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First report of foregut microbial community in proboscis monkeys: Are diverse forests a reservoir for diverse microbiomes?

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Abstract

Foregut fermentation is well known to occur in a wide range of mammalian species and in a single bird species. Yet, the foregut microbial community of free-ranging, foregut-fermenting monkeys, i.e., colobines, has not been investigated so far. We analyzed the foregut microbiomes in six free-ranging individuals of proboscis monkeys (*Nasalis larvatus*) from two different tropical habitats with varying plant diversity (mangrove and riverine forests), from a semi-free-ranging setting with supplemental feeding, and from captivity, using high-throughput sequencing based on 16S ribosomal RNA genes. We found a decrease in foregut microbial diversity from a diverse natural habitat (riverine forest) to a low diverse natural habitat (mangrove forest), to human-related environments. Of a total of 2,700 bacterial operational taxonomic units (OTUs) detected in all environments, only 153 OTUs were shared across all individuals, dominated by Firmicutes and Proteobacteria in the relative abundance. This indicates that these OTUs are candidates that is not influenced by diet or habitat. The relative abundance of the habitat-specific microbial communities showed a wide range of differences among living environments, although such bacterial communities appeared to be dominated by Firmicutes and Bacteroidetes, suggesting that those phyla are key to understanding the adaptive strategy in proboscis monkeys living in different habitats.

Introduction

A variety of mammalian herbivores and a single-known avian herbivore digest plant material with the help of commensal microbiomes in a forestomach (Stevens and Hume, 1998; Mackie, 2002). Unlike hindgut fermenters, which have enlarged fermentation compartments in the cecum and/or colon, foregut fermenters have a pregastric fermentation chamber (Stevens and Hume, 1998). Given recent developments in sequencing technology, hindgut microbial diversity and community structure based on large amplicon libraries of 16S ribosomal RNA (rRNA) genes, mostly using fecal DNA, have been widely investigated in various vertebrate taxa (Ley et al., 2008; Muegge et al., 2011). Microbiomes of the foregut have been less studied. This is because, although it is relatively easy to sample feces both in the wild and in captivity, it is difficult to collect pregastric contents. Nonetheless, several studies have investigated the foregut microbial community in captive and free-ranging foregut-fermenting animals, revealing a universal trend in foregut microbial communities at the phylum level: Firmicutes and Bacteroidetes are generally dominant across different animal taxa such as artiodactyl, rodents, colobines, sloths, macropod, and avian foregut fermenters (Pope et al., 2010; Samsudin et al., 2011; Dai et al., 2012; Godoy-Vitorino et al., 2012; Gruninger et al., 2014; Ishaq and Wright, 2014; Kohl et al., 2014; Li et al., 2014; Roggenbuck et al., 2014; Zhou et al., 2014; Cersosimo et al., 2015; Henderson et al., 2015; Amato et al., 2016; Dill-McFarland et al., 2016; Salgado-Flores et al., 2016; Shinohara et al., 2016). However, the foregut microbial community in free-ranging colobine monkeys has not been investigated so far.

Foregut-fermenting colobine monkeys have complex, multi-chambered stomachs where the commensal microbiome detoxifies defensive plant chemicals and digests plant cell walls (Bauchop and Martucci, 1968), thus making an important contribution to the colobine's digestion. In primates, the distal gut microbiome varies, even within a species, with diet (Arumugam et al., 2011) and/or living conditions. Compared with their free-ranging conspecifics, captive primates generally have reduced gut microbial diversity, which has been associated with gut dysbiosis (Amato et al., 2013). Additionally, fecal microbiome patterns in captive primates are comparable to those in humans, most likely as a consequence of artificial ("Western") diets (Amato et al., 2016; Clayton et al., 2016). Therefore, microbial studies in free-ranging colobine monkeys living in natural habitats compared with captive monkeys have the potential to provide a full picture of the microbial diversity in colobine foreguts.

To understand the forestomach microbial patterns of colobines in relation to their diet and living-environment, we first analyzed the foregut microbiome in endangered proboscis monkeys (*Nasalis larvatus*) living in multiple natural habitats in comparison with that of a free-ranging but provisioned individual as well as a captive specimen. The proboscis monkey is endemic to Borneo, the largest island in Asia. They are the only colobine species in which an apparent rumination of stomach contents has been observed under free-range conditions (Matsuda et al., 2011), with a natural diet of varying proportions of leaves (38%–73%), fruits (11%–50%), and flowers (3%–8%) (Bennett and Sebastian, 1988; Yeager, 1989; Matsuda et al., 2009; Boonratana, 2013). They are suitable for the investigation of foregut microbial diversity and community

structure in relation to different feeding habits, because they inhabit various forest types along rivers with great differences in dietary diversity, such as low-diversity mangrove forests and high-diversity peat swamps and riverine forests (Yeager, 1989; Boonratana, 2003; Matsuda et al., 2009; Feilen and Marshall, 2014). Here, we report the foregut microbial communities in six proboscis monkeys living in riverine and mangrove forests, as well as under provision and captive conditions. We expected that foregut microbial diversity would decrease from a diverse natural habitat (riverine forest) to a lower diverse natural habitat (mangrove) to captive conditions.

Results and discussion

We analyzed 16S rRNA gene sequencing-based bacterial composition of the pregastric contents collected from six adult male proboscis monkeys living in different environments in Sabah, Borneo, and Malaysia. Two free-ranging monkeys lived in a riverine forest, another two lived in a mangrove forest, one semi-free-ranging monkey lived in a mangrove forest where it was provisioned with artificial diet items, and one monkey was maintained in a zoological collection.

Based on the rarefaction curves, the number of operational taxonomic units (OTUs) showed that the species richness of the foregut microbiome of monkeys living in the riverine forest was about twice as high as that in monkeys living in other conditions (Table 1, Figure 1A, Figure S1A). In accordance with Shannon's H' of the plant diversity of the forests, the number of plant species and plant parts consumed in the natural habitats of free-ranging monkeys (riverine forest, 188 plant species

consumed with H' 4.3; mangrove forest, seven plant species consumed with H' 2.5) (Table 1), the highest and lowest H' in microbial diversity were observed in the samples from riverine ($H' = 6.0$) and mangrove ($H' = 2.7$) forests, respectively. Proboscis monkeys living in mangrove forest subsist primarily on leaves and unripe fruits of a single plant species, *Sonneratia caseolaris* (Boonratana, 2003; Matsuda et al., in press), whereas monkeys living in riverine forest have a more generalist diet. Similarly, three-toed sloths (*Bradypus variegatus*), which consume primarily only one plant species, have lower diversity of the foregut microbial community than do two-toed sloths (*Choloepus hoffmanni*), which consume a broader diet (Dill-McFarland et al., 2016). These findings support the concept that the variety of nutrients, carbohydrate substrates, and indigestible compounds derived from a diverse diet can shape a variety of feeding niches for microbial taxa and/or functional groups, as suggested by the hindgut microbial diversity of free-ranging howler monkeys (Amato et al., 2013). On the other hand, proboscis monkeys from the provisioned and captive populations, with a dietary diversity (in number of individual diet items) and OTU species richness as low as those in monkeys from the mangrove forest, had relatively high microbial H' diversity. This is likely related to the greater variety of nutrient contents in the diet items, which contained leaves (as in the natural diet) and vegetables raised for human consumption.

More than 99.0% of the sequencing reads were assigned at the phylum level. The five most abundant phyla in the foregut were Bacteroidetes (8.5%–47% of bacterial reads), Firmicutes (16%–82%), and Proteobacteria (1.5%–68%), followed by Actinobacteria (1.4%–4.7%) and Spirochaetes (1.0%–3.1%) (Figure 1B), indicating that

141 the foregut microbial community does not deviate substantially from that previously
142 found in captive colobines (Zhou et al., 2014; Amato et al., 2016) or other foregut
143 fermenters (Pope et al., 2010; Dai et al., 2012; Godoy-Vitorino et al., 2012; Gruninger et
144 al., 2014; Ishaq and Wright, 2014; Kohl et al., 2014; Li et al., 2014; Roggenbuck et al.,
145 2014; Cersosimo et al., 2015; Henderson et al., 2015; Dill-McFarland et al., 2016;
146 Salgado-Flores et al., 2016; Shinohara et al., 2016). Cyanobacteria reads, possibly
147 derived from plant chloroplast DNA (Clayton et al., 2016), were generally detected in
148 the free-ranging individuals in this study (1.8%–5.8%, but 0.4% in the captive
149 individual). Additionally, many more Tenericutes [reported to include potential human
150 pathogens (Yildirim et al., 2010)] reads were found in the captive individual (3.7%, but
151 0.1%–0.5% in free-ranging individuals), possibly related to a more humanized diet or
152 close contact with humans in captivity. Indeed, the provisioned (semi-free-ranging)
153 individual showed an intermediate pattern in this respect (1.6%). These tendencies did
154 not change with polymerase chain reaction (PCR) time or 16S region (Figure S1).
155 A total of 2,700 bacterial OTUs were detected after the individual bacterial
156 compositions of each environment were subsampled and merged (Dataset S3). Only
157 153 OTUs were found across all samples from proboscis monkeys (Figure 2A),
158 indicating that they are the core bacterial community that is not influenced by diet or
159 habitat. These microbial community members were generally dominated by Firmicutes
160 and Proteobacteria, though it would be difficult to generalize their patterns across the
161 different habitats due to the high individual variation even within the individuals living
162 in the same habitat, e.g., < 60% of the reads of M3 were Proteobacteria while it only

163 comprises < 5% of the reads in M4. On the other hand, 1,081 OTUs were neither shared
164 among all environments nor specific to particular environments (Figure 2A).
165 Interestingly, the relative abundance of these OTUs was about one-third in all
166 individuals (32%–38%) except for the provisioned individual (71%) (Figure 2B),
167 indicating that much more of the microbiome of the provisioned individual originated
168 from both free-ranging and captive-like bacteria and that, therefore, it would show an
169 intermediate pattern in the principal coordinates analysis plot based on the unweighted
170 distances (Figure S2).

171 Finally, 743 OTUs were found only in samples from free-ranging monkeys
172 living in riverine forest, 160 only in samples from free-ranging monkeys living in
173 mangrove forest, 181 only in samples from the semi-free-ranging provisioned monkey,
174 and 382 only in samples from the captive monkey (Figure 2A). The relative abundance
175 of the habitat-specific microbial community showed a wide range of differences among
176 living environments (Figure 2B). The lowest abundance of a specific community (1.1%)
177 was found in the individuals living in the mangrove forest, indicating that there are
178 almost no mangrove-specific bacteria, and the highest was found in the captive
179 individual (38%). The habitat-specific bacterial community consisted mostly of
180 Firmicutes and Bacteroidetes in the relative abundance, suggesting that OTUs which
181 belong to these phyla are candidates to understand the adaptive strategy in proboscis
182 monkeys living in different habitats.

183 Around half of the sequence reads were assigned to known genera. Fifteen of
184 these genera were commonly observed in some environments (>1%) (Table 2).

185 *Actinobacillus* (Proteobacteria) was common in both free-ranging and provisioned
186 individuals (3.8%–25%), whereas *Mitsuokella* (Firmicutes) was only abundant in free-
187 ranging individuals (28% in riverine forest, 31.7% in mangrove forest). Various
188 Firmicutes genera were detected in the captive individual, i.e., *Oscillospira* (14%)
189 followed by [*Eubacterium*], *Bulleidia*, *Lactobacillus,* and *Ruminococcus* (1.7%–5.4%).
190 Contrary to the finding that both *Prevotella* and *Bacteroides* (Bacteroidetes) are
191 dominant in the fecal microbiome in humans and captive primates (Clayton et al., 2016;
192 Hale et al., 2018), *Bacteroides* was rarely found in the foregut microbiome of all
193 individuals (<0.01%) in this study, although *Prevotella* was broadly found in all
194 individuals (5.9%–37%). *Prevotella* was higher in the foregut of free-ranging proboscis
195 monkeys in riverine forest, who had a more diverse (and possibly better) diet than those
196 living in mangrove forest, consistent with the fact that *Prevotella* increases in the
197 foregut of cattle fed more energy-rich diets (McCann et al., 2014). A similar foregut
198 microbial pattern (more *Prevotella* but less *Bacteroides*) has been reported not only in a
199 captive golden snub-nosed monkey (*Rhinopithecus roxellana*), which is one of the
200 species phylogenetically closest to the proboscis monkey (Zhou et al., 2014), but also in
201 other foregut-fermenting animals such as moose (*Alces alces*) (Ishaq and Wright, 2014)
202 and roe deer (*Capreolus pygargus*) (Li et al., 2014). More metagenomic and functional
203 analyses of these bacterial groups will help in the understanding of the specialized
204 physiology of leaf-eating foregut fermenters.
205 To our knowledge, this is the first indication of a relationship among foregut
206 microbial and diet and habitat diversity in free-ranging, foregut-fermenting animals. Our

results may not be representative of the entire proboscis monkey population due to the small sample size ($N = 6$) with high individual variation within the same environmental condition. Effects of diet on both foregut and hindgut microbial patterns have previously been shown in various free-ranging and captive animals (Dill-McFarland et al., 2016; Borbon-Garcia et al., 2017; Hale et al., 2018), although these results are rarely shown together with information on the living environment of the animals. The geographic region Sundaland, which includes our study sites, is a large reservoir of endemic tropical plant and animal species such as the proboscis monkey (Myers et al., 2000). Primates of this region have suffered significantly from loss of forest (Wich et al., 2012; Ancrenaz et al., 2014; Abram et al., 2015; Bernard et al., 2016) due to small- and large-scale conversion of forest to oil palm plantations (Sodhi et al., 2004; Woodruff, 2010; Abram et al., 2014). Apart from the response of animals and plants to such impacts on their environment, the response of microbes is still poorly understood, with the exception of the effects of conversion of Amazonian rainforest to agricultural lands on soil bacterial communities (Rodrigues et al., 2013). Thus, there may be the potential to build upon the preliminary data that we generated for more detailed investigations testing the novel concept that diverse forests such as riverine forest provide not only various food sources but also, indirectly, a diverse microbiome for resident animals. The findings that the loss of microbial diversity in the animal foregut in this study and in the hindgut in a previous study (Amato et al., 2013) is linked to forest diversity in some species of endangered primates suggest the expansion of conservation priorities in biodiversity hotspots. One of the serious problems when

primates and other animals are pushed into degraded habitats with lower diversity is forest destruction (Estrada et al., 2017), which can have dysbiotic effects (Honda and Littman, 2012) on gastrointestinal microbial patterns that are associated with gastrointestinal distress (Amato et al., 2016) in threatened primates.

Sequencing analysis of this study was according to a previous study (Hayakawa et al., 2018) and descriptive information of the materials and methods was available in Supporting Information Appendix S1. The sequencing data have been deposited in the DDBJ database with accession number DRA006759.

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Author contributions

IM conceptualized the initial idea, SKSSN DJS DARS RS IM performed the sampling,

SKSSN DJS DARS RS BG AT arranged the sampling in the wild/zoo, TH AS SF HI performed the experiment, TH performed and interpreted the statistical analysis and TH MC IM drafted the manuscript. All authors contributed to the final version of the manuscript.

Conflict of Interest

The authors have declared that no competing interests exist.

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387

388 Table 1. Diversity indices in the forestomach commensal microbiome of proboscis

389 monkeys

Habitat	Habitat diversity (H')	Plants consumed[a]		Sample ID	V1–V2		V3–V4	
		No. of species	No. of parts		No. of *OTUs	H'	No. of OTUs	H'
Riverine	4.3[a]	188	259	R1	1,903	6.03	962	5.30
forest				R2	1,687	3.95	871	3.51
Mangrove	2.5[a]	7	8	M3	778	3.24	501	2.35
forest				M4	887	2.66	508	2.23
Provisioned		18[b]	25[b]	P5	952	4.80	601	4.31
Captive		6	6	C6	782	4.91	610	4.53

390 [a]Matsuda et al. (in press), [b]Tangah (2012), *OTU, operational taxonomic unit.

391 Table 2. Relative abundance of commonly observed genera of forestomach commensal

392 microbiome of proboscis monkeys

Phylum	Genus	Riverine	Mangrove	Provisioned	Captive
Actinobacteria	*Bifidobacterium*	3.8%	**24.7%**	6.3%	–
Bacteroidetes	*Prevotella*	**11.5%**	5.9%	**37.3%**	**12.9%**
	YRC22	–	–	5.1%	–
Firmicutes	*[Eubacterium]*	–	–	–	2.6%
	Bulleidia	–	–	–	1.7%
	Butyrivibrio	–	–	1.3%	–
	Lactobacillus	–	–	-	2.9%
	Mitsuokella	**28.2%**	**31.7%**	–	–
	Oscillospira	–	–	–	**14.4%**
	RFN20	3.0%	1.7%	–	–
	Ruminococcus	1.2%	–	–	5.4%
Proteobacteria	*Actinobacillus*	3.8%	**24.7%**	6.3%	–
Spirochaetes	*Treponema*	1.6%	1.3%	2.5%	1.2%

393 Only genera with >1% relative abundance are shown. Values >10% are highlighted in

394 bold.

395

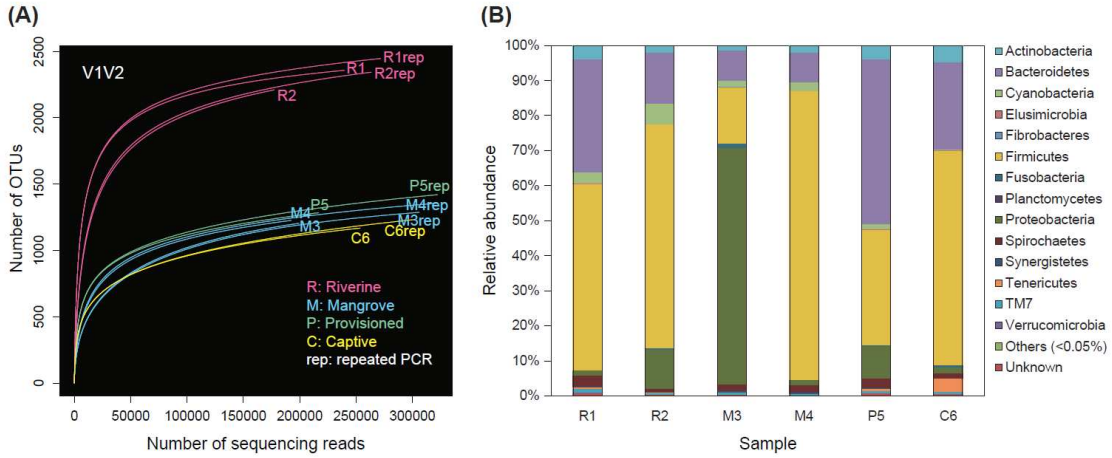

Figure 1. (A) Rarefaction curve of operational taxonomic units and (B) relative abundance of microbial flora and taxonomic assignments at the phylum level from proboscis monkeys living in different environments based on the V1–V2 region of the 16S rRNA gene. Phyla represented by less than 0.05% in any samples were merged in the category "Others."

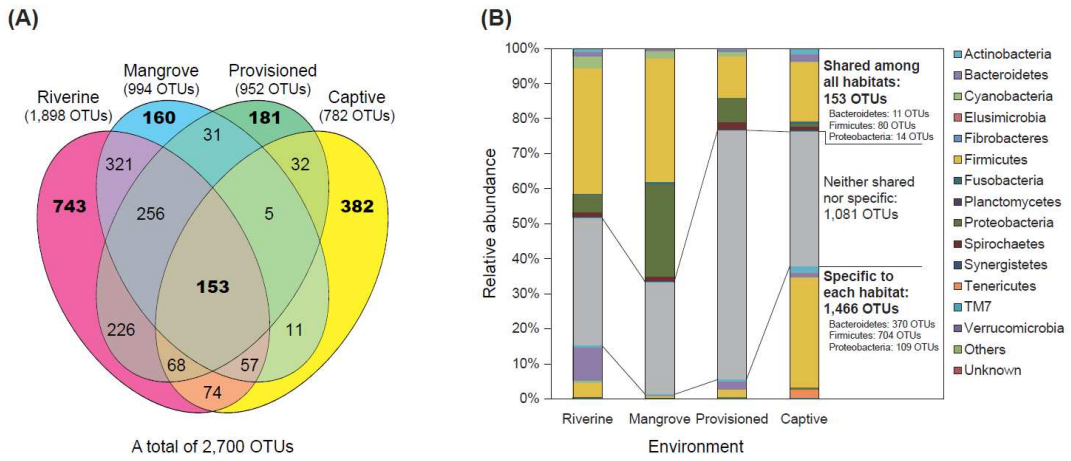

Figure 2. (A) Venn diagram of the detected bacterial operational taxonomic units (OTUs) in forestomach contents collected from proboscis monkeys living in four different environments (riverine, mangrove, provisioned, and captive) and (B) the relative abundance of their microbial flora and taxonomic assignments with degree of sharing of bacterial species. The number of OTUs that belongs to each category is also shown.

410 **Electronic supplementary material**

411 Appendix S1 Descriptive information regarding the methods used within this study.

412

413 Dataset S1. Operational taxonomic units (OTUs), assigned taxa, nucleotide sequences,

414 and number of sequencing reads based on the 16S V1–V2 region.

415 Attached different data file.

416

417 Dataset S2 Operational taxonomic units (OTUs), assigned taxa, nucleotide sequences,

418 and number of sequencing reads based on the 16S V3–V4 region.

419 Attached different data file.

420

421 Dataset S3 Operational taxonomic units (OTUs), assigned taxa, nucleotide sequences,

422 and number of subsampled and merged sequencing reads based on the 16S V1–V2

423 region.

424

425 Attached different data file.

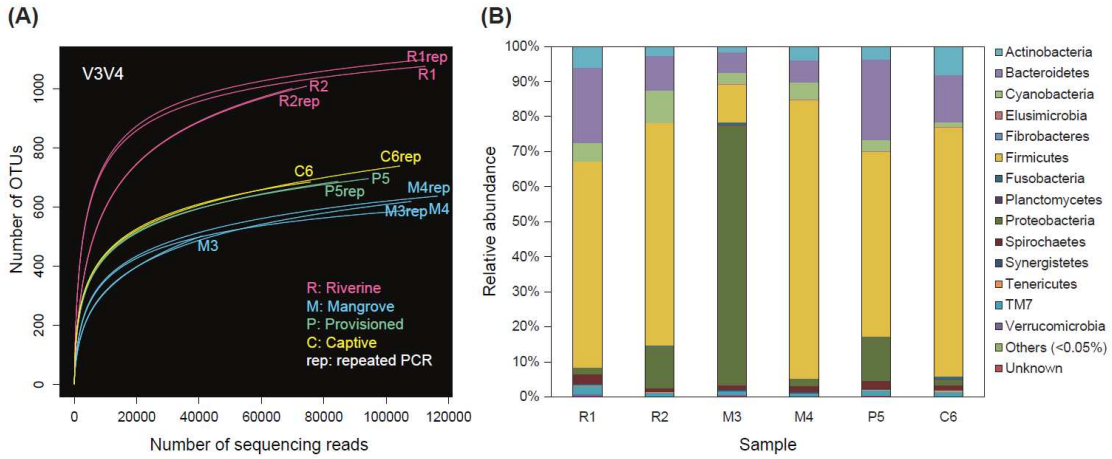

Figure S1. (A) Rarefaction curve of operational taxonomic units (OTUs) and (B) relative abundance of microbial flora and taxonomic assignments at the phylum level from proboscis monkeys living in different environments based on the V3–V4 region of the 16S rRNA gene. Phyla represented by less than 0.05% in any samples were merged in the category "Others."

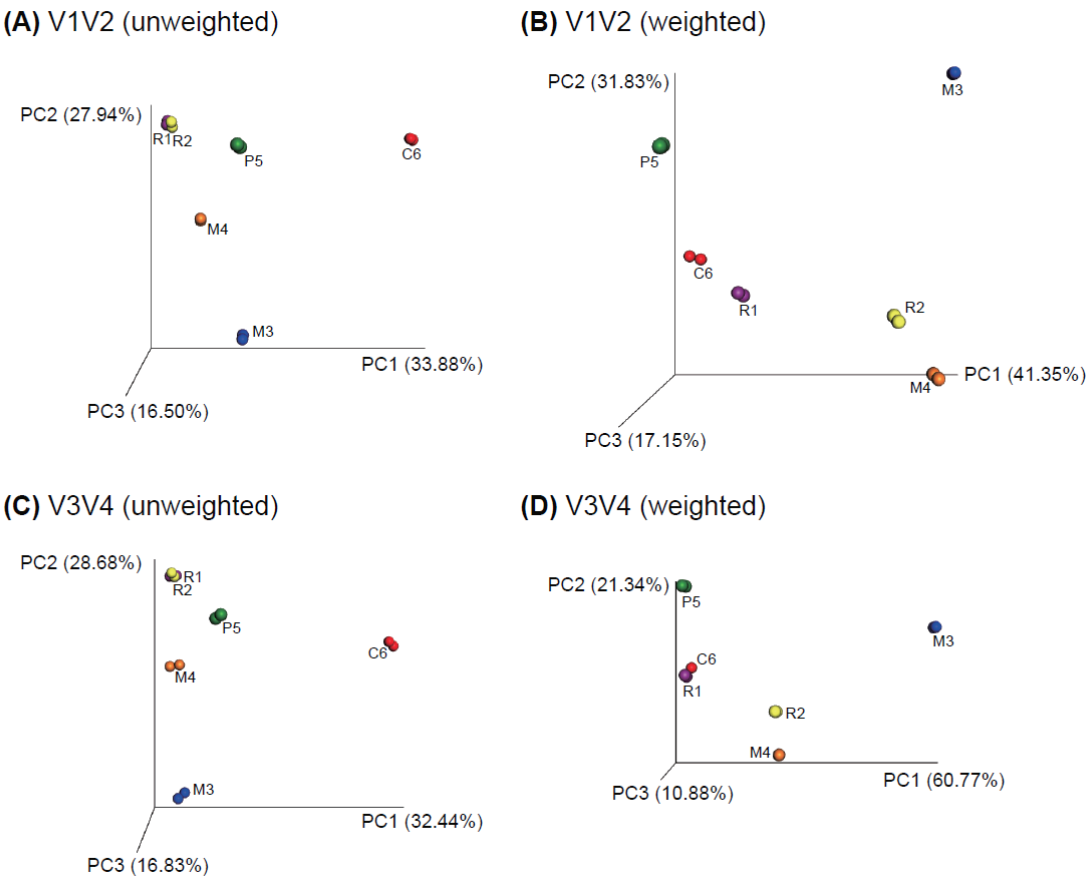

Figure S2. Principal coordinates analysis plots using UniFrac distances. Two circles of the same color indicate the first and repeated polymerase chain reaction results from the same sample. Note that P5 (a provisioned individual) was located between free-ranging individuals (R1, R2, M3, M4) and a captive individual (C6) in the plots based on the unweighted distances, indicating that P5 had an intermediate pattern of microbial community. (A) Based on the unweighted distances in the 16S V1–V2 region. (B) Based on the weighted distances in the 16S V1–V2 region. (C) Based on the unweighted distances in the 16S V3–V4 region. (D) Based on the weighted distances in the 16S V3–V4 region.