ethanol preserved microbial communities were alike in composition and diversity, and both preservation methods yielded microbial compositions similar to fresh, never preserved, immediately extracted fecal samples. Finally, we found differences in DNA concentration and microbial diversity by week; however, the majority of these differences are attributed to week 1, the only week in which a 96-well plate PowerSoil DNA Isolation kit was used for extraction rather than a 50-prep PowerSoil DNA Isolation kit.

4.1. DNA concentration and purity

The low DNA concentrations recorded in week 1 were surprising and may be related to extraction method. The protocol and reagents used in the 50-prep and 96-well plate kits are nearly identical; however, week 1 results were distinct across all preservation methods (Fig. 1). Week 8 DNA concentrations were significantly higher in frozen samples than all other preservation methods and most earlier time points. DNA concentrations can increase as a result of bacterial growth, which increases the amount of DNA within a sample, or increased cell lysis, which increases the accessibility of DNA during extraction. While there are some unique environmental bacteria that are capable of growing at extremely low temperatures (e.g. −15 °C) (Mykytczuk et al., 2013), fecal bacterial growth at −20 °C or −80 °C is unlikely. Increased cell lysis is a more feasible explanation in this case. Each frozen sample only went through one freeze–thaw cycle; therefore, freeze–thaw cell lysis does not account for the increased DNA concentrations over time. However, lysis could occur in samples subjected to prolonged freezing. One study on bacteria in frozen breast milk reported a decrease in colony counts over nine months (Ahrabi et al., 2013). In other words, the number of viable bacterial cells decreased over time in the freezer — likely due to cell lysis. This lysis may have rendered the DNA more accessible

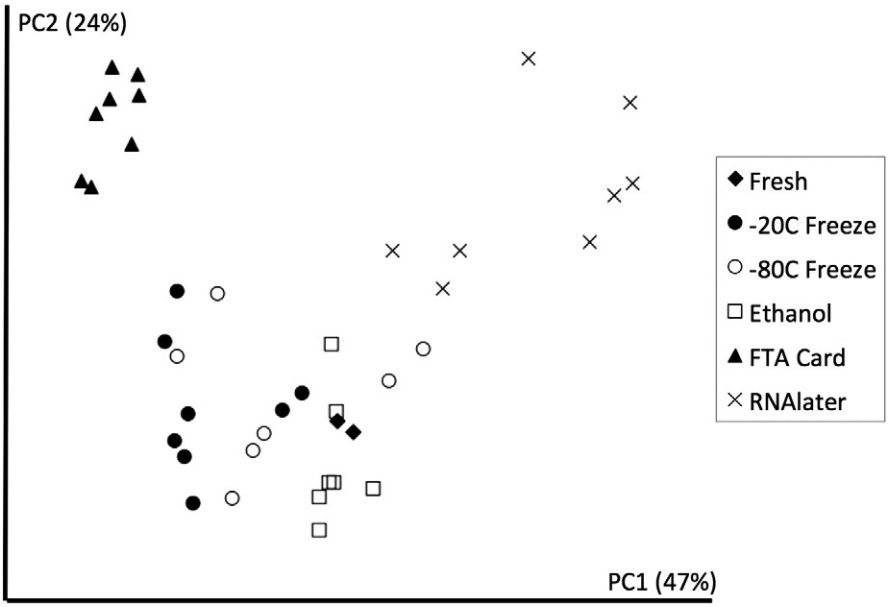

Fig. 7. Principal coordinate analysis by week on weighted UniFrac distances. Weighted UniFrac metrics account for microbial species richness and evenness.

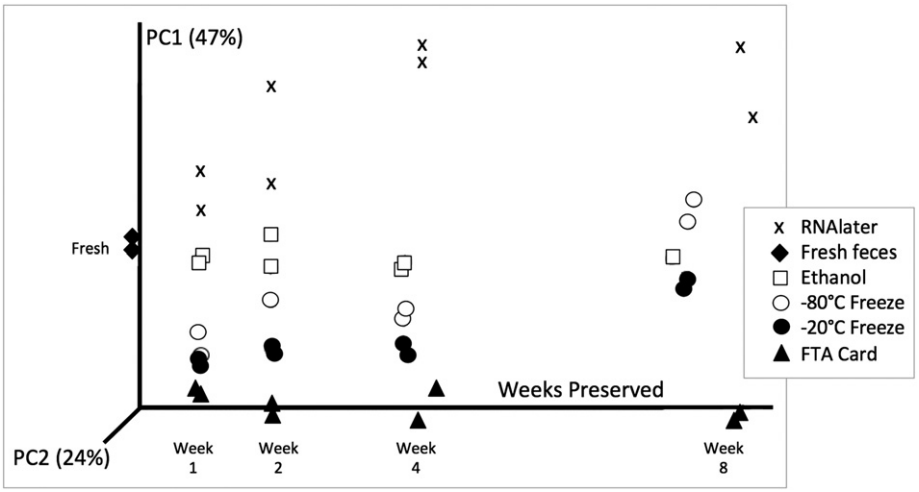

Fig. 8. Principal coordinate analysis by week on weighted UniFrac distances. Microbial communities remain relatively stable within a preservation method over 8 weeks.

to extraction. DNA extraction methods vary greatly in cell lysis efficiency, but even the most robust methods fail to achieve 100% cell lysis (Anderson and Lebepe-Mazur, 2003; Li et al., 2007; Mackenzie et al., 2015; Salonen et al., 2010; Scupham et al., 2007; Vishnivetskaya et al., 2014; Yuan et al., 2012). Thus, if a sample undergoes some cell lysis prior to extraction (i.e. due to prolonged freezing), it may appear to have a higher DNA concentration compared to a sample that has not been subject to conditions that induce lysis (i.e. samples frozen for a short period of time).

In contrast, the DNA concentration of samples preserved in RNAlater significantly decreased between week 2 and week 8. RNAlater manufacturer instructions indicate that samples can be stored at room temperature (25 °C) for one week, at 4 °C for one month, and indefinitely at −20 °C (www.lifetechnologies.com). In this study, samples were stored in RNAlater at room temperature for 8 weeks to simulate "field conditions." The decline in DNA concentrations over time may have been due to DNA degradation associated with non-optimal storage conditions.

FTA cards produced the lowest DNA concentrations, but also began extractions with the least amount of fecal material. FTA card A260/A280 ratios were also below normal range; however, there was no relationship between DNA concentration/purity and amplification success, microbial diversity, or microbial composition. All FTA cards amplified and produced over 14,000 sequence reads. Results similar to ours have been reported in previous studies; feces preserved with FTA cards yielded low DNA concentrations but amplified with little PCR inhibition (Nechvatal et al., 2008). In another study on fecal preservation in primates, DNA concentration was not a good indicator of target DNA amount (i.e. host versus microbial), and DNA purity was not correlated with amplification success (Bubb et al., 2011). In our study, only 2 samples did not amplify well. One was an ethanol sample from week 8; the other was a sample frozen at −20 °C from week 2. Neither of these samples had low DNA concentrations or low A260/A280 ratios. In fact, frozen samples (−20 °C and −80 °C) yielded the highest DNA concentrations and purities. Fresh samples also had high DNA concentrations and purities.

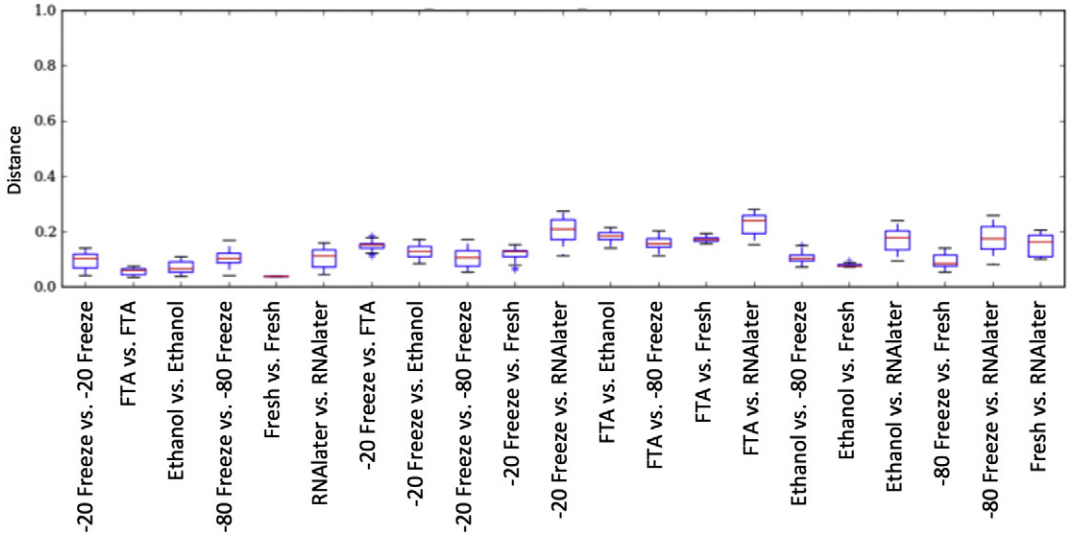

Fig. 9. Weighted UniFrac distance comparison boxplots within and between preservation methods. The distance within the following preservation methods: freezing at −20 °C, freezing at −80 °C, and immersion in ethanol, was not significantly different than the distance between these preservation methods and fresh samples respectively. The distance within FTA card and RNAlater-preserved samples was significantly less than the distance between fresh samples and FTA or RNAlater-preserved samples respectively.

Table 2
Summary of results. All of the major findings from this study are reported by preservation method and week.

	DNA		Microbial diversity	Microbial composition				
	Concentration	**Purity**	**Shannon diversity index**	**Relative abundance of OTUs**	**Group significance test**	**Supervised learning**	**PCoA**	**Distance boxplots**
By preservation method	FTA cards: lowest	Freezing > FTA cards	FTA cards: highest	FTA cards: highest *Firmicutes*	FTA cards: highest *Firmicutes* lowest *Proteobacteria* lowest *Cyanobacteria*	Fresh, frozen, and ethanol = most similar. Samples commonly misassigned between these 3 methods	Unweighted analysis: fresh samples cluster with ethanol and RNAlater. FTA cards cluster separately	Freezing and ethanol: most similar to fresh.
	Freezing > Ethanol or RNAlater		RNAlater > ethanol or freezing at −80 °C	RNAlater: lowest *Bacteroidetes*	RNAlater: lowest *Bacteroidetes* (genus *Prevotella*) lowest *Ruminococcaceae*	RNAlater and FTA cards = most dissimilar. All samples were assigned perfectly to these methods	Weighted analysis: fresh samples cluster with ethanol and freezing. FTA cards and RNAlater cluster separately	FTA cards and RNAlater least similar to Fresh.
			Ethanol > freezing at −20 °C		Ethanol: highest *Cyanobacteria*			
By week	Week 1: lowest	No differences by week	Week 1: highest	No differences by week			Stable microbial composition/ abundance over time	

4.2. Microbial species diversity

In contrast to the concentration and purity results, FTA cards (with low DNA concentrations) exhibited the highest microbial diversity, while frozen samples (with high DNA concentrations) exhibited the lowest microbial diversity. Some studies suggest that high DNA concentrations are critical to recovering and detecting rare microbial species in fecal samples (Anderson and Lebepe-Mazur, 2003; Scupham et al., 2007). Other studies – like ours – found no correlation between DNA concentration and microbial diversity (Salonen et al., 2010).

Fresh (week 0) samples exhibited the lowest microbial diversity. This unexpected finding may be a consequence of DNA degradation or microbial enrichment – in which a few microbes multiply and outcompete other microbes present in the feces – prior to extraction. We believe these explanations to be unlikely. Although a previous study notes that fecal storage at room temperature or 4 °C for just 8 h results in a significant reduction of microbial diversity (Ott et al., 2004), another study on fecal storage noted no significant changes in microbial composition or diversity when fecal samples were stored at room temperature for up to 2 weeks (Lauber et al., 2010). In our case, there was approximately 3 h between fresh fecal collection and DNA extraction. Feces were on ice throughout this period unless they were being actively divided into aliquots for this study. Once all aliquoting was complete, DNA extraction began immediately on the "fresh" (week 0) samples. The likelihood of substantial changes in the microbial community during this period in these conditions is low.

Another possible explanation for these results is that fresh samples do not undergo any forms of chemical or mechanical cell lysis prior to DNA extraction. In contrast, all preserved samples undergo direct or indirect cell lysis during the preservation process: FTA cards chemically lyse microbial cells immediately after feces are applied to the card; freezing results in some mechanical cell lysis during the freeze/thaw process; RNAlater and ethanol cause some chemical lysis during preservation. Cell lysis allows "access" to microbial DNA that otherwise cannot be extracted and sequenced. While all samples undergo both chemical and mechanical cell lysis during extraction, perhaps the additional lysis that occurs in preserved – but not fresh – samples results in increased DNA extraction and thus increased microbial diversity from these samples. If this is the case, then fresh, never-preserved samples may not be representative of the real gut microbial community. The fact that FTA card samples – which undergo a powerful and immediate cell lysis upon application to the card – have the greatest microbial diversity potentially provides further support for the idea that greater cell lysis results in greater microbial diversity values. Alternately, FTA cards may be biased against common gut microbial species. Because the Shannon diversity index account for both species richness and evenness, this bias may result in falsely elevated diversity values for FTA cards.

In terms of DNA concentration and microbial diversity, week 1 stood out as an anomaly — with the lowest DNA concentrations and highest microbial diversity compared to all other weeks. As noted previously, week 1 was the only week in which a 96-well plate PowerSoil DNA Isolation kit was used rather than a 50-prep kit. Our results suggest that the difference in extraction kits may be responsible for the discrepant results of week 1. Many studies have reported that DNA extraction method (i.e. brand of kit or protocol) has a strong effect on microbial composition and diversity (Anderson and Lebepe-Mazur, 2003; Martin-Laurent et al., 2001; McOrist et al., 2002; Salonen et al., 2010; Scupham et al., 2007). In this case, both PowerSoil DNA Isolation kits are touted as being highly similar, with the 96-well plate kit simply offering a high-throughput option (MoBio.com). However, perhaps small differences in timing and protocols lead to larger differences in DNA concentration and microbial diversity.

4.3. Microbial species composition

Despite the differences noted in microbial diversity and DNA concentration by week, microbial species composition was relatively stable across weeks within each preservation method. Additionally, fresh samples, which also yielded low microbial diversity, clustered most closely with frozen and ethanol samples in weighted PCoA. FTA card and RNAlater samples (in the weighted analysis) did not cluster with the rest of the samples, indicating differences in the microbial communities preserved by these methods. Contrastingly, a previous study on fecal preservation method in captive western lowland gorillas reported that RNAlater and frozen samples had more similar microbial communities than ethanol preserved samples (Vlčková et al., 2012). This finding could be related to several differences between our studies: primate species, host diet, methodology, or a difference in microbial species and their interactions with the various preservation methods.

Several preservation methods demonstrated unique biases in microbial preservation. FTA cards preserved the highest relative abundances of OTUs in the *Firmicutes* phylum while RNAlater preserved the lowest relative abundances in Phylum *Bacteroidetes* (genus *Prevotella*). Ethanol

preserved the greatest OTU frequencies of *Cyanobacteria*. Other studies have similarly reported biases in microbial preservation (Vlčková et al., 2012), and these biases should be carefully considered prior to the selection of a fecal preservation method. For example, *Prevotella* abundance is associated with carbohydrate and simple sugar digestion (Wu et al., 2011). Captive black howler monkeys exhibit higher relative abundances of *Prevotella* than wild black howler monkeys due to a captive diet consisting of fruits, cereal, and primate pellets compared to the wild diet consisting primarily of leaves (Amato et al., 2013). RNAlater (stored at room temperature as in this study) would not be a recommended fecal preservation method for a study comparing gut microbiota of wild and captive black howler monkeys, due to the importance of *Prevotella* in differentiating these groups.

Finally, distance boxplot results confirm that ethanol-preserved and frozen samples yield microbial communities most similar to fresh (week 0) samples while FTA card and RNAlater preserved samples were least like fresh samples. These results raise several intriguing questions: FTA cards are dissimilar to fresh samples and also have the highest microbial diversity. Is this because FTA cards become contaminated during the drying process? (Cards are left on a lab bench to dry for up to 24 h.) Are air or dust microbes landing on these cards and adding to the microbial communities within these samples? Or, is the chemical matrix on the FTA card so effective at cell lysis that FTA cards are most effective at preserving DNA from the gut microbiota, and all other methods are less effective? Future studies are needed to address these questions. Specifically, a formulated gut microbial community with a known composition and diversity can be subjected to the same preservation methods used in this study. Then we can determine if or how preservation method alters microbial communities, and if increased cell lysis that some samples undergo (e.g. FTA card samples) results in a microbial diversity and composition most similar to or different from the original formulated community.

5. Conclusions

Our results lead us to several conclusions: 1) Preservation methods can exhibit bias toward or against some microbial groups, so methods should be selected carefully after considering scientific question, host species, and dietary ecology. 2) Freezing (when feasible) or fecal preservation in ethanol results in microbial communities most similar to fresh fecal samples. However, further testing is needed to determine if the additional lysis offered by FTA card preservation provides a better representation of the gut microbial community composition and diversity. 3) Long term (8 weeks) preservation of fecal samples had little effect on microbial composition and diversity over time. Overall, particularly for gut microbial studies in herbivorous primates, we recommend the use of ethanol as a preservation method. Samples preserved in ethanol can be stored long term at room temperature without significant alterations to the microbial community. Although ethanol is not the most convenient preservation method for field studies, ethanol-preserved samples effectively maintain the integrity of the fecal microbial community.

Acknowledgments

We would like to thank the Columbian Park Zoo for kindly providing us with spider monkey fecal samples. Krista Nichols, Ching Ching Wu, and Tsang-Long Lin generously provided laboratory space for the molecular work. We also thank Richard D. Howard for his support of this project, and review of this manuscript. Bong Suk-Kim's guidance and Gaenna Rogers' assistance in the laboratory were greatly appreciated. This project was funded by Morris Animal Foundation, Purdue University College of Veterinary Medicine Veterinary Scholars Program, San Diego Zoo Global, the Offield Family Foundation, and the Earth Microbiome Project. We also thank the Howard Hughes Medical Institute for their support. VLH was supported by a Purdue University Andrews Fellowship and a Purdue Research Foundation Research Grant.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.03.021.

References

Adams, J.R., Kelly, B.T., Waits, L.P., 2003. Using faecal DNA sampling and GIS to monitor hybridization between red wolves (*Canis rufus*) and coyotes (*Canis latrans*). Mol. Ecol. 12, 2175–2186.
Ahrabi, A.F., Handa, D., Codipilly, C.N., Shah, S., Potak, D., Schanler, R.J., 2013. Effects of prolonged freezing on the integrity of human milk. J. Fed. Am. Soc. Exp. Biol. 27, 629 (624).
Amato, K., Yeoman, C., Kent, A., Righini, N., Carbonero, F., Estrada, A., Gaskins, H., Stumpf, R., Yildirim, S., Torralba, M., Gillis, M., Wilson, B., Nelson, K., White, B., Leigh, S., 2013. Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. ISME J. 7, 1344–1353.
Anderson, K.L., Lebepe-Mazur, S., 2003. Comparison of rapid methods for the extraction of bacterial DNA from colonic and caecal lumen contents of the pig. J. Appl. Microbiol. 94, 988–993.
Backhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A., Semenkovich, C.F., Gordon, J.I., 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. U. S. A. 101, 15718–15723.
Bahl, M.I., Bergström, A., Licht, T.R., 2012. Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. FEMS Microbiol. Lett. 329, 193–197.
Bittinger, K., 2014. qiimer: Work with QIIME Output Files in R. R package Version 0.9.2.
Breiman, L., 2001. Random forests. Mach. Learn. 45, 5–32.
Bubb, A., Ehlers, K., Kotze, A., Grobler, J.P., 2011. The effect of sample age and storage method on DNA yield and microsatellite amplification from baboon (*Papio ursinus*) faecal samples. Eur. J. Wildl. Res. 57, 971–975.
Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R., 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26, 266–267.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010b. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. **ISME J.**
Carroll, I.M., Ringel-Kulka, T., Siddle, J.P., Klaenhammer, T.R., Ringel, Y., 2012. Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. PLoS One 7, e46953.
Cavigelli, S.A., 1999. Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed femurs, *Lemur catta*. Anim. Behav. 57, 935–944.
Cho, I., Blaser, M.J., 2012. The human microbiome: at the interface of health and disease. Nat. Rev. Genet. 13, 260–270.
Chung, H., Pamp, S.J., Hill, J.A., Surana, N.K., Edelman, S.M., Troy, E.B., Reading, N.C., Villablanca, E.J., Wang, S., Mora, J.R., Umesaki, Y., Mathis, D., Benoist, C., Relman, D.A., Kasper, D.L., 2012. Gut immune maturation depends on colonization with a host-specific microbiota. Cell 149, 1578–1593.
Dehnhard, M., Naidenko, S., Frank, A., Braun, B., Goritz, F., Jewgenow, K., 2008. Non-invasive monitoring of hormones: a tool to improve reproduction in captive breeding of the Eurasian lynx. Reprod. Domest. Anim. 43, 74–82.
Delsuc, F., Metcalf, J.L., Parfrey, L.W., Song, S.J., González, A., Knight, R., 2013. Convergence of gut microbiomes in myrmecophagous mammals. Mol. Ecol. 23, 1301–1317.
Dominianni, C., Wu, J., Hayes, R., Ahn, J., 2014. Comparison of methods for fecal microbiome biospecimen collection. BMC Microbiol. 14, 103.
Frantzen, M., Silk, J., Ferguson, J., Wayne, R., Kohn, M., 1998. Empirical evaluation of preservation methods for faecal DNA. Mol. Ecol. 7, 1423–1428.
Galili, T., 2010. Post Hoc Analysis for Friedman's Test (R Code) vol. 2014 (R-statistics.com).
Gilbert, J.A., Folker, M., Dion, A., Pavan, B., Brown, C.T., Christopher, T.B., Narayan, D., Jonathan, A.E., Dirk, E., Dawn, F., Wu, F., Daniel, H., Janet, J., Rob, K., James, K., Eugene, K., Kostas, K., Joel, K., Nikos, K., Rachel, M., Alice, M., Christopher, Q., Jeroen, R., Alexander, S., Ashley, S., Rick, S., 2010. Meeting report: the terabase metagenomics workshop and the vision of an Earth microbiome project. Stand. Genomic Sci. 3, 243–248.
González-Zamora, A., Arroyo-Rodríguez, V., Chaves, O.M., Sánchez-López, S., Stoner, K.E., Riba-Hernández, P., 2009. Diet of spider monkeys (*Ateles geoffroyi*) in Mesoamerica: current knowledge and future directions. Am. J. Primatol. 71, 8–20.
Hollander, M., Wolfe, D.A., 1999. Wilcoxon–Nemenyi–McDonald-Thompson test, Nonparametric Statistical Methods. John Wiley & Sons, Inc., New York.
Hooper, L.V., Littman, D.R., Macpherson, A.J., 2012. Interactions between the microbiota and the immune system. Science 336, 1268–1273.
Hothorn, T., Hornik, K., Wiel, M.A.v.d., Zeileis, A., 2006. A Lego system for conditional inference. Am. Stat. 60, 257–263.

Hothorn, T., Bretz, F., Westfall, P., 2008a. Simultaneous inference in general parametric models. Biom. J. 50, 346–363.
Hothorn, T., Hornik, K., Wiel, M.A.v.d., Zeileis, A., 2008b. Implementing a class of permutation tests: the coin package. J. Stat. Softw. 28, 1–23.
Khan, M., Altmann, J., Isani, S., Yu, J., 2002. A matter of time: evaluating the storage of fecal samples for steroid analysis. Gen. Comp. Endocrinol. 128, 57–64.
Knights, D., Costello, E., Knight, R., 2011. Supervised classification of human microbiota. FEMS Microbiol. Rev. 35, 343–359.
Lauber, C.L., Zhou, N., Gordon, J.I., Knight, R., Fierer, N., 2010. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiol. Lett. 307, 80–86.
Ley, R., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., Gordon, J.I., 2008. Evolution of mammals and their gut microbes. Science 320, 1647–1651.
Li, F., Hullar, M.A.J., Lampe, J.W., 2007. Optimization of terminal restriction fragment polymorphism (TRFLP) analysis of human gut microbiota. J. Microbiol. Methods 68, 303–311.
Littman, D.R., Pamer, E.G., 2011. Role of the commensal microbiota in normal and pathogenic host immune responses. Cell Host Microbe 10, 311–323.
Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71, 8228–8235.
Mackenzie, B.W., Waite, D.W., Taylor, M.W., 2015. Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences. Front. Microbiol. 6.
Martin, F.-P.J., Dumas, M.-E., Wang, Y., Legido-Quigley, C., Yap, I., Tang, H., Zirah, S., Murphy, G., Cloarec, O., Lindon, J., Sprenger, N., Fay, L., Kochhar, S., van Bladeren, P., Holmes, E., Nicholson, J., 2007. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. Mol. Syst. Biol. 3.
Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G., Catroux, G., 2001. DNA extraction from soils: old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. 67, 2354–2359.
McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2011. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6, 610–618.
McOrist, A.L., Jackson, M., Bird, A.R., 2002. A comparison of five methods for extraction of bacterial DNA from human faecal samples. J. Microbiol. Methods 50, 131–139.
Mowry, R.A., Gompper, M.E., Beringer, J., Eggert, L.S., 2011. River otter population size estimation using noninvasive latrine surveys. J. Wildl. Manag. 75, 1625–1636.
Muller-Graf, C.D.M., Woolhouse, M.E.J., Packer, C., 1999. Epidemiology of an intestinal parasite (*Spirometra* spp.) in two populations of African lions (*Panthera leo*). Parasitology 118, 407–415.
Murphy, M.A., Waits, L.P., Kendall, K.C., Wasser, S.K., Higbee, J.A., Bogden, R., 2002. An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conserv. Genet. 3, 435–440.
Mykytczuk, N.C.S., Foote, S.J., Omelon, C.R., Southam, G., Greer, C.W., Whyte, L.G., 2013. Bacterial growth at −15 °C; molecular insights from the permafrost bacterium *Planococcus halocryophilus* Or1. ISME J. 7, 1211–1226.
Nechvatal, J.M., Ram, J.L., Basson, M.D., Namprachan, P., Niec, S.R., Badsha, K.Z., Matherly, L.H., Majumdar, A.P.N., Kato, I., 2008. Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. J. Microbiol. Methods 72, 124–132.
Nelson, T., Rogers, T., Carlini, A., Brown, M., 2013. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. Environ. Microbiol. 15, 1132–1145.
Nielsen, M.K., Vidyashankar, A.N., Andersen, U.V., DeLisi, K., Pilegaard, K., Kaplan, R.M., 2010. Effects of fecal collection and storage factors on strongylid egg counts in horses. Vet. Parasitol. 167, 55–61.
Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. vegan: Community Ecology Package. R package Version 2.0-10.
Ott, S., Musfeldt, M., Timmis, K., Hampe, J., Wenderoth, D., Schreiber, S., 2004. In vitro alterations of intestinal bacterial microbiota in fecal samples during storage. Diagn. Microbiol. Infect. Dis. 50, 237–245.
Petersen, C., Round, J.L., 2014. Defining dysbiosis and its influence on host immunity and disease. Cell. Microbiol. 16, 1024–1033.
RCoreTeam, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rietmann, S., Walzer, C., 2014. Parasitological examination of common hippopotamus (*Hippopotamus amphibius*) faeces in the Gamba Complex of Protected Area in Gabon. Wien. Tierarz. Monats. 101, 66–73.
Rochelle, P.A., Cragg, B.A., Fry, J.C., Parkes, R.J., Weightman, A.J., 1994. Effect of sample handling on estimation of bacterial diversity in marine sediments by 16S rRNA gene sequence analysis. FEMS Microbiol. Ecol. 15, 215–225.
Roesch, L., Casella, G., Simell, O., Krischer, J., Wasserfall, C., Schatz, D., Atkinson, M., Neu, J., Triplett, E., 2009. Influence of fecal sample storage on bacterial community diversity. Open Microbiol. J. 3, 40–46.
Round, J., Mazmanian, S., 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. 9, 313–323.
Salonen, A., Nikkila, J., Jalanka-Tuovinen, J., Immonen, O., Rajilic-Stojanovic, M., Kekkonen, R.A., Palva, A., de Vos, W.M., 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J. Microbiol. Methods 81, 127–134.
Scupham, A.J., Jones, J.A., Wesley, I.V., 2007. Comparison of DNA extraction methods for analysis of turkey cecal microbiota. J. Appl. Microbiol. 102, 401–409.
Sekirov, I., Russell, S.L., Antunes, L.C.M., Finlay, B.B., 2010. Gut microbiota in health and disease. Physiol. Rev. 90, 859–904.
Shutt, K., Setchell, J.M., Heistermann, M., 2012. Non-invasive monitoring of physiological stress in the Western lowland gorilla (*Gorilla gorilla gorilla*): validation of a fecal glucocorticoid assay and methods for practical application in the field. Gen. Comp. Endocrinol. 179, 167–177.
Stoops, M.A., Anderson, G.B., Lasley, B.L., Shideler, S.E., 1999. Use of fecal steroid metabolites to estimate the pregnancy rate of a free-ranging herd of tule elk. J. Wildl. Manag. 63, 561–569.
ThermoScientific, 2011. NanoDrop Spectrophotometers: Assessment of Nucleic Acid Purity. http://www.nanodrop.com.
Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., Gordon, J.I., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1131.
UCLA, 2014. Friedman Test vol. 2014. UCLA: Statistical Consulting Group.
Uenishi, G., Fujita, S., Ohashi, G., Kato, A., Yamauchi, S., Matsuzawa, T., Ushida, K., 2007. Molecular analyses of the intestinal microbiota of chimpanzees in the wild and in captivity. Am. J. Primatol. 69, 367–376.
Vallet, D., Petit, E.J., Gatti, S., Levrero, F., Menard, N., 2008. A new 2CTAB/PCI method improves DNA amplification success from faeces of Mediterranean (Barbary macaques) and tropical (lowland gorillas) primates. Conserv. Genet. 9, 677–680.
Villers, L., Jang, S., Lent, C., Lewin-Koh, S.-C., Norosoarinaivo, J., 2008. Survey and comparison of major intestinal flora in captive and wild ring-tailed lemur (*Lemur catta*) populations. Am. J. Primatol. 70, 175–184.
Vishnivetskaya, T.A., Layton, A.C., Lau, M.C.Y., Chauhan, A., Cheng, K.R., Meyers, A.J., Murphy, J.R., Rogers, A.W., Saarunya, G.S., Williams, D.E., Pfiffner, S.M., Biggerstaff, J.P., Stackhouse, B.T., Phelps, T.J., Whyte, L., Sayler, G.S., Onstott, T.C., 2014. Commercial DNA extraction kits impact observed microbial community composition in permafrost samples. FEMS Microbiol. Ecol. 87, 217–230.
Vlčková, K., Mrázek, J., Kopečný, J., Petrželková, K.J., 2012. Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (*Gorilla gorilla gorilla*). J. Microbiol. Methods 91, 45–51.
Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267.
Wu, G.D., Lewis, J.D., Hoffmann, C., Chen, Y.-Y., Knight, R., Bittinger, K., Hwang, J., Chen, J., Berkowsky, R., Nessel, L., Li, H., Bushman, F.D., 2010. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol. 10.
Wu, G.D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.Y., Keilbaugh, S.A., Bewtra, M., Knights, D., Walters, W.A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F.D., Lewis, J.D., 2011. Linking Long-term dietary patterns with gut microbial enterotypes. Science 334, 105–108.
Xenoulis, P.G., Gray, P.L., Brightsmith, D., Palculict, B., Hoppes, S., Steiner, J.M., Tizard, I., Suchodolski, J.S., 2010. Molecular characterization of the cloacal microbiota of wild and captive parrots. Vet. Microbiol. 146, 320–325.
Yuan, S., Cohen, D.B., Ravel, J., Abdo, Z., Forney, L.J., 2012. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS ONE 7, e33865.