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Using the gut microbiota as a novel tool for examining colobine primate GI health

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ABSTRACT

Primates of the Colobinae subfamily are highly folivorous. They possess a sacculated foregut and are believed to rely on a specialized gut microbiota to extract sufficient energy from their hard-to-digest diet. Although many colobines are endangered and would benefit from captive breeding programs, maintaining healthy captive populations of colobines can be difficult since they commonly suffer from morbidity and mortality due to gastrointestinal (GI) distress of unknown cause. While there is speculation that this GI distress may be associated with a dysbiosis of the gut microbiota, no study has directly examined the role of the gut microbiota in colobine GI health. In this study, we used high-throughput sequencing to examine the gut microbiota of three genera of colobines housed at the San Diego Zoo: doucs (*Pygathrix)* ($N = 7$), colobus monkeys (*Colobus)* ($N = 4$), and langurs (*Trachypithecus)* ($N = 5$). Our data indicated that GI-healthy doucs, langurs, and colobus monkeys possess a distinct gut microbiota. In addition, GI-unhealthy doucs exhibited a different gut microbiota compared to GI-healthy individuals, including reduced relative abundances of anti-inflammatory *Akkermansia.* Finally, by comparing samples from wild and captive Asian colobines, we found that captive colobines generally exhibited higher relative abundances of potential pathogens such as *Desulfovibrio* and *Methanobrevibacter* compared to wild colobines, implying an increased risk of gut microbial dysbiosis. Together, these results suggest an association between the gut microbiota and GI illness of unknown cause in doucs. Further studies are necessary to corroborate these findings and determine

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cause-and-effect relationships. Additionally, we found minimal variation in the diversity and composition of the gut microbiota along the colobine GI tract, suggesting that fecal samples may be sufficient for describing the colobine gut microbiota. If these findings can be validated in wild individuals, it will facilitate the rapid expansion of colobine gut microbiome research.

1. Introduction

Mutualistic microbial communities in the gastrointestinal (GI) tract, known as the gut microbiota, make important contributions to the nutrition of all mammals (Mackie, 2002). One of the primary abilities of the gut microbiota is to convert indigestible plant structural compounds such as cellulose into short-chain fatty acids (SCFA), which can be absorbed directly by the host and used for energy (Flint et al., 2012). SCFAs produced by the gut microbiota can supply hosts with up to 70% of their daily energy needs, facilitate nutrient absorption, and prevent the accumulation of potentially toxic metabolic by-products (Flint et al., 2008; Neish, 2009). The gut microbiota also regulates xenobiotic metabolism (Bjorkholm et al., 2009). Therefore, mammals rely on their gut microbiota to digest food items with large amounts of plant structural carbohydrates and toxins.

Within the Order Primates an immense diversity of dietary specializations exist. Among these, the ability to consume large proportions of leaves during some periods of the year has evolved multiple times (Campbell et al., 2011). Some form of folivory is represented by prosimians (indriids, bamboo lemurs, and sportive lemurs), New World monkeys (howler monkeys), Old World monkeys (colobines), and apes (gorillas). Leaves generally contain high quantities of structural carbohydrates such as cellulose and hemicellulose as well as secondary metabolites such as tannins and phenolics (Norconk et al., 2009). Therefore, while a range of behavioral and physiological adaptations aid folivorous primates in exploiting a leafy diet (Lambert, 1998), the gut microbiota is also believed to play a prominent role in facilitating the use of these hard-to-digest food items.

Among folivorous primates, colobines are of special interest due to their sacculated foregut, which allows for pregastric fermentation of food items by the gut microbiota (Davies and Oates, 1994; Chivers and Hladik, 1980). Specifically, foregut fermentation occurs in the saccus, and in some genera, the presaccus, which consist of enlarged, sacculated fermentation chambers, proximal to the gastric regions (Chivers, 1994; Lambert, 1998). These unique rumen-like adaptations to folivory are hypothesized to have evolved as a mechanism to avoid competition with frugivorous apes (Chivers, 1994). Some species of colobines are known to consume diets composed of greater than 90% leaves in the wild (Kirkpatrick, 1998)

Despite their ability to utilize hard-to-digest, low-quality food items, nearly a third of the 78 colobine species listed by the IUCN are classified as endangered or critically endangered (IUCN, 2015). The wild populations of Delacour's langurs (*Trachypithecus delacouri*) and Tonkin snub-nosed monkeys (*Rhinopithecus avunculus*) are estimated at only 250 individuals while the Cat Ba langur (*Trachypithecus poliocephalus poliocephalus*) population is estimated at 60 individuals (IUCN, 2015). Due to their capacity to maintain protected, breeding populations of primate taxa, zoos and sanctuaries have become an important tool for the conservation of primates like these. However, gastrointestinal (GI) illness is one of the major challenges associated with housing colobines in captivity (Hill, 1964; Ullrey, 1986; Calle et al., 1995; Ensley et al., 1982; Heldstab, 1988; Hollihn, 1973; Janssen, 1994; Loomis and Britt, 1983; Overskei et al., 1992; Shelmidine et al., 2013; Sutherland-Smith et al., 1998). Common GI issues reported in captive colobines include diarrhea, vomiting, bloat, and weight loss (Agoramoorthy et al., 2004; Davies and Oates, 1994; Edwards, 1997; Nijboer and Clauss, 2006; Sutherland-Smith et al., 1998). Therefore, while a number of zoos and sanctuaries around the world are currently home to a variety of colobine species, these clinical conditions can represent a barrier to the establishment of successful captive breeding populations.

The underlying causes of GI illness in captive colobines are often unresolved. Nevertheless, it has long been speculated that the specialized diet and gut physiology of colobines contributes to their susceptibility (Crissey and Pribyl, 1997; Ruempler, 1998). Specifically, while wild colobines consume fiber-heavy diets dominated by leafy browse, captive primates, including colobines, are generally provided with considerably lower-fiber diets with less leafy browse (Nijboer and Dierenfeld, 1996; Oftedal et al., 1991). Just as low-fiber diets can lead to acidosis and GI distress in ruminants, they may lead to GI illness in colobines as well (Lambert, 1998).

Interestingly, though, some colobines are more sensitive than others. In particular, doucs (*Pygathrix* spp.) and red colobus (*Procolobus* sp.) are reported to frequently develop GI problems of unknown cause in captivity (Gijzen et al., 1966; Ruempler, 1998; Struhsaker, 2010; Janssen, 1994). These differences in susceptibility are unlikely to be attributable to inter-species variation in diet since the major components of all colobine diets are young and mature leaves, seeds, and in the case of some snub-nosed monkeys (*Rhinopithecus* spp.), lichens (Guo et al., 2007; Harris and Chapman, 2007; Rawson, 2006; Ryan et al., 2012; Ulibarri, 2013; Workman, 2009; Xiang et al., 2007; Zhuo et al., 2006). Furthermore, intra-specific differences in diet composition across time and space are greater than inter-specific differences (Chapman et al., 2002). Instead, sensitivity to GI illness may be related to GI morphology and the associated gut microbiota since doucs and red colobus (as well as *Piliocolobus, Rhinopithecus, Nasalis*) have a four-chambered foregut while other species have a two- or three-chambered

foregut (*Colobus, Semnopithecus, Trachypithecus*, and *Presbytis*) (Caton, 1998). Recent research indicates that shifts in the gut microbial communities of other mammals can lead to gastrointestinal distress (Rooks et al., 2014; Suchodolski et al., 2012), and captive primates maintain gut microbial communities that are distinct from, and often less diverse than, their wild counterparts (Amato et al., 2013), which may make them more susceptible to invasion by potential GI pathogens.

To determine the relationship between the gut microbiota and captive colobine, specifically douc, GI health, here we examine the gut microbiota of three genera of colobines housed at the San Diego Zoo: doucs (*Pygathrix nemaeus)*, black and white colobus monkeys (*Colobus guereza* and *C. angolensis)*, and langurs (*Trachypithecus francoisi* and *T. cristatus)*. A past postmortem survey at the San Diego Zoo, indicated gastroenterocolitis as one of the three major contributors to death in doucs (Janssen, 1994), but clinical examination of sick doucs generally does not provide treatable diagnoses. Therefore, our goal was to determine whether an altered gut microbiota might be associated with GI illness of unknown cause in more recent postmortem examinations of San Diego doucs. We hypothesized that doucs possess a gut microbial community distinct from other colobines, which may make them more susceptible to GI illness. Specifically, we predicted that doucs would exhibit lower gut microbial diversity, increased relative abundances of potential microbial pathogens (e.g. *Shigella*), and reduced relative abundances of microbes that are known to protect against GI pathogens and/or inflammation (e.g. *Bacteroides*) (Round and Mazmanian, 2009). We also hypothesized that doucs exhibiting a history of vomiting and diarrhea, and GI lesions at necropsy (GI-unhealthy) would possess a distinct gut microbial community compared to individuals without these traits (GI-healthy). We expected the microbial communities of GI-unhealthy doucs to exhibit even lower gut microbial diversity than that of GI-healthy doucs and more marked changes in the relative abundances of potentially detrimental or beneficial microbes.

To understand the impact of captivity on the gut microbiota of Asian colobines in a broader context, we also compared bacterial communities of samples from wild Asian colobines collected in China (*Rhinopithecus*) and Vietnam (*Pygathrix*) to samples collected from captive Asian colobines at the Beijing Zoo and the Wildlife Rescue Center of Fanjingshan National Nature Reserve in China (*Rhinopithecus*) as well as the San Diego Zoo (*Pygathrix*). We hypothesized that captive Asian colobines would possess a distinct gut microbial community compared to wild Asian colobines. Specifically, we expected captive colobines to exhibit reduced gut microbial diversity, lower relative abundances of cellulose-degrading bacteria that can provide hosts with energy in the form of short-chain fatty acids (e.g. *Butyrivibrio*) (Louis et al., 2004), and higher relative abundances of potentially pathogenic bacteria.

2. Material and methods

San Diego Zoo data collection

Included in this study are 16 colobines ($N = 7$ doucs, $N = 4$ colobus, $N = 5$ langurs) that were submitted to the San Diego Zoo Institute for Conservation Research Wildlife Disease Laboratories for postmortem examination between 2002 and 2011 (Table 1). These colobines were housed at the San Diego Zoo and died or were humanely euthanized due to poor health. All animals had complete post-mortem evaluations comprising gross evaluation followed by microscopic analysis. Following prosection, organs were fixed in 10% buffered formalin, processed, sectioned at 5 μm, stained with hematoxylin and eosin and examined by a veterinary pathologist. Additional special stains of the gastrointestinal tract were utilized to detect metaplastic changes and identify pathogens and included Steiner silver, gram, Periodic Schiff acid, and Alcian blue stains. Lumen contents and adjacent unmanipulated mucosal samples with adherent overlying ingesta were harvested with sterile technique along the length of the gastrotintestinal tract, from each sacculated stomach compartment (presaccus, saccus, tubus, pylorus) through the small and large intestines to the distal colon (duodenum, jejunum, ileum, cecum, colon) although all sample sites were not collected for every individual (sample information and metadata https://qiita.ucsd.edu/study/description/1453). Samples were placed into sterile containers, snap frozen in liquid nitrogen, and stored at −80 °C. Clinical histories from each animal were correlated with post-mortem findings to assess each animal's health. When gastrointestinal pathogens were visualized microscopically, PCR targeting suspected agents (i.e. attaching and effacing *E. coli*, *Megabacteria*, *Helicobacter*, *Campylobacter*, invasive amoeba) was performed to genotype pathogens. Samples for microbiome analysis were shipped to the University of Colorado Boulder, where they were stored at −20 °C until processing. Sample collection and processing was approved by the University of Colorado Boulder IACUC (Protocol 1203.04).

Doucs were categorized into two groups: GI-healthy and GI-unhealthy (of unknown cause). GI-healthy animals had no history of chronic vomiting/diarrhea and no recent episodes of vomiting/diarrhea at the time of death (per submission form). Their gastrointestinal tracts were not determined to be a contributor to the morbidity or mortality of the animal based on histological examination. GI-unhealthy animals had a history of chronic vomiting/diarrhea with recent episodes at the time of death. Their gastrointestinal tracts were determined histologically to be a contributor to this clinical presentation and the morbidity or mortality of the animal. Five out of the seven doucs, one out of the five langurs, and none of the four colobus monkeys were classified as GI-unhealthy (Table 1). However, four of the langurs had leafy foreign bodies.

16S rRNA amplification, sequencing, and sequence processing

The mucosa/luminal side of each tissue sample was swabbed, and DNA extracted using the MO BIO PowerSoil DNA extraction kit according to EMP standard protocols (http://www.earthmicrobiome.org/emp-standard-protocols/). PCR targeting the V4 region of the 16S rRNA bacterial gene was performed with the 515F/806R primers, utilizing the protocol described in Caporaso et al. (2012). Amplicons were barcoded and pooled in equal concentrations for sequencing. The

Table 1
Summary of samples utilized.

Host common name	Host scientific name	Sampling site	GI health	GI site	Number of individuals
Douc	*Pygathrix nemaeus*	San Diego Zoo, USA	Healthy	Multiple	2
Douc	*Pygathrix nemaeus*	San Diego Zoo, USA	Unhealthy	Multiple	5
Douc	*Pygathrix nemaeus*	Son Tra Nature Reserve, Vietnam	Unknown-healthy	Fecal	12
Langur	*Trachypithecus francoisi*	San Diego Zoo, USA	Healthy[a]	Multiple	2
Langur	*Trachypithecus francoisi*	San Diego Zoo, USA	Unhealthy[a]	Multiple	1
Langur	*Trachypithecus cristatus*	San Diego Zoo, USA	Healthy[a]	Multiple	2
Colobus monkey	*Colobus guereza*	San Diego Zoo, USA	Healthy	Multiple	2
Colobus monkey	*Colobus angolensis*	San Diego Zoo, USA	Healthy	Multiple	2
Snub-nosed monkey	*Rhinopithecus brelichi*	Fanjingshan National Nature Reserve, China	Unknown-healthy	Fecal	7
Snub-nosed monkey	*Rhinopithecus brelichi*	Beijing Zoo and Wildlife Rescue Center of Fanjingshan National Nature Reserve, China	Unknown-healthy	Fecal	8

[a] Four of the five sampled langurs had leafy foreign bodies.

amplicon pool was purified with the MO BIO UltraClean PCR Clean-up kit and sequenced on the Illumina HiSeq2000 sequencing platform at the BioFrontiers Institute Next-Generation Genomics Facility at University of Colorado, Boulder, USA.

The single-end sequencing reads from the 515F primer were quality-checked using the default settings for the split_ libraries_ fastq.py function in QIIME v1.9.0. Sequences were clustered into representative bacterial OTUs using the sortmerna/sumaclust implementation of open-reference OTU-picking at 97% sequence similarity. Taxonomy was assigned using the RDP classifier and the Greengenes 13_8 database. On average we obtained 75,913 reads/sample (range: 1–409 298 reads/sample), including the wild samples (see below). Any OTUs representing less than 0.00005% of the total dataset were filtered out as recommended for Illumina-generated sequencing data (Bokulich et al., 2013). Greengenes Proteobacteria OTU 4448331 was removed from all samples since it has been shown to bloom in response to suboptimal sample storage conditions and is unlikely to be a major influence on the gut microbiota *in vivo* (Salter et al., 2014) (See supplemental material, Appendix A). All sequences that were unassigned or assigned to chloroplasts or mitochondria were also removed. Data were rarefied to 4871 reads per sample before analysis.

18S rRNA amplification, sequencing, and sequence processing

We amplified a fragment of 18S rRNA that is taxonomically informative for microbial eukaryotic lineages (Amaral-Zettler et al., 2009). A blocking primer specific to mammals was used to minimize amplification of host DNA (GCCCGTCGCTACTACC-GATTGGIIIIITTAGTGAGGCCCT C3 Spacer) as described in the EMP 18S protocol. All primers for amplification and sequencing are available at (http://www.earthmicrobiome.org/emp-standard-protocols/18s/).

We used a curated version of the Silva 111 database as the reference database (original: http://www.arb-silva.de/documentation/release-108/; Pruesse et al., 2007, curated version available at qiime.org/home_static/dataFiles.html, Yilmaz et al., 2014). Sequences not corresponding to eukaryotic 18S were removed from the dataset prior to analysis by excluding reads that failed to align to the eukaryotic portion of Silva 111 at a low similarity threshold (70% sequence similarity) with PyNAST (Caporaso et al., 2010) within QIIME. We utilized the open reference OTU picking workflow in QIIME as described by (Rideout et al., 2014). Taxonomy was assigned using the PR2 database (Guillou et al., 2013) using the QIIME script parallel_ assign_ taxonomy_ blast.py. The dataset was further filtered to exclude all sequences assigned to vertebrate animals, as these likely correspond to the primate host, as well as sequence reads assigning to plant or insect. We obtained an average of 7942 reads/samples (range: 0–32,739 reads/sample). We filtered out low abundance OTU's (<0.0005% of reads in the total dataset) as recommended for Illumina generated data (Bokulich et al., 2013).

Statistical analysis of microbiome data (16S rRNA and 18S rRNA)

Although multiple swabs were available for each host individual at each body site, microbial communities at each body site were similar between replicates (Supplementary Information). Therefore, only one random swab from each site on each individual was included in statistical analyses. Because not all stomach sites were sampled for each individual, we randomly chose a stomach sample for data analysis for each individual. Depending on the comparisons performed, data from different GI sites, individuals, GI-health status groups, and/or colobine genus were combined, as indicated below. To understand patterns in the microbiota across host genera, we limited our analyses to those individuals that were considered GI-healthy. To understand patterns in the microbiota in response to GI disease, we utilized data from doucs only (GI-healthy, GI-unhealthy).

Patterns in alpha and beta diversity were visualized using QIIME and Emperor. Because alpha diversity data (Chao1; Chao, 1987) followed a normal distribution, we tested for significant differences in microbial diversity across individuals, gut sites (controlling for genus and health status), host genera (controlling for individual and gut site), and GI-health status (controlling for individual and gut site) using analysis of variance (ANOVA, Vegan package, R software, version 3.0.2). For 16S data, we tested for significant differences in microbial community composition across individuals, gut sites (controlling for genus and health status), host genera (controlling for individual and gut site), and host GI-health status (controlling for individual and gut site) using permutational analysis of variance (PERMANOVA (adonis), vegan package, R software,

version 3.0.2). Beta diversity statistics were performed using both unweighted and weighted UniFrac distances between samples. For 18S data, because of the small number of OTUs in some samples, we did not use distance measures to describe variation in composition across samples (in samples with only 1 OTU, distance cannot be measured). Instead, all 18S results are based on relative abundance of microbial taxa. Significant changes in the relative abundances of individual bacterial and eukaryotic taxa were tested across gut sites, host genera, and host health status using a series of Kruskal–Wallis tests (R software), and *p*-values were corrected using family-wide error rates (FDR, R software). For both 16S rRNA and 18S rRNA datasets, we detected significant differences in alpha and beta diversity across individuals in all subsets of data and across gut sites for GI-healthy colobines, and therefore these factors were controlled for in subsequent relevant models. Because Kruskal–Wallis tests do not allow the inclusion of multiple variables, we could not control for host individual identity when testing for the effects of host genus or host GI-health status on the relative abundance of each microbial taxa. However, for six of the eight microbial taxa whose relative abundances changed in response to host GI-health status and whose distributions approximated a normal distribution, ANOVA tests that controlled for host individual identity produced significant results that generally matched the results of the uncontrolled Kruskal–Wallis tests, suggesting that results were not a product of pseudo-replication. Additionally, Kruskal–Wallis tests using data from only one gut site per individual gave similar results (albeit with less statistical power) compared to the tests with more samples included. Probability of correct classification of health states was estimated using a supervised learning approach implementing the R package randomForest (Liaw and Wiener, 2002).

Wild data collection and 16S analysis

Fecal samples were collected from 12 wild doucs (*Pygathrix nemaeus*) in Son Tra Nature Reserve, Da Nang, Vietnam from March–May 2013 (Table 1). Samples were placed on ice for transport and kept frozen at a maximum of −20 °C until processing at the University of Minnesota (Saint Paul, MN, USA). DNA was extracted using the established method of Yu and Morrison (2004) with some modifications. Specifically, two rounds of bead-beating were performed in the presence of NaCl and sodium dodecyl sulfate, followed by ammonium acetate and isopropanol precipitations. Precipitated nucleic acids were treated with DNase-free RNase (Roche). DNA was purified using the QIAmp® DNA Stool Mini Kit (QIAGEN, Valencia, CA). DNA quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Massachusetts, USA). The V4 hypervariable region of the 16S rRNA gene was amplified using the 515F/806F primer combination (Caporaso et al., 2012). The 16S rRNA amplification protocol from the Earth Microbiome Project was adopted to perform the PCR amplification (Gilbert et al., 2010). Sequence data were generated using an Illumina MiSeq.

Between April 2010 and July 2013, fecal samples were collected from 7 wild Guizhou snub-nosed monkeys (*Rhinopithecus brelichi*) in Fanjingshan National Nature Reserve, Guizhou, China and from 8 captive Guizhou snub-nosed monkeys at the Beijing Zoo and the Wildlife Rescue Center of Fanjingshan National Nature Reserve in China (Table 1). Samples were immediately preserved using FTA cards (Whatman Inc., Florham Park, NJ, USA). DNA extraction was performed using a MoBio PowerSoil DNA extraction kit at Purdue University (West Lafayette, IN, USA) or the Zhejiang Institute of Microbiology (Hangzhou, China). PCR amplification and sequencing were completed at the University of Colorado Boulder following the same protocols described for the San Diego Zoo samples. Sequence data were generated for half the samples using an Illumina MiSeq and for half using an Illumina HiSeq.

Sequence data from all wild samples were trimmed to a common 100 bp length and processed and analyzed using the same methodology described for the San Diego samples. OTUs were picked using the open reference method in conjunction with the San Diego Zoo dataset to allow for direct comparison and data were rarefied to 16,736 reads per sample before analysis. Fecal samples from China and Vietnam were compared only to colon samples from colobines (*Pygathrix, Trachypithecus*) at the San Diego Zoo. Although variation in sample processing as well as body site lead to some discrepancy among the three datasets, it is unlikely that the effects of these variables are greater than the effect of captivity on the gut microbiota (Lozupone et al., 2013).

All raw sequence data are available at EBI under accession numbers ERP016286 (San Diego Zoo Samples), ERP016358 (wild douc samples), ERP016329 (wild and captive snub-nosed monkey samples), and ERP016285 (wild and captive snub-nosed monkey samples).

3. Results

Individual host identity

Individual colobines exhibited distinct gut bacterial communities both in terms of diversity ($F_{15,66} = 8.6, p < 0.01$) and composition (weighted: $F_{15,81} = 6.8, r^2 = 0.61, p < 0.01$; unweighted: $F_{15,81} = 3.9, r^2 = 0.47, p < 0.01$; Fig. 1). This pattern was consistent regardless of whether GI-unhealthy individuals were included or not. Similarly, we discovered distinct microbial eukaryotic communities across individual hosts. Diversity of microbial eukaryotes varied significantly by individual (chao1: $F_{14,38} = 2.5, p = 0.01$) as did the relative abundance of several eukaryotes.

GI site

Surprisingly, we did not detect differences in gut bacterial diversity or composition across GI sites. Similarly, we did not observe significant differences in microbial eukaryotic diversity across GI sites. Relative abundances of microbial eukaryotes were also similar across GI sites.

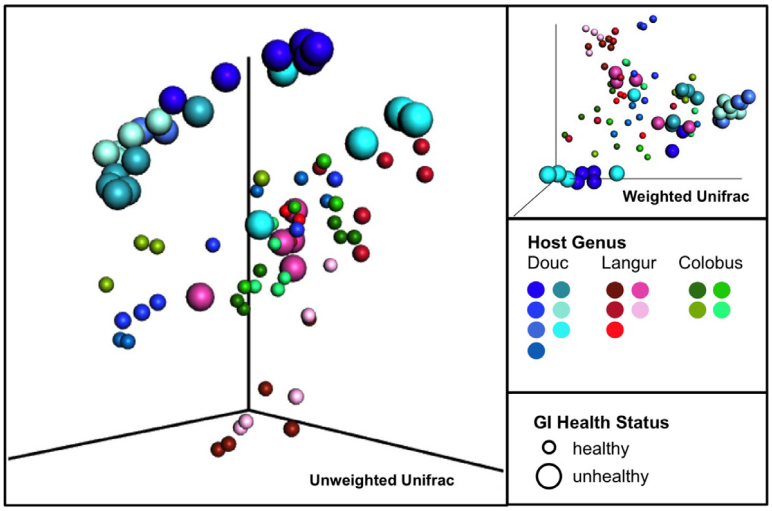

Fig. 1. Differences in microbial community composition across gastrointestinal sites between individuals. The PCoA plots using unweighted UniFrac distances and weighted UniFrac distances include GI-healthy colobus monkeys (green hues), GI-healthy and GI-unhealthy langurs (red hues), and GI-healthy and GI-unhealthy doucs (blue hues). Different shades of color indicate different individuals. The size of the points indicate the health status of the individual. Both plots demonstrate clustering of bacterial and archaeal communities by host genus, by individual, and within doucs, by GI health status. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Host genus

Our data demonstrated that while bacterial diversity is similar, the composition of the colobine gut microbiota differs among host genera (weighted: $F_{2,49} = 4.0$, $r^2 = 0.14$, $p < 0.01$; unweighted: $F_{2,49} = 2.9$, $r^2 = 0.11$, $p < 0.01$; Fig. 2). In particular, GI-healthy doucs exhibited higher relative abundances of Coxiellaceae, genus p-75-a5 of Erysipelotrichaceae, genus YRC22 of Paraprevotellaceae, and *Turicibacter* and lower relative abundances of Coriobacteraceae, Streptococcaeceae *Odoribacter*, and *Akkermansia* compared to other GI-healthy colobines. GI-healthy colobus monkeys had higher relative abundances of Rhodocyclaceae, Coriobacteraceae, Enterococcaceae, Streptococcaceae, Bifidobacteraceae, genus TG5 of Dethiosulfovibrionaceae, *Eubacterium, Faecalibacterium*, and *Methanobrevibacter* and lower relative abundances of genus YRC22 of Paraprevotellaceae and *Turicibacter* than GI-healthy doucs and langurs while GI-healthy langurs had higher relative abundances of *Odoribacter, Sarcina*, and *Akkermansia* and lower relative abundances of Bifidobacteraceae, Enterococcaceae, genus p-75-a5 of Erysipelotrichaceae, *Prevotella*, and *Faecalibacterium* than GI-healthy doucs and colobus monkeys.

In addition, we detected significant differences in microbial eukaryotic diversity across colobine genera (chao1: $F_{2,27} = 7.5$, $p < 0.01$). Specifically, colobus monkeys hosted a significantly higher diversity of microbial eukaryotes (chao1: mean 13.8 ± -7.8 OTUs) than doucs (chao1: mean 8.3 ± -3.2 OTUs) or langurs (chao1: mean 4.7 ± -3.7 OTUs). There were also significant differences in the relative abundances of microbial eukaryotic taxa across host genera (Fig. 2). Because the sample size was very small for the 18S rRNA dataset, we had reduced statistical power. Therefore, we discuss results that are significant with and without FDR corrected p-values, as well as trends in the data that are not significant. GI-healthy doucs had higher relative abundances of the Archamoebae Endolimax (FDR $p = 0.008$) and the fungi Saccharomycetales (uncorrected $p = 0.08$) compared to GI-healthy colobus and langurs. Although not significant, GI-healthy doucs had lower relative abundances of Blastocystis (subtypes 1, 2, 3, and 12) (Fig. 2(D)). Overall, all monkey genera included Blastocystis, Entamoeba, Tetratrichomonas, and Saccharomycetales, which are common in the primate gut (Alfellani et al., 2013; Parfrey et al., 2014).

Douc GI-health status

As hypothesized, we observed distinct bacterial communities in GI-unhealthy and GI-healthy doucs. GI-unhealthy doucs had similar gut microbial diversity compared to GI-healthy doucs, but gut microbiota composition differed between the two groups (weighted: $F_{1,37} = 3.9$, $r^2 = 0.10$, $p < 0.01$; unweighted: $F_{1,37} = 4.1$, $r^2 = 0.10$, $p < 0.01$; Fig. 3). Specifically, GI-unhealthy doucs possessed higher relative abundances of a variety of microbes, including Coriobacteraceae, Peptostreptococcaceae, *Succinovibrio, Bulleidia*, *Pastuerella*, *Eubacterium, Campylobacter*, *Megasphaera*, *Succiniclasticum, Selenomonas, Streptococcus, Acidaminococcus*, and *Phascolarctobacterium* and lower relative abundances of the YRC22 genus of Paraprevotellaceae, *Bilophila*, and *Turicibacter* compared to GI-healthy doucs.

The bacterial composition of GI-unhealthy doucs was not only distinct from that of healthy doucs but also from the other two colobine species (colobus and langur) regardless of health status (Fig. 1; weighted: $F_{1,81} = 9.1$, $r^2 = 0.10$, $p < 0.01$; unweighted: $F_{1,81} = 5.5$, $r^2 = 0.06$, $p < 0.01$). These communities were distinct enough that using a supervised learning approach, we were able to correctly classify all but one GI-unhealthy douc to correct GI-health status with greater than 50% probability (Fig. 4, upper panel). Notably, this one individual (ID# 44923) was also the only individual that was difficult to classify based on clinical history and histology. However, removing this individual from the original model did not greatly

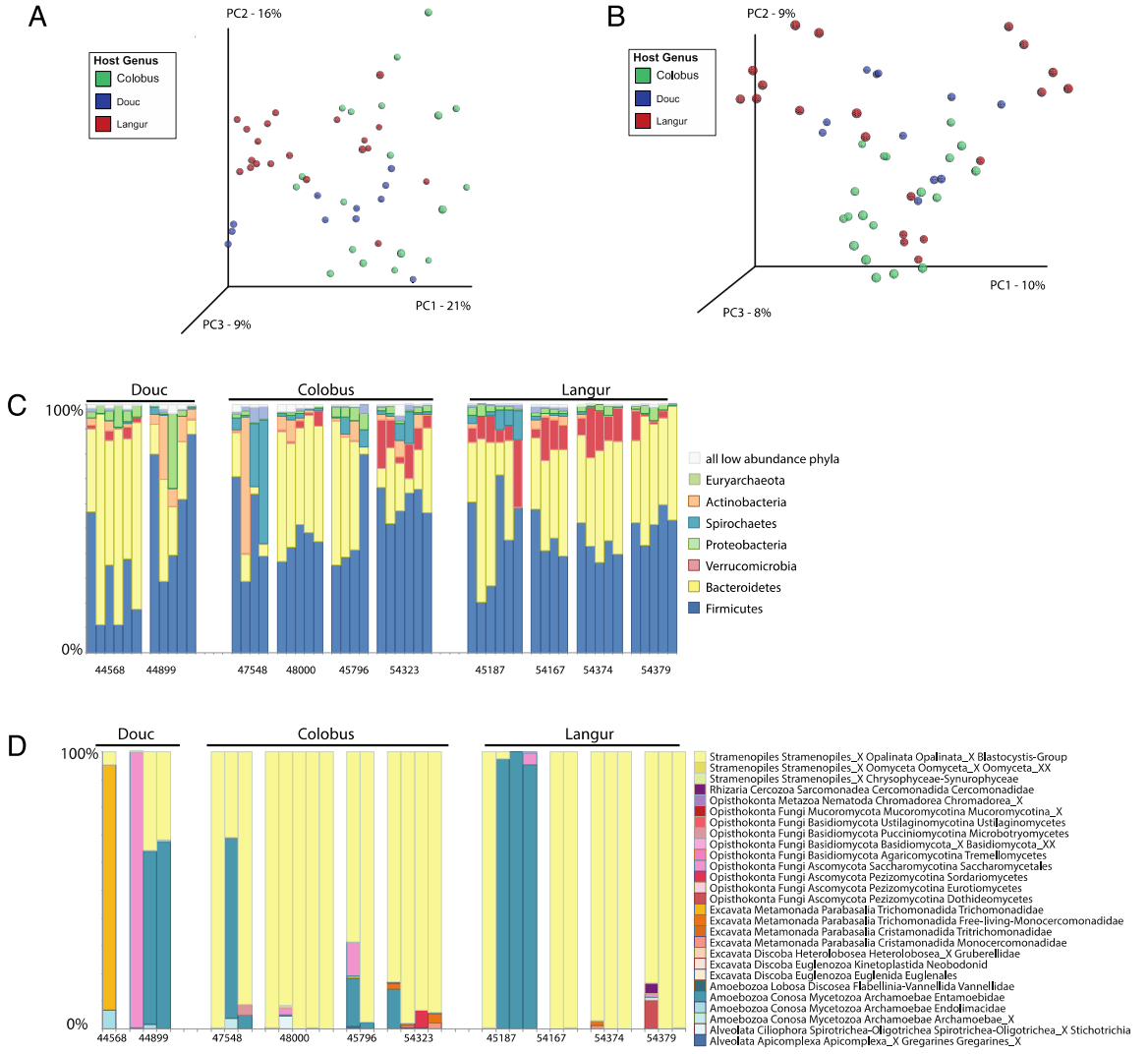

Fig. 2. Gut microbial communities of three genera of GI-healthy Asian colobines. PCoA plots demonstrate clustering of bacterial and archaeal communities by host genus using (A) weighted UniFrac distances and (B) unweighted UniFrac distances. Taxonomy plots also illustrate differences in microbial community composition among host genera both in terms of (C) bacterial and archaeal phyla, as well as (D) microbial eukaryotes. Data in taxonomy plots are clustered by individual host ID number. Multiple columns for each individual represent distinct GI sites (not every individual has a sample for every site).

affect the results (weighted: $F_{1,76} = 10.7$, $r^2 = 0.12$, $p < 0.01$; unweighted: $F_{1,76} = 6.0$, $r^2 = 0.07$, $p < 0.01$). Using this larger dataset with all animals, we were able to examine individual body sites separately. We found that the stomach (weighted: $F_{1,13} = 2.1$, $r^2 = 0.15$, $p = 0.02$; unweighted: $F_{1,13} = 1.8$, $r^2 = 0.13$, $p < 0.01$), cecum (weighted: $F_{1,12} = 2.8$, $r^2 = 0.20$, $p < 0.01$; unweighted: $F_{1,12} = 1.5$, $r^2 = 0.12$, $p = 0.02$), and colon (weighted: $F_{1,14} = 2.2$, $r^2 = 0.15$, $p < 0.01$; unweighted: $F_{1,14} = 1.9$, $r^2 = 0.13$, $p < 0.01$) had distinct gut microbial composition in sick doucs compared to the same body sites in GI-healthy doucs, colobus monkeys, and langurs while the small intestine did not exhibit any marked differences.

We also detected differences in the gut microbial eukaryotic communities according to douc GI-health status (Fig. 3). GI-unhealthy doucs tended to have higher eukaryotic diversity than GI-healthy doucs (chao1: $F_{1,25} = 4.1$, $p = 0.05$; GI-unhealthy: 8.3 ± -3.2 OTUs, GI-healthy: 14.0 ± -5.5 OTUs). Although less predictive than bacterial/archaeal OTUs, eukaryotic profiles were also informative for health classification (Fig. 4, lower panel), with GI-unhealthy doucs harboring a higher relative abundance of Endolimax (Kruskal–Wallis: FDR $p = 0.019$, mean relative abundance GI death vs. not: 0% vs. 1.7%). GI-unhealthy doucs also had a notably high relative abundance of Blastocystis subtype 3 (36% GI-healthy vs. 0% GI-unhealthy, not significant).

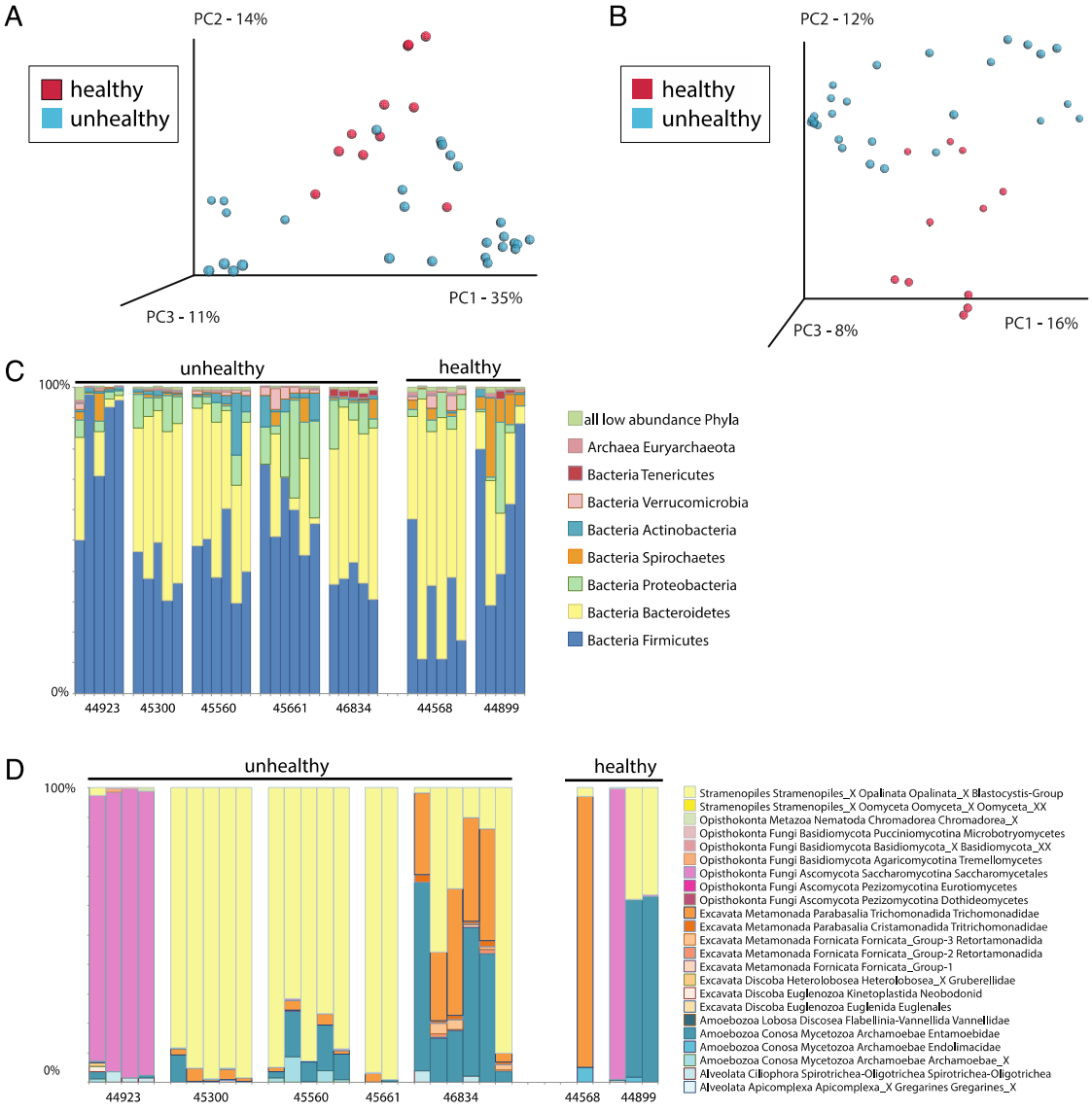

Fig. 3. Gut microbial communities of GI-healthy and GI-unhealthy doucs. PCoA plots demonstrate clustering of bacterial and archaeal communities by douc GI-health status using (A) weighted UniFrac distances and (B) unweighted UniFrac distances. Taxonomy plots also illustrate differences in microbial community composition among GI-healthy and GI-unhealthy individuals both in terms of (C) bacterial and archaeal phyla, as well as (D) microbial eukaryotes. Data in taxonomy plots are clustered by individual host ID number. Multiple columns for each individual represent distinct GI sites (not every individual has a sample for every site).

Wild vs. captive Asian colobines

Although bacterial diversity between wild colobine feces and captive colobine colons was similar, the bacterial community was distinct (weighted: $F_{1,45} = 5.7, r^2 = 0.11, p < 0.01$; unweighted: $F_{1,45} = 7.3, r^2 = 0.14, p < 0.01$; Fig. 5). Wild colobines exhibited higher relative abundances of *Dehalobacterium, Oscillospira, Atopobium, Blautia, Coprobacillus, Desulfotomaculum, Clostridium*, and *Ruminococcus* and lower relative abundances of Euryarchaeota, Planctomycetes, Fibrobacteres, Peptostreptococcaeceae, Victivallaceae, the YRC22 and CF231 genera of Paraprevotellaceae, *Parabacteroides, Prevotella, Epulopiscium, Bacteroides, Desulfovibrio, Butyricimonas, Methanobrevibacter, Phascolarctobacterium*, and *Dialister*. There was also a non-significant trend for wild colobines to have lower relative abundances of *Akkermansia* compared to captive colobines.

4. Discussion

In this study, we set out to determine whether GI illness of unknown cause in the douc population at the San Diego Zoo is associated with a distinct gut microbiota. Specifically, we tested three hypotheses: (1) GI-healthy doucs exhibit a distinct

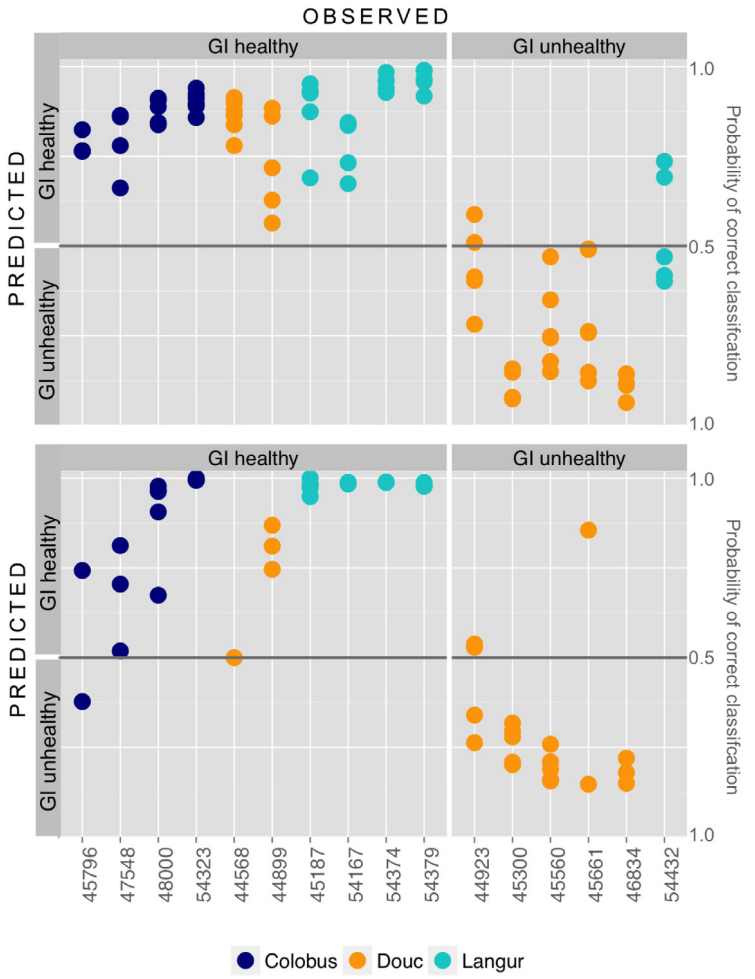

Fig. 4. Classification of health status using a supervised learning approach. Values indicate the probability of classification to the correct (observed) GI health status for each sample from each individual based on bacterial (upper panel) and eukaryotic (lower panel) OTU data.

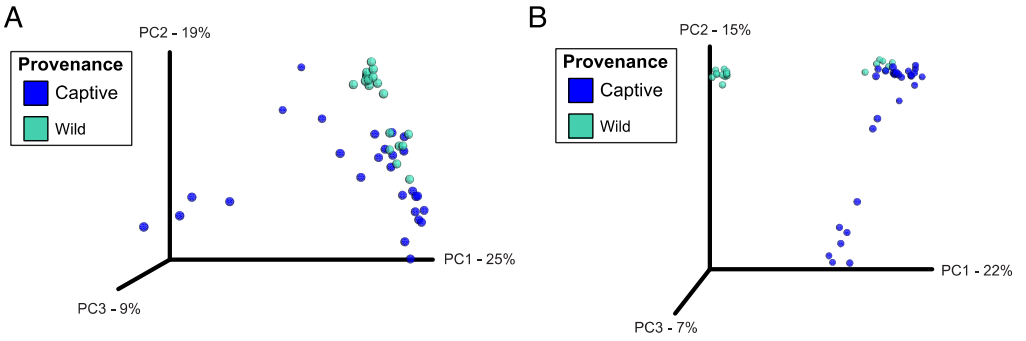

Fig. 5. Fecal and gut microbial communities of wild and captive Asian colobines, respectively. PCoA plots demonstrate clustering of bacterial and archaeal communities by host provenance using (A) weighted UniFrac distances and (B) unweighted UniFrac distances.

gut microbiota compared to other Asian colobine genera at the San Diego Zoo, (2) the gut microbiota of GI-unhealthy doucs are distinct from that of GI-healthy doucs, and (3) captive colobines generally possess a less diverse gut microbiota with fewer potentially beneficial microbes and more potentially detrimental microbes compared to wild colobines. Our results, summarized in Table 2, provide preliminary support for most of these hypotheses.

When we compared GI-healthy doucs to GI-healthy langurs and colobus, we found significant differences in both bacterial/archaeal gut communities as well as microbial eukaryotic communities among all three primate genera. Some microbial patterns suggest that doucs may be more susceptible to GI illness than other Asian colobines in captive settings. For exam-

Table 2
Summary of results. ✓: significant difference detected; ×: no significant difference detected; na: test could not be performed. All comparisons utilize captive individuals only, with the exception of the captive-wild comparison. Tests for Individual and GI site were performed twice, once with only GI-healthy individuals and once with all individuals. Only host genera for which both wild and captive samples were available were used for the captive-wild comparison, but all individuals were included regardless of health status.

	Bacteria/Archaea		Eukaryotes	
	Diversity	Composition	Diversity	Composition
Individual	✓	✓	✓	✓
GI site	×	×	×	×
Genus	×	✓	✓	✓
GI health	×	✓	✓	✓
Captivity	×	✓	na	na

ple, doucs had lower relative abundances of *Akkermansia. A. muciniphila* is a mucin-degrader that is suggested to have an important gut barrier and anti-inflammation functions in mice (Everard et al., 2013). In terms of microbial eukaryotes, GI-healthy doucs, colobus, and langurs hosted typical communities including *Entameoba, Blastocystis, Tetratrichomonas*, and *Saccharomycetales*, but doucs had a notably lower relative abundance and diversity of *Blastocystis* subtypes 2 and 12. In contrast, GI-unhealthy doucs had a higher relative abundance of subtype 3, which has been implicated in irritable bowel-like symptoms in humans (Tan et al., 2008). However, the significance of these findings are unknown at this time. Whether this difference might contribute to GI illness must be examined in more detail.

Next logical steps are to determine the cause and effect relationships and determine which host-associated factors may shape the douc gut microbiota. While each colobine genus we sampled is fed a slightly different diet at the San Diego Zoo, differences in the gut microbiota across colobine genera are most likely a result of host phylogenetic relationships and associated physiological differences since host phylogeny has a stronger impact on the taxonomic composition of the mammalian gut microbiota than host diet (Ley et al., 2008). Additionally, no evidence exists that suggests the douc diet is more or less diverse or seasonally variable than that of other colobines (Chapman et al., 2002; Davies and Oates, 1994; Ulibarri, 2013). Instead, we speculate that douc gut morphology contributes to a distinct relationship between host diet and the gut microbiota. Specifically, doucs possess a sacculated foregut with four chambers while colobus have three chambers, and langurs have two (Davies and Oates, 1994). Therefore, a more complex GI morphology (i.e. presence of a presaccus) could lead to increased sensitivity to dietary shifts. Unique characteristics of the muscle and epithelial tissue associated with the presaccus in four-chambered-foregut colobines like doucs have been hypothesized to confer a "gastric mill" function (Caton, 1998), which grinds food into small particles, while colobines that lack a presaccus, such as langurs, have larger teeth and faster chewing rates for breaking down food before it is swallowed (Wright et al., 2008). As a result, colobines can be considered either "digestive folivores" or "ingestive folivores" (Wright et al., 2008). As "digestive folivores" doucs may have a more specialized gut microbiota that is more sensitive to changes in host dietary input compared to "ingestive folivores" such as black and white colobus and langurs. If this is the case, similar dietary shifts could have markedly distinct effects on the gut microbiota and digestive health of each group of colobines. Further research with more colobine taxa is necessary to provide additional insight.

Our data also indicated that GI-unhealthy doucs possessed distinct gut microbial communities compared to GI-healthy doucs. For example, GI-unhealthy doucs possessed higher relative abundances of genera of potential pathogenic species, most notably *Campylobacter* (Anderson et al., 1993; Islam et al., 2006; Vilardo et al., 2006). In addition, GI-unhealthy doucs exhibited higher diversities of microbial eukaryotes. While these patterns could point to parasitosis as a potential cause of douc GI distress, histological analyses did not identify parasitosis as a potential primary etiology in any of the cases. Furthermore, differences in relative abundances of several common microbial eukaryotic taxa in GI-unhealthy doucs compared to GI-healthy doucs are difficult to interpret because all of the taxa are generally considered commensal, although as discussed above, Blastocystis subtypes may have different pathogenicity. Additionally, some research suggests captive primates may be infected by *Tetratrichomonas* strains from other animals (Smejkalova et al., 2012). Whether these strains can have a negative impact on primate health is not currently understood, and further speciation is required to identify presence and abundance of potential pathogens.

Finally, previous studies show that captive nonhuman primates have reduced gut microbial diversity and different relative abundances of both potentially beneficial and potentially pathogenic taxa compared to wild nonhuman primates, which could increase susceptibility to GI illness (Amato et al., 2013; Hale et al., in preparation). In this study, although we did not detect reduced bacterial/archaeal diversity in captive Asian colobines, captive Asian colobines exhibited increased relative abundances of *Desulfovibrio* and *Methanobrevibacter*, among other taxa. *Desulfovibrio* can convert dihydrogen in the gut into hydrogen sulfide, a cytotoxic, a genotoxic gas that has been linked to inflammatory bowel disease and colorectal cancer in humans (Carbonero et al., 2012; Medani et al., 2011). In humans, methanogens like *Methanobrevibacter* have also been linked with irritable bowel syndrome, constipation, and small intestinal bacterial overgrowth (Pimentel et al., 2012). Therefore, increased relative abundances of these genera in captive colobines may increase the risk of GI illness. In addition, it is interesting to note that wild colobines in this dataset had higher relative abundances of a variety of microbes that

produce short-chain fatty acids such as acetate and butyrate, which can reduce GI inflammation and improve GI health in mice (Donohoe et al., 2011). Finally, high relative abundances of *Methanobrevibacter* in captive colobines may signal horizontal microbial transmission between from humans to colobines since this genus is most commonly detected in the human gut (Nakamura et al., 2011).

While more knowledge regarding microbial function and impacts on host health are necessary to interpret our results further, this study demonstrates the utility of high-throughput sequencing of the bacterial/archaeal 16S rRNA gene for exploring patterns in colobine health. In general, differences in the gut microbiota were associated with captivity in Asian colobines and GI illness in doucs. Additionally, as described above, doucs were classified as GI-healthy or GI-unhealthy using clinical symptoms as well as histology of gut epithelial tissue. Not only did we find significant microbial differences between GI-healthy and GI-unhealthy doucs, but the microbial data mirrored the GI-health categorization in that the single douc that was difficult to classify as GI-healthy or GI-unhealthy was also difficult to classify based on its gut microbiota.

It is important to remember that our sample size is not large in terms of the number of individuals that were sampled at the San Diego Zoo, and some potentially confounding factors could not be controlled for such as diet and antibiotic treatment prior to death. We also were not able to sample GI-unhealthy individuals prior to illness, making host genotype a potential confound in this small dataset. Furthermore, while we controlled for pseudo-replication in both alpha and beta diversity analyses, this was not possible for Kruskal–Wallis tests examining patterns in individual microbial taxa. As a result, further studies are necessary to confirm the observed patterns. Additionally, more research with higher taxonomic resolution examining microbial diversity, composition, and function is to understand which microbes, if any, directly cause GI illness, or susceptibility to illness, in Asian colobines. This information will allow us to better explore which factors lead to detrimental changes in the gut microbiota and ultimately which host–gut microbe interactions might result in GI disease.

This study also contributes important data for the general study of the colobine gut microbiota. Often fecal samples are used to describe the gut microbial communities of mammals, including humans. This methodology is particularly useful when studying primates since collection is completely non-invasive. Few opportunities outside of captive primate facilities exist to collect samples directly from the GI tract, and even fewer target critically endangered species. However, because colobines possess a specialized gut morphology with a sacculated foregut, it has been unclear whether fecal samples accurately represent host–gut microbe interactions for these species. This dataset provided a unique opportunity to examine the gut microbiota along the entire GI tract of several species of colobines. Our finding that the gut microbiota does not vary along the GI tract suggests that fecal samples may be sufficient for understanding the physiology and ecology of colobines in a microbial context. These findings have the potential to rapidly advance wild colobine microbiome research since fecal samples are currently collected by many research groups for a number of other analyses. However, due to small sample sizes and potential differences among colobine species, additional data will be critical for validating our findings. Additionally, it is possible that low-fiber diets in captivity or sampling handling during necropsy lead to a homogenization of the colobine gut. Therefore, GI tract data collected opportunistically from wild mortalities will be ideal for confirming the suitability of fecal samples for research describing the gut microbiota of wild colobines.

Overall, the insights garnered from this study indicate that describing the gut microbiota may be a key factor in supporting Asian colobine conservation efforts. Colobine numbers in the wild are declining at a rapid rate with no signs of reprieve (IUCN, 2015), and while captivity offers colobines protection from external threats such as hunting and habitat destruction (Mittermeier et al., 2009), it is also associated with a distinct gut microbiota that may influence susceptibility to GI illness. Knowledge regarding the role of the gut microbiota in contributing to colobine GI illness in captivity would allow the development of optimized diets and potential therapies to avoid these morbidities, and would ultimately improve the effectiveness of captive breeding programs. Considering this, the current study serves as a critical first step toward the successful application of new research tools in the context of primate conservation.

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Appendix A. Supplementary material

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.gecco.2016.06.004.

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