Journal of Microbiological Methods 130 (2016) 180–188

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Effects of field conditions on fecal microbiota

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ARTICLE INFO

Article history:
Received 14 July 2016
Received in revised form 23 September 2016
Accepted 23 September 2016
Available online 26 September 2016

Keywords:
Environmental conditions
Fecal microbial community
Fecal preservation
Gut microbiota
Primates

ABSTRACT

Gut microbiota can provide great insight into host health, and studies of the gut microbiota in wildlife are becoming more common. However, the effects of field conditions on gut microbial samples are unknown. This study addresses the following questions: 1) How do environmental factors such as sunlight and insect infestations affect fecal microbial DNA? 2) How does fecal microbial DNA change over time after defecation? 3) How does storage method affect microbial DNA? Fresh fecal samples were collected, pooled, and homogenized from a family group of 6 spider monkeys, *Ateles geoffroyi*. Samples were then aliquoted and subjected to varying light conditions (shade, sun), insect infestations (limited or not limited by netting over the sample), and sample preservation methods (FTA — Fast Technology for Analysis of nucleic acid — cards, or freezing in liquid nitrogen then storing at −20 °C). Changes in the microbial communities under these conditions were assessed over 24 h. Time and preservation method both effected fecal microbial community diversity and composition. The effect size of these variables was then assessed in relation to fecal microbial samples from 2 other primate species (*Rhinopithecus bieti* and *R. brelichi*) housed at different captive institutions. While the microbial community of each primate species was significantly different, the effects of time and preservation method still remained significant indicating that these effects are important considerations for fieldwork.

1. Introduction

In 1984, R.J. Putnam wrote in *Facts from Feces*, "For any frustrated field mammalogist seeking observational data on their elusive study animals, dung may represent the most readily available and easily collected source of information upon which they may fall back in despair" (Putnam, 1984). Today, it is not with despair but with renewed vigor that wildlife scientists use feces as a window into the health of elusive and threatened animals around the world (Amato et al., 2013; Amato et al., 2015; Amato et al., 2016; Clayton et al., 2016; Nelson et al., 2013; Uenishi et al., 2007; Villers et al., 2008; Xenoulis et al., 2010). Technology has enabled us to 'see the world' in a grain of feces: Recent advances in next-generation sequencing and bioinformatics software now allow us to analyze and compare entire gut microbial communities efficiently and effectively. As we have learned from previous studies on the gut microbiota, this complex community plays a critical role in host immune development and defense (Cho and Blaser, 2012; Chung et al., 2012; Hooper et al., 2012; Levy et al., 2015; Littman and Pamer, 2011), disease (Petersen and Round, 2014; Round and Mazmanian, 2009; Sekirov et al., 2010; Shreiner et al., 2015), digestion (Backhed et al., 2004; Martin et al., 2007; Turnbaugh et al., 2006), dietary adaptation (Ley et al., 2008), reproduction (Rosengaus et al., 2011; Sharon et al., 2010), and behavior (Buffington et al., 2016; Forsythe et al., 2010; Heijtz et al., 2011).

There are, of course, anatomical and physiological reasons why fecal microbial DNA may not be representative of the gut microbial community. For example, in foregut fermenters such as ruminants and colobine monkeys, foregut bacteria is subsequently subjected to glandular digestion; and these bacteria may not be represented in the distal gastrointestinal (GI) tract or feces (Kay and Davies, 1994). (However, we

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

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http://dx.doi.org/10.1016/j.mimet.2016.09.017
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recently found no significant differences in the colobine microbial community at different locations along the GI tract (Amato et al., 2016).) Directly sampling the foregut requires highly invasive procedures such as orogastric lavage, endoscopy, or abdominal surgery. These procedures are not always feasible — particularly in wildlife or endangered species. Additionally, microbes in feces consist primarily of gut luminal bacteria, and adherent mucosal bacterial populations are distinct from luminal bacteria and less well represented in the feces (Eckburg et al., 2005). Despite these limitations, fecal bacterial DNA is commonly used as a proxy for 'gut microbiota' in many mammalian species (Ley et al., 2008; Muegge et al., 2011).

Nevertheless, field conditions introduce a range of factors that have the potential to affect the gut microbiota, making it unclear whether all fecal samples collected from wild animals are truly representative of the gut microbial community. Although many studies guide fecal sample collection for short-term storage in highly controlled conditions such as hospitals or laboratories (Carroll et al., 2012; Dominianni et al., 2014; Lauber et al., 2010; Nechvatal et al., 2008; Ott et al., 2004; Roesch et al., 2009; Sinha et al., 2015; Wu et al., 2010), few studies have examined fecal preservation methods under longer-term field conditions without electricity or freezers (Frantzen et al., 1998; Hale et al., 2015; Song et al., 2016; Vlčková et al., 2012). And none, to our knowledge, have examined how the fecal microbial community changes over time in response to field environmental conditions. This raises several important questions that need to be answered for future gut microbial studies in wildlife: 1) How do environmental factors such as sunlight and insect infestations affect fecal microbial DNA? 2) How does fecal microbial DNA change over time (24 h) after defecation? 3) How does a 'field-friendly' sample storage method like FTA cards compare to the 'gold-standard' of freezing fecal samples in terms of preserving microbial DNA?

In our study, we examined variation in the fecal microbiota of primates in response to multiple field conditions. We hypothesized that environment, time, and storage method would all significantly alter the gut microbial profile. Specifically, we hypothesized that direct sunlight would kill many microbes and degrade the microbial DNA — thus decreasing the diversity of samples exposed to sun. In regards to insect infestations, we predicted that samples without netting to prevent the direct contact with insects would exhibit significantly altered microbial composition due to the addition of insect-specific microbes to the fecal samples or through accelerated decomposition. We expected the microbial profiles to change increasingly over 24 h due to both microbial overgrowth of some microbial species and DNA degradation of other species. Finally, based on a previous study, we predicted that FTA cards would preserve a stable but potentially biased representation of the gut microbiota (Hale et al., 2015).

We focused our study on the spider monkey, *Ateles geoffroyi*, an herbivorous species native to Central and South America (Cuarón et al., 2008; González-Zamora et al., 2009). The natural spider monkey diet primarily consists of fruits and leaves (González-Zamora et al., 2009). Our study animals were housed at the Columbian Park Zoo (Lafayette, IN, USA), and fed a diet of fresh fruits and vegetables along with primate pellet. To assess the effect size of field condition variables versus other biological or environmental variables, we also examined fecal microbiota of 2 other captive monkey species (*Rhinopithecus bieti* and *Rhinopithecus brelichi*) from 2 different locations (Beijing Zoo and Wildlife Rescue Center of Fanjingshan National Nature Reserve). *Rhinopithecus* species are highly foliverous foregut fermenters, unlike *A. geoffroyi* that have simple stomachs, no pre-gastric fermentation, and are more frugivorous (Ley et al., 2008). In captivity, the *R. bieti* and *R. brelichi* monkeys received a diet of fresh leaves and leafy greens along with occasional fruits, eggs, peanuts, and steamed corn meal cakes mixed with protein/vitamin powder. Physiologically, biogeographically, and dietarily, *A. geoffroyi* monkeys are quite distinct from *R. bieti*, and *R. brelichi* and we expected these critical differences to be reflected in the gut microbiota.

2. Methods

2.1. Fecal collection and processing

Fecal samples were collected in September 2013 from a group of 6 adult spider monkeys (*Ateles geoffroyi*) at the Columbian Park Zoo (Lafayette, IN, USA). The monkeys were co-housed, and none of them were on antibiotics within 12 months of the sampling date. All *A. geoffroyi* fecal samples collected for this study were fresh (<1 h old). Feces were collected in a 50 ml sterile plastic screw top tube (TedPella, Redding, CA, USA) using a sterile metal spatula. Samples 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.

We conducted a 24-h experiment to determine if or how the fecal microbial DNA changed over time when subjected to varying light conditions (shade versus sun), insect infestations (limited or not limited by netting over the sample), and sample preservation methods (FTA cards versus freezing in liquid nitrogen then storing at −20 °C). The samples were divided into 6 plastic weighing plates (The Lab Depot, Inc., Dawsonville, GA) with 12 g of feces per plate. Four plates, designated A, B, C, and D, were placed at 4 different locations on the Purdue University campus. Plates A and C were placed on grass exposed to direct sunlight throughout the day. Plates B and D were placed on soil in full shade (i.e. under foliage) throughout the day. Two additional plates, CN and DN, were fully covered by black plastic screen mesh (1.5 mm) that was secured to the weighing plates with duct tape. The screen netting was added to plates CN and DN to minimize insect infestation in these fecal samples (Fig. 1). Plate CN (i.e. C + netting) was placed next to Plate C in direct sunlight whereas plate DN (i.e. D + netting) was placed next to Plate D in full shade. Two additional aliquots (0.25 g each) were

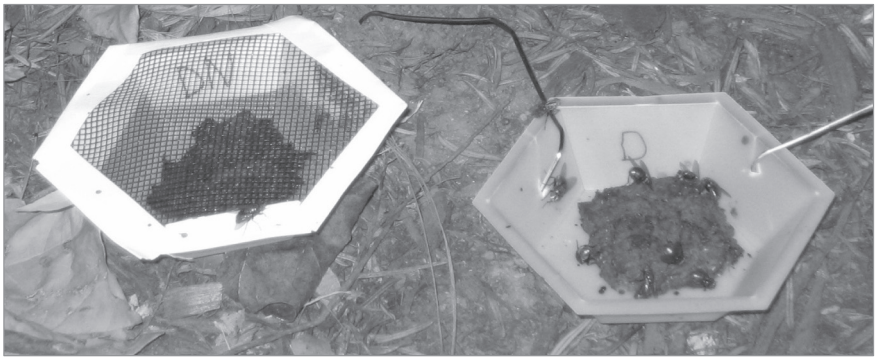

Fig. 1. Left: Plate DN is covered with screen mesh netting to prevent insect infestation. Right: In Plate D, multiple flies are noted on the feces. Beetles and beetle larvae were also observed in plates without netting. Note: Plate D is staked into the ground with paperclips to prevent wind from flipping the plate.

taken from the homogenized feces. These aliquots were never subjected to any form of preservation (e.g. freezing or FTA cards) and are denoted as "fresh" samples. DNA from these 2 aliquots was extracted within 3 h of collection (see details below).

Immediately before weighing plates were taken to their respective field locations, 0.5 g of feces were removed from each plate. This time point was considered "0 h" from fecal collection. Half of this amount (0.25 g feces) was sealed in a cryotube, snap frozen in liquid nitrogen, then stored at −20 °C until DNA extraction. The other half of the feces (0.25 g) was applied to an FTA card (Whatman Inc., Florham Park, NJ) using a sterile cotton swab (Dynarex, Orangeburg, NY, USA). FTA cards were air dried at room temperature for 12–24 h and then stored at room temperature in individual Ziploc bags with MiniPax desiccant packets (Multisorb Technologies Inc., Buffalo, NY, USA). Desiccant packets were replaced as necessary. This process was repeated at 2, 12, and 24 h from the "0 h" time point. At each time point, 0.5 g of feces were removed from each weighing plate; half was snap frozen and stored at −20 °C; the other half was applied to an FTA card, dried, and stored in a Ziploc bag. Feces were collected from the center of each fecal pile (rather than the outside edges) via sterile cotton swab, and previously sampled areas were avoided. After 24 h, all remaining feces and weigh plates were disposed of appropriately.

Additionally, for comparative purposes, fecal samples of 5 captive Yunnan snub-nosed monkeys (*Rhinopithecus bieti*) and 4 captive Guizhou snub-nosed monkeys (*R. brelichi*) were collected from the Beijing Zoo (Hebei Province, China) and the Wildlife Rescue Center of Fanjingshan National Nature Reserve (Guizhou Province, China) respectively. All of the *R. bieti* were co-housed and ranged in age from 2 to 14 years old. Two of the *R. brelichi* were co-housed and the other 2 were housed individually. *R. brelichi* individuals ranged in age from 6 to 17 years old. A few of the *Rhinopithecus* monkeys were sampled more than once over the course of 10 days. All *Rhinopithecus* samples were collected in July 2013 within 2 h of defecation and preserved using FTA cards (Whatman Inc., Florham Park, NJ, USA) as described above.

DNA extraction, PCR amplification, and DNA 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. For samples preserved by freezing, 0.25 g of feces per sample were used for DNA extraction. For samples preserved on FTA cards, a 2 mm Harris Uni-Core biopsy punch (TedPella, Redding, CA, USA) was used to make 20 punches in each FTA sample circle. These 20 punches were then used for DNA extraction. Extraction of the *A. geoffroyi* samples was performed using a PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) at Purdue University in West Lafayette, IN. Extraction of the *R. bieti* and *R. brelichi* samples was performed using a PowerSoil DNA isolation kit at the Zhejiang Institute of Microbiology in Hangzhou, China.

All *A. geoffroyi*, *R. bieti,* and *R. brelichi* samples then went through the remainder of sample processing together. Each sample was amplified by targeting the V4 region of the 16S rRNA gene using universal bacterial primers 515F and 806R (Caporaso et al. 2012). Illumina adaptors and unique Golay barcodes were incorporated as part of the primer construct for each sample. Equal concentrations of all amplicons were then pooled for sequencing. The amplicon pool was cleaned using a MoBio UltraClean PCR Clean-Up Kit (MoBio Laboratories, Carlsbad, CA, USA). Samples were paired-end sequenced on an Illumina MiSeq. MiSeq reads, were trimmed to 100 base pairs. All sequencing was performed at the BioFrontiers Institute Next-Generation Genomics Facility at University of Colorado, Boulder, USA.

Sample processing, sequencing and core amplicon data analysis were performed by the Earth Microbiome Project (www.earthmicrobiome.org), and all amplicon and metadata has been made public through the data portal (https://qiita.ucsd.edu/).

2.2. Microbial taxonomic assignment

16S rRNA amplicon sequences were processed using Quantitative Insights Into Microbial Ecology (QIIME — version 1.9.1) software that allows analysis of microbial communities (Caporaso et al., 2010a). Default parameters within QIIME were used for de-multiplexing, quality-filtering, and clustering amplicon sequences. A total of 1,813,776 reads were obtained after filtering (mean reads per individual: 29,254; standard deviation: 8621). Sequences were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity. Open reference picking was used to assign sequences to OTUs and cluster sequences against the Greengenes (version 13_8, release date August 2013 (McDonald et al., 2011)) reference dataset (http://greengenes.secondgenome.com). Sequences that did not match this dataset at 97% were then clustered into de novo OTUs at 97% sequence similarity using UCLUST within QIIME. Representative sequences for all OTUs were then aligned to Greengenes reference alignment using PyNAST (Caporaso et al., 2010b). De novo OTUs were assigned taxonomies using RDP classifier (Wang et al., 2007) and the Greengenes reference dataset (version 13_8, release date August 2013) with an 80% confidence threshold. All samples were rarified at 6637 reads. This read number allowed us to exclude samples that clearly failed to amplify while still including at least 1 replicate from each sampling condition and time. Rarefaction uses a standardized effort to compare microbial species (OTU) richness in each fecal sample (Hughes and Hellmann, 2005). Samples that contained fewer than 6637 reads were excluded from the analysis. Of 62 samples, a single sample was excluded for having too few reads. This sample was exposed to full sun for 12 h then preserved by freezing.

2.3. Statistical analyses

We compared alpha diversity of gut microbiota by calculating the Shannon diversity and Chao1 indices along with species richness (observed OTUs) in QIIME. Alpha diversity values were compared using a nested mixed effects model with repeated measures in RStudio (version 0.99.465). Fixed effects included fecal preservation method (i.e. FTA cards versus freezing), hours from defecation to fecal collection (i.e.

Table 1
Shannon, Chao1 and observed OTU values by fecal preservation method, hours to collection, light condition and netting status.

Field condition variables	Treatments	Shannon Diversity Index (Mean, SD)	Chao1 (Mean, SD)	Observed OTUs (Mean, SD)
Preservation method	FTA	8.08 (0.25)	4179 (796)	2551 (573)
	Frozen	769 (0.49)	4095 (1173)	2545 (553)
	p-Value	<0.01	0.79	0.93
Hours to collection	0–2 h	7.76 (0.45)	4250 (972)	2529 (542)
	12–24 h	8.02 (0.38)	4021 (1014)	2569 (584)
	p-Value	<0.01	0.42	0.71
Light condition	Sun	7.84 (0.53)	4187 (917)	2601 (586)
	Shade	7.94 (0.32)	4091 (1070)	2498 (535)
	p-Value	0.43	0.65	0.57
Netting status	Netting	7.74 (0.51)	4361 (1068)	2574 (557)
	No netting	7.97 (0.37)	4023 (942)	2536 (566)
	p-Value	0.05	0.5	0.87

"Hours to Collection": 0–2 h combined, 12–24 h combined), lighting condition (sun versus shade) and netting status (i.e. netting versus no netting). Plate (A, B, C, D) was included a random effect.

UniFrac distances were used to assess beta diversity within QIIME (Lozupone and Knight, 2005). UniFrac measures distances between microbial communities based on the species they contain and the phylogenetic relationships between those species. Principal Coordinate Analysis (PCoA) was used to visualize UniFrac distances. Each point in a PCoA plots represents the microbial community from a single sample. Samples with the most similar microbial communities cluster together. A weighted PCoA accounts for both microbial species and relative abundance. An unweighted PCoA only accounts for microbial species (presence/absence) but not abundance. An analysis of variance (PERMANOVA) was then performed on the weighted UniFrac distance matrix to partition variation in the matrix by field condition variables. Data were nested by plate (A, B, C, D), and analyses were conducted in RStudio (version 0.99.465).

Supervised learning analyses using the Random Forests method were performed in QIIME to determine if groups could be differentiated by microbial composition (OTUs) (Breiman, 2001; Knights et al., 2011). Groups were analyzed based on the following variables: hours from defecation to fecal collection (i.e. "Hours to Collection": 0, 2, 12, 24 h; and hours to collection with 0–2 h samples combined and 12–24 h samples combined), sample preservation method (i.e. FTA cards versus freezing), netting status (i.e. netting versus no netting), and light condition (i.e. sun versus shade). These analyses were performed after filtering out OTUs observed in fewer than 1% of the samples. 80% of the data were used as a training set, and 20% of the data were used as a test set in an iterative process. 1000 decision trees were generated based on groups and OTUs. Results from these analyses produced an error ratio and a confusion matrix. The error ratio is the error of random guessing over the sum of the error in the test sets. The confusion matrix is a table that indicates how many times samples were incorrectly classified based on OTU composition.

We compared OTU frequencies between groups using a Kruskal-Wallis test with FDR corrected *p*-values (group_significance.py script in QIIME). The Kruskal-Wallis test is a non-parametric ANOVA (Analysis of Variance) that determines if there are significant differences in OTU abundance between groups. The FDR (False Discovery Rate) correction accounts for multiple comparisons. Groups included hours from defecation to fecal collection (i.e. "Hours to Collection": "fresh" samples, 0–2 h samples combined, 12–24 h samples combined), and sample preservation method (i.e. FTA cards versus freezing).

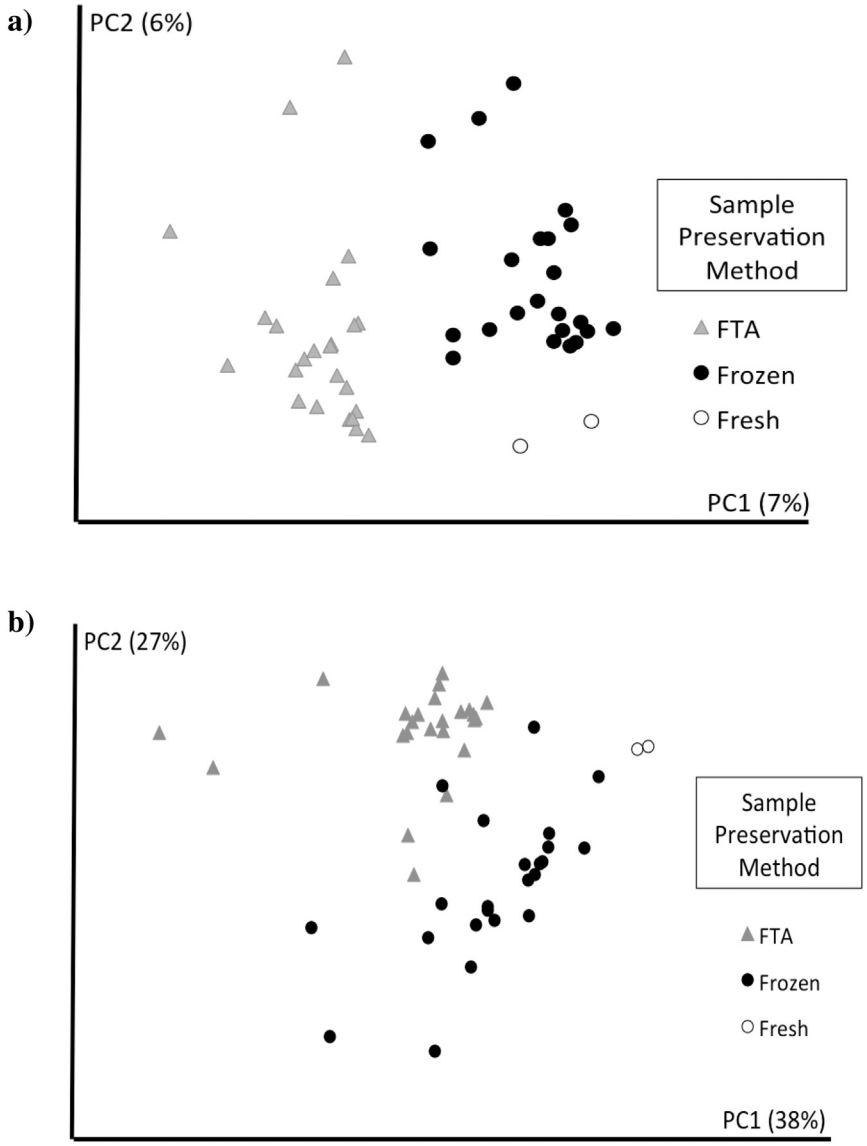

Fig. 2. Principal Coordinate Analysis on a) unweighted and b) weighted UniFrac metrics "fresh" samples – immediately extracted, never preserved – are included for reference.

To assess effect size of field condition variables in relation to other biological/environmental variables like species or provenance, we used the weighted UniFrac distance matrix to compare distances within *A. geoffroyi* samples to distances between *A. geoffroyi* and *R. bieti*/*R. brelichi* samples. Distances were assessed using a two-sided Student's *t*-test with 999 Monte Carlo permutations and the nonparametric Bonferroni correction for multiple comparisons.

3. Results

3.1. Microbial diversity

A repeated measures mixed model was used to compare microbial diversity (Shannon, Chao1, observed OTUs) by hours to fecal collection, sample preservation method, light condition, and netting status. The Shannon index but not the Chao1 or observed OTUs differed significantly by fecal preservation method (Table 1) — with FTA cards yielding significantly higher diversity than frozen samples. To analyze "hours to collection," samples from 0 to 2 h were combined and samples from 12 to 24 h combined. Again, The Shannon index but not Chao1 or observed OTUs differed significantly over time (Table 1) with samples collected between 12 and 24 h exhibiting significantly greater microbial diversity than samples collected between 0 and 2 h. There was no significant difference in any alpha diversity metric relative to light condition or netting status.

3.2. Microbial composition

Principal Coordinate Analysis (PCoA) based on unweighted and weighted (Fig. 2) UniFrac distances indicate that samples clustered by sample preservation method. Fresh, immediately extracted samples were included in the "Sample Preservation Method" PCoA (Fig. 2) for reference. These fresh samples cluster closer to frozen samples. Further visualization with weighted PCoA by "hours from collection" (0, 2, 12, 24 h), light condition (sun versus shade), and netting status (netting versus no netting) revealed no strong clustering patterns (Fig. 3). Samples collected at 12 and 24 h trail to the left, suggesting that microbial communities in these samples may change through time, particularly after 12 h of environmental exposure. As such, samples collected at 0 and 2 h were combined into 1 group, and samples collected at 12–24 h were combined into another group for the remainder of the analyses. PERMANOVA results based on the weighted UniFrac distance matrix indicated that fecal preservation method and time (0–2 h or 12–24 h) were both associated with significant differences in the microbial communities (preservation method pseudo-F = 33.32; $p = 0.001$; time pseudo F = 4.35; $p = 0.016$) while light condition and netting status were not (light condition pseudo-F = 1.35; $p = 0.092$; netting status pseudo F = 0.96; $p = 0.332$).

Supervised learning error ratios for analyses by netting status, hours to collection (0, 2, 12, 24 h), and light condition were all <2 (netting status: 1.00; hours to collection: 1.23; light condition: 1.44). When "hours to collection" samples were reanalyzed with 0–2 h combined and 12–24 h combined, a significant error ratio (2.02) resulted, indicating a difference in OTU composition based on time. Error ratios <2 indicate that groups do not differ significantly. For sample preservation method, the error ratio (error of random guessing over the sum of the error in the test sets) was 14.03, indicating strong clustering by preservation method. The confusion matrix on preservation method indicated that all FTA samples and all frozen samples were correctly assigned based on OTU composition (Table 2a). Both fresh samples were misassigned as frozen samples, indicating the similarity in microbial composition between fresh and frozen samples. The confusion matrix for "hours to collection" also showed a relatively strong separation of samples based on time — with only a 13% misassignment rate for 0–2 h samples and a 35% misassignment rate for 12–24 h samples (Table 2b).

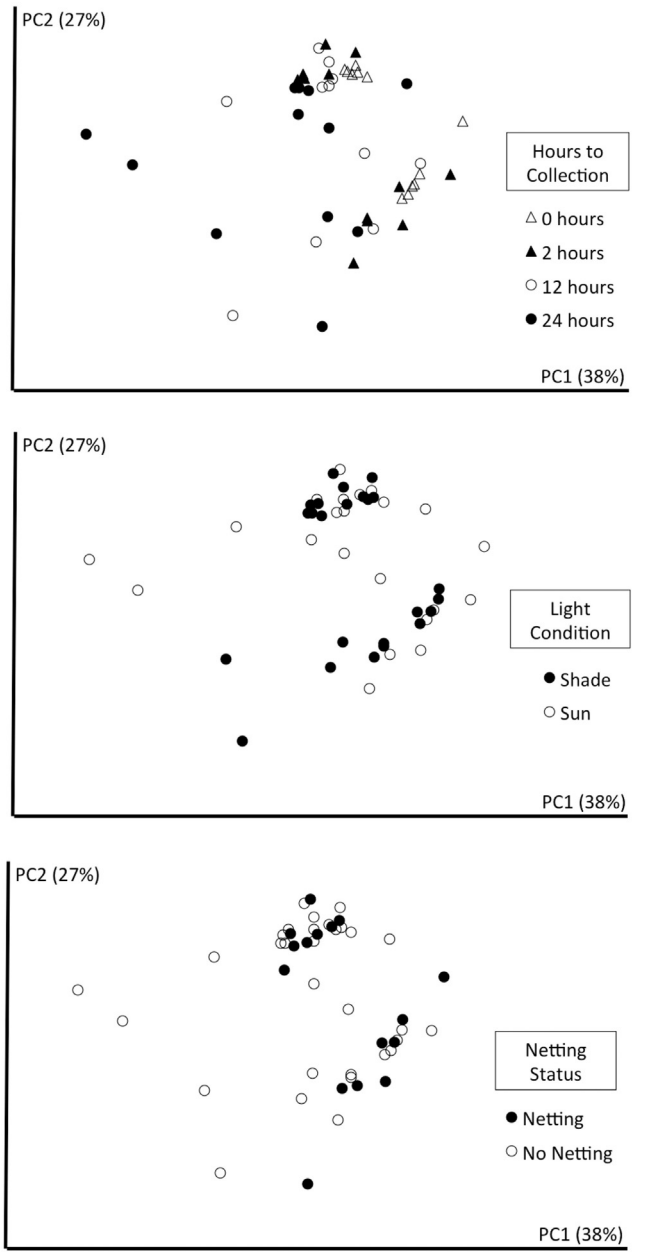

Fig. 3. Principal Coordinate Analysis on weighted UniFrac distances. Variables tested include hours from defecation to fecal collection (top), light condition — sun versus shade (middle), and netting status (bottom). Netting was used to cover some samples to prevent insect infestation.

Microbial composition differed across many variables including sample preservation method and time (Fig. 4). In terms of preservation method, FTA cards preserved a higher relative abundance of an OTU in the Ruminococcaceae family and a lower relative abundance of an OTU in the *Dialister* genus (see Supplementary material; group_significance.py results, Table S1). Two unclassified bacteria and several *Prevotella* genera OTUs were also differentially abundant between the FTA and Frozen groups. In regard to hours-to-collection, an OTU in the phylum Bacteroidetes (class Bacteroidales) decreased significantly over time suggesting that the DNA of this OTU gradually degrades after defecation (see Supplementary material; group_significance.py results, Table S2).

3.3. Effect size of field condition variables

To determine how the effects of preservation method and time compared to the effects of species and provenance, we examined the *A.*

Table 2
Confusion matrices based on a) preservation method and b) hours-to-collection. These matrices are produced by supervised learning analysis. Rows indicate the true assignment of each sample. Columns indicate predicted assignment of each sample based on OTU composition. The "class error" column lists rate of misassignment for samples within each group. In a) "fresh" samples were immediately extracted and never subjected to a preservation method.

a)

True/predicted	Fresh	Frozen	FTA	Class error
Fresh	0	2	0	1
Frozen	0	23	0	0
FTA	0	0	24	0

b)

True/predicted	0–2 h	12–24 h	Class error
0–2 h	21	3	0.13
12–24 h	8	15	0.35

geoffroyi fecal microbial communities in comparison to fecal microbial communities from *R. bieti* and *R. brelichi* (Fig. 5). Due to difference in housing condition, location, and diet, we cannot evaluate whether microbial differences within or between *R. bieti* and *R. brelichi* are due to biological or environmental differences. However, these samples do serve as a reference point for the *A. geoffroyi* samples. When microbial composition (richness) alone was visualized among the 3 primate species (Fig. 5a), the microbial communities of each monkey species were highly distinct and differences based on preservation method and time were not obvious. However, when microbial richness and abundance were assessed together (weighted UniFrac: Fig. 5b), the effects of preservation method and time became apparent.

These effects were quantified using the weighted UniFrac distance matrix. Larger distances between groups of samples indicates less similar microbial communities. We found that distance within all *A. geoffroyi* samples preserved on FTA cards was significantly less than the distance between *A. geoffroyi* samples preserved on FTA cards versus samples preserved by freezing ($t = -15.7$, nonparametric Bonferroni-corrected $p = 0.015$). The distance within FTA-preserved *A. geoffroyi* samples was also significantly less than the distance between these samples and the *R. bieti*/*R. brelichi* samples ($t = -64.5$, nonparametric Bonferroni-corrected $p = 0.015$). Similarly, the distance within *A. geoffroyi* samples preserved 0–2 h after defecation was significantly less than the distance between 0 and 2 h samples and samples collected 12–24 h after defecation ($t = -7.1$, nonparametric Bonferroni-corrected $p = 0.015$); and the distance within the same 0–2 h samples was also significantly less than the distance between the *R. bieti* and *R. brelichi* samples ($t = -81.2$, nonparametric Bonferroni-corrected $p = 0.015$).

4. Discussion

The fecal microbial community is dynamic over time in the field. Our results indicate that the fecal flora may be altered by time and sample preservation method and that field condition variables are important to consider in field studies due to their effect size.

4.1. Effect of preservation method

Fresh and frozen samples clustered together in compositional analyses, suggesting that freezer preserved microbial communities are more similar to fresh samples than FTA-card-preserved microbial communities. In support of our hypothesis, microbial communities preserved on FTA cards were found to be distinct when compared to frozen or freshly extracted samples. It is unclear why FTA cards preserve greater microbial diversity and a unique microbial composition compared to freezing, but the alpha diversity results suggest that this difference is driven by abundance/evenness rather than simple presence or absence (observed_OTUs) or rare species (which are given more weight in the Chao1 index). Perhaps, the chemical matrix of the FTA card preferentially lyses microbes in the family *Ruminococcaceae*. Or, perhaps FTA cards, while "sterile," may contain trace microbial DNA, as has been reported previously in other laboratory reagents or kits (Salter et al., 2014). Blank extractions of the FTA cards were not performed to eliminate this possibility; however, there was also no consistent evidence of FTA card contamination when all taxa from this study were compared to a list of 93 common contaminants (see Supplementary material, Table S3) (Salter et al., 2014). Additionally, the 4 potential contaminants identified in >3 samples (*Streptococcus*, *Corynebacterium*, *Pseudomonas*, *Acinetobacter*), were all genera capable of colonizing the gut and were not necessarily contaminants (Corbella et al., 1996; Donskey, 2006; Jiang et al., 2015; Matamoros et al., 2013; Rooks et al., 2014; Sartor and Mazmanian, 2012). Finally, another study that examined FTA

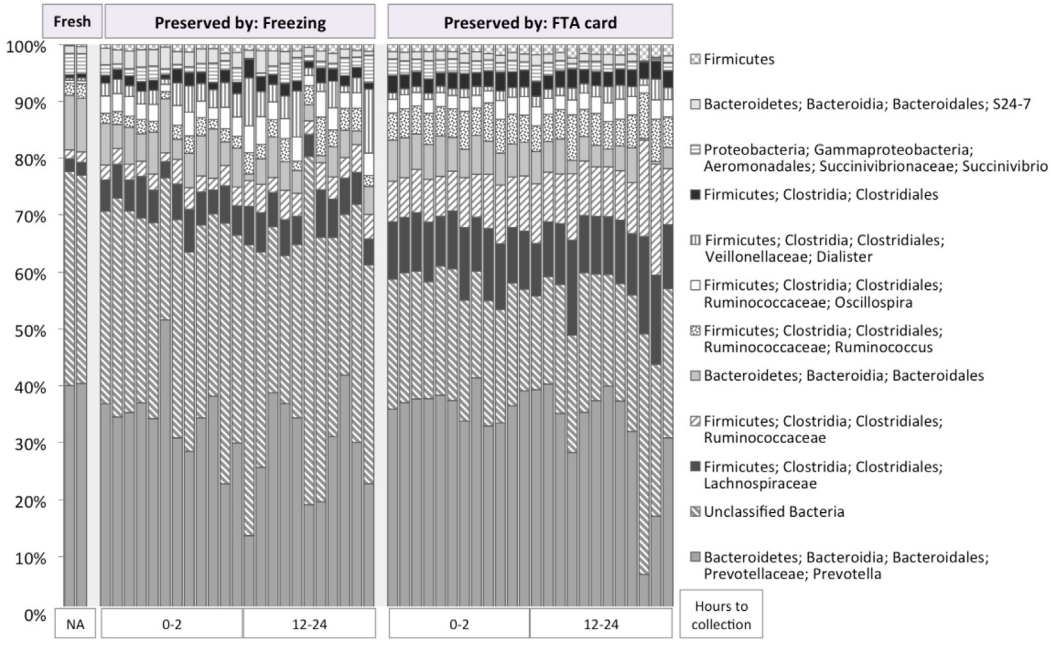

Fig. 4. Relative abundance of OTU by preservation method and time.

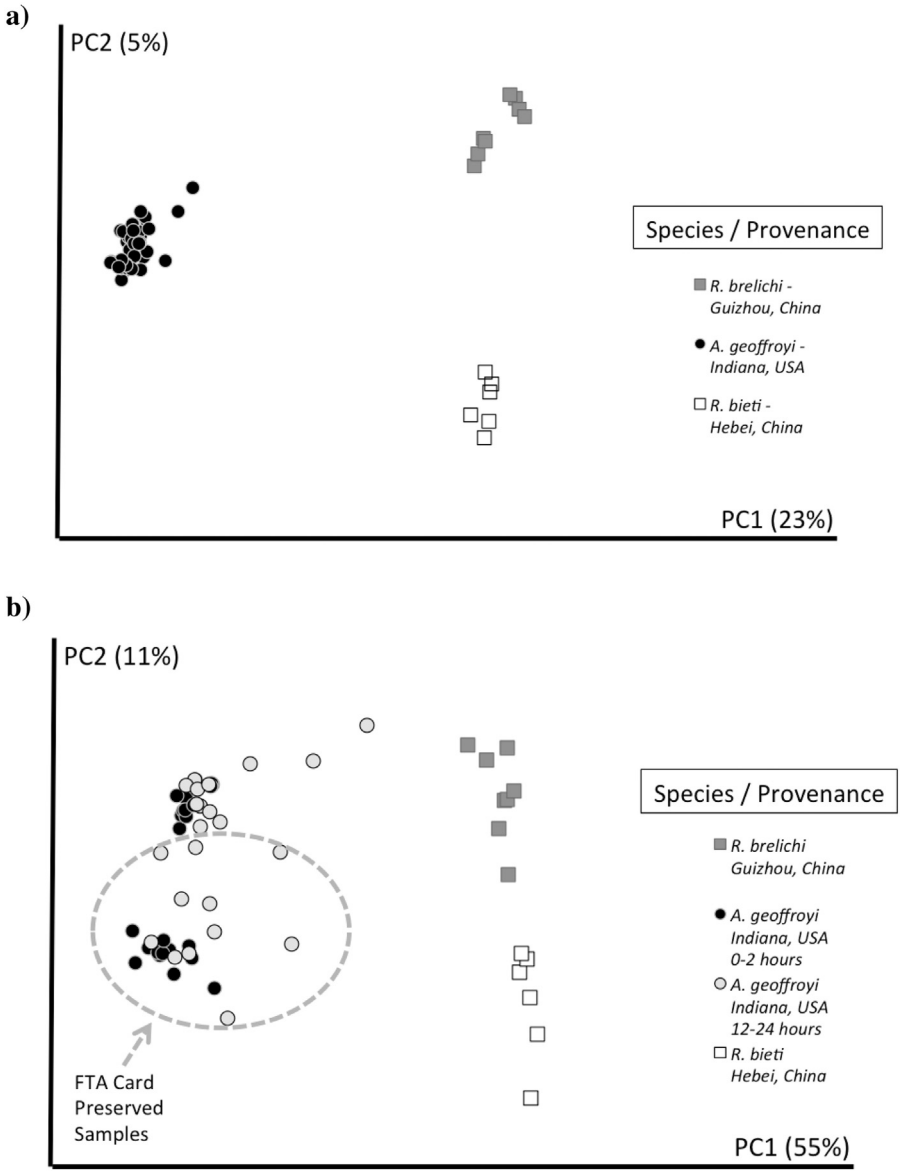

Fig. 5. Principal Coordinate Analysis on a) unweighted and b) weighted UniFrac distances comparing effects of preservation method and time to effects of species/provenance. Small circles represent samples involved in the field condition experiment. In panel b, small black circles represent samples that were collected between 0 and 2 h after defecation; small grey circles represent samples that were collected 12–24 h after defecation. The large dashed grey circle indicates all field condition experiment samples that were preserved on FTA cards. The remaining field condition samples were preserved by freezing.

cards as a method of preserving microbial DNA in stool, found almost no evidence for DNA contamination of FTA cards as the blank FTA extractions failed PCR or yielded no measurable DNA and fewer than 100 reads after amplification (Song et al., 2016).

It is possible that FTA cards may have become contaminated during the drying process, but this is not very likely, given that the opportunity for contamination was limited to air microbes that landed on FTA cards while they dried on a lab bench. The large number of microbes present in feces compared to the small number of microbes in the air would likely overwhelm and prevent any signature of air microbes in an FTA sample. Another possible explanation is that the immediate and powerful chemical lysis microbes undergo on FTA cards resulted in highly effective microbial DNA preservation. Frozen samples undergo limited mechanical and no chemical lysis during the freeze/thaw process. Fresh samples undergo no mechanical or chemical lysis prior to extraction. Thus, it is possible that the chemical lysis step performed by FTA cards allowed more DNA to be accessed and extracted than samples that were frozen or samples that were fresh and never preserved. If this is true, then FTA card samples offer the best representation of the gut microbial community. However, further experiments are needed before FTA cards replace freezers as the new 'gold standard.'

4.2. Effect of hours to collection

Opposite to what we predicted, microbial diversity was significantly greater in samples collected between 12 and 24 h as compared to samples collected between 0 and 2 h, suggesting that microbial overgrowth of a one or a few species and DNA degradation of other species do not play a major role in altering the fecal microbial profile over time in the field. Additionally, either "rare" (initially undetectable) fecal microbiota species continue multiplying over time in each sample, or contaminants from the environment are causing the increased diversity in the 12–24 h samples. Our findings contradict one laboratory study that reported significant losses in microbial diversity in feces stored at room temperature over 24 h (Ott et al., 2004). However, in the Ott et al. study, samples were individually stored and sealed in screw cap cups (Ott et al., 2004) rather than exposed to the outside environment, as in our study. Ott et al. (2004) attribute the loss in diversity in their study to

oxygen exposure and bacterial "starvation." Many gut microbes, particularly butryate-producers, are anaerobic (Barcenilla et al., 2000); thus, prolonged exposure to aerobic conditions results in bacterial cell death. Without butyrate as an energy source, other fecal bacteria "starve," die, and degrade, ultimately leading to reduced microbial diversity (Ott et al., 2004). We did not find evidence for this phenomenon in our study.

In supervised learning analyses, significant grouping is noted by "hours-to-collection" when 0–2 h samples are combined and 12–24 h samples are combined. Our findings are similar to another study on fecal sample storage that reported significant changes in microbial community structure between 12 and 24 h after fecal collection, but very little change in the microbial community prior to 12 h (Roesch et al., 2009). Specifically, Roesch et al. (2009) noted significant decreases in *Bacteroidetes* OTUs over time. Our results support this finding: One abundant OTU in the phylum *Bacteroidetes* was in greater abundance in fresh and 0–2 h samples compared to 12–24 h samples.

4.3. Effect of insects and light conditions

Also opposite to what we predicted, netting status (excluding or allowing insect infestation) and light conditions (sun versus shade) did not cause any significant alterations in microbial diversity or composition. This was somewhat surprising. In samples that were not covered by netting, pronounced insect infestations were observed — flies, beetles, and maggots crawled throughout and covered every surface of the fecal sample. However, due to the volume of feces collected for each sampling period (0.25 g), fecal bacteria likely overwhelmed the relatively smaller contribution of "insect bacteria" in the DNA extraction and amplification process. As such, this argues against insect contamination as the driver for increased diversity in the 12–24 h samples. Contaminants from other sources are still feasible. In relation to light, perhaps the sun served as desiccating agent in our study, essentially preserving the fecal samples. Alternately, the sun may have inactivated or killed bacteria in the fecal samples; however, the microbial DNA was still present and amplifiable.

4.4. Effect size of field condition variables

Field condition variables including fecal preservation method and time remained significant based on distance matrix quantifications in the presence of fecal samples from monkeys of other species/provenances. This suggests that methodology (preservation method, time) in fecal sample collection is critical to experiment validity and that these effects could potentially overwhelm subtler microbial differences due to variables like species or provenance.

5. Conclusions

We conclude with several recommendations for future gut microbial studies in wildlife, particularly herbivorous primates: 1) FTA cards are convenient and effective at preserving fecal microbes, and they provide less variable results than frozen samples. However, samples preserved on FTA cards have microbial communities distinct from fresh samples and samples preserved by freezing. Until further testing determines why FTA cards preserve such unique microbial communities, freezing is recommended, particularly if the intention is to compare microbial communities between other samples preserved by freezing. 2) Fecal samples should be obtained as fresh as possible, and not older than 12 h for the most representative examination of the gut microbial community. 3) Microbial community alterations were observed with several experimental variables and these effects were similar in scale to effects associated with differences in species/provenance. Experimental method validation is highly advised prior to field studies and caution is advised in comparing samples collected at different times or using differing methods.

Acknowledgements

We are very grateful to the Columbian Park Zoo, Beijing Zoo and the Wildlife Rescue Center of Fanjingshan National Nature Reserve for providing the monkey feces used in this study. Krista Nichols kindly provided laboratory space for the molecular work at Purdue University. We also offer sincere thanks to Richard D. Howard for his support, guidance, and review of this manuscript. Our gratitude additionally goes to Bong Suk-Kim and Gaenna Rogers and Qikun Zhang for their help and guidance in the laboratory. The suggestions provided by the reviewer for this manuscript were also extremely insightful and helpful. This project was funded by San Diego Zoo Global, the Offield Family Foundation, the Earth Microbiome Project, Morris Animal Foundation and the Margot Marsh Biodiversity Foundation. 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.2016.09.017.

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