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

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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.

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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