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Development, diet and dynamism: longitudinal and cross-sectional predictors of gut microbial communities in wild baboons

Tiantian Ren,[1†] Laura E. Grieneisen,[2†] Susan C. Alberts,[3,4] Elizabeth A. Archie[2,3*] and Martin Wu[1]**

[1]*Department of Biology, University of Virginia, Charlottesville, VA 22904, USA.*

[2]*Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46617, USA.*

[3]*Institute of Primate Research, National Museums of Kenya, Nairobi, Kenya.*

[4]*Department of Biology, Duke University, Durham, NC 27708, USA.*

Summary

Gut bacterial communities play essential roles in host biology, but to date we lack information on the forces that shape gut microbiota between hosts and over time in natural populations. Understanding these forces in wild primates provides a valuable comparative context that enriches scientific perspectives on human gut microbiota. To this end, we tested predictors of gut microbial composition in a well-studied population of wild baboons. Using cross-sectional and longitudinal samples collected over 13 years, we found that baboons harbour gut microbiota typical of other omnivorous primates, albeit with an especially high abundance of *Bifidobacterium*. Similar to previous work in humans and other primates, we found strong effects of both developmental transitions and diet on gut microbial composition. Strikingly, baboon gut microbiota appeared to be highly dynamic such that samples collected from the same individual only a few days apart were as different from each other as samples collected over 10 years apart. Despite the dynamic nature of baboon gut microbiota, we identified a set of core taxa that is common among primates, supporting the hypothesis that microbiota codiversify with their host species. Our analysis identified two tentative enterotypes in adult baboons that differ from those of humans and chimpanzees.

Received 28 July, 2014; revised 18 March, 2015; accepted 18 March, 2015. For correspondence. *E-mail earchie@nd.edu; Tel. (574) 631 0178; Fax (574) 631 8149. **E-mail mw4yv@virginia.edu; Tel. (434) 924 4518; Fax (434) 982 5626. †These authors contributed equally to the manuscript.

Introduction

Vertebrate gut microbiota play important roles in host biology, including immune regulation, energy acquisition, vitamin synthesis and disease risk (Turnbaugh *et al.*, 2006; Hooper *et al.*, 2012; Bengmark, 2013; Morgan *et al.*, 2013). There is mounting evidence that variation in the functions of gut microbiota is mediated by variation in gut microbial composition both within and between hosts (Turnbaugh *et al.*, 2009; Greenblum *et al.*, 2011; Hooper *et al.*, 2012; Bengmark, 2013; Iida *et al.*, 2013; Karlsson *et al.*, 2013; Koeth *et al.*, 2013; Markle *et al.*, 2013; Viaud *et al.*, 2013). However, to date, most such evidence comes from research on humans and captive animal models, leaving large gaps in our understanding of the forces that shape gut microbial composition in wild vertebrates, both between individuals and within the same individual over time. Filling these gaps is especially important for wild primates, in part because such information helps reveal which of the forces that shape human gut microbiota are common across primates, and which are unique to humans and perhaps a consequence of modern human lifestyles (Yildirim *et al.*, 2010; Degnan *et al.*, 2012; Amato, 2013; Moeller *et al.*, 2014).

To help address these gaps, we used cross-sectional and longitudinal sampling to characterize distal gut bacterial communities over an unusually long, 13 year time span in a well-studied population of wild baboons. Baboons provide an especially relevant comparative system for understanding variation in human gut microbiota because of their relatively close evolutionary relationship to humans, and because baboons lead a terrestrial, savannah-dwelling lifestyle that is thought to resemble the ecology of early humans (DeVore and Washburn, 1963; Codron *et al.*, 2008; Sponheimer *et al.*, 2013). Specifically, we worked with the Amboseli Baboon Research Project (ABRP) in Kenya (Alberts and Altmann, 2012), where longitudinal, individual-based research on demography, developmental milestones, social relationships, diet and climate provided an especially rich context within which to understand individual-level variation in the

Table 1. Sample size information including the number of individuals and fecal samples used in analyses of the dataset rarefied to 1500 reads.

Individual	Sex	Number of fecal samples	Range of years samples were collected	Age range or age at time of sample collection (years)
BEAM	M	10	1994–2001	5.95–13.16
DUNLIN	F	10	1996–1999	0.72–3.69
OKOT	M	10	1996–1998	1.32–2.81
LEBANON	M	8	1997–2009	0.17–12.16
OCEAN	M	8	1997–2000	0.6–3.78
VIXEN	F	8	1994–1998	17.06–20.84
DRONGO	F	7	1996–2009	6.99–19.88
ECHO	F	7	1995–2001	3.43–9.39
VANGA	M	7	1995–2001	2.54–9.31
GOLON	M	6	1996–1999	17.76–20.63
LAWYER	M	6	2001–2001	1.12–1.98
OXYGEN	F	6	2000–2001	1.05–2.43
HONEY	F	3	1999–2000	1.85–3.13
AMIGO	M	1	1998	15.07
CABANA	F	1	1999	0.53
CEDAR	M	1	1998	2.86
DYNAMO	M	1	1998	0.94
HEKO	F	1	1997	14.27
LADHA	F	1	1998	4.57
LARK	F	1	1997	9.71
LAZA	F	1	1998	6.9
PLATO	M	1	1998	8.6
VOGUE	F	1	1998	1.08
VORTEX	F	1	1997	10.32

structure of gut microbial communities. Our main objectives were to: (i) characterize the basic structure of gut microbiota in wild baboons, (ii) gain a multivariate understanding of the relative importance of development, social relationships, diet and climate in predicting gut microbial community structure, (iii) understand patterns of longitudinal change in baboon gut microbial communities and (iv) test whether gut microbiota in wild baboons contain a core set of microbial taxa and enterotypes. Throughout, we discuss our results in the context of what is known about human and other primate gut microbiota.

Results and discussion

General patterns in the baboon gut microbial profile

We analysed distal gut microbial composition using 107 fecal samples from 24 baboons collected between 1994 and 2009 (Table 1). For 13 baboons, we analysed multiple samples (range = 3–10 samples per baboon; time span between longitudinal samples ranged from 2 days to 13 years). From these 107 samples, we generated 358 428 high-quality 16S rRNA reads, yielding an average of 3350 reads per sample and 7201 total operational taxonomic units (OTUs) using a 97% identity cut-off. This dataset was rarefied to 1500 ($n = 107$ samples from 24 baboons; Table 1) and 3000 reads per sample ($n = 54$ samples from 17 baboons; Table S1). The 1500- and 3000-read datasets were highly similar in microbial composition at the OTU level (Pearson correlation coefficient = 0.983, $P = 0.001$). Furthermore, Mantel tests correlating compositional dissimilarity in samples rarefied to 1500 versus 3000 reads revealed a high level of congruence between these datasets (Mantel tests for Bray–Curtis dissimilarities: $r = 0.996$, $P = 0.001$; unweighted UniFrac: $r = 0.978$, $P = 0.001$; weighted UniFrac: $r = 0.996$, $P = 0.001$). As a result, the results we present in the main text rely on the 1500-read dataset; where appropriate, we repeat analyses using the 3000-read dataset and present them as Supporting Information.

Taxonomic assignment revealed representatives from 11 bacterial phyla and 90 bacterial genera. Similar to other mammalian gut microbial communities, the four most common phyla included Firmicutes (48.8% of reads), Actinobacteria (17.2%), Bacteroidetes (7.2%) and Proteobacteria (4.1%). However, compared with humans and other primates (Ley *et al*., 2008; Yildirim *et al*., 2010), samples from wild baboons harboured a much higher percentage of Actinobacteria, of which 97.8% were assigned to the genus *Bifidobacterium*, and a relatively smaller proportion of Bacteroidetes (Fig. 1). *Bifidobacterium* is dominant in the gut flora of breastfed human infants (Turroni *et al*., 2012), where it is thought to play a role in the digestion of the complex carbohydrates in human milk. Accordingly, in humans, the percentage of *Bifidobacterium* decreases dramatically with age and it comprises only 3–6% of the adult gut flora. In baboons, grasses and other fibre-rich foods were common in the diet, and *Bifidobacterium* spp. may be important in digesting the high fibre content of these foods.

Notably, nearly one-fifth (20.8%) of reads were unclassified and potentially novel at the phylum level. These unclassified OTUs were unlikely to be sequencing artefacts because the vast majority (86.1%) appeared in more than one sample, and their distribution among samples was indistinguishable from that of classified OTUs (Supporting Information Fig. S1). Moreover, 54.0% of these unclassified OTUs were ≥ 90% identical to OTUs found in other mammalian fecal samples (Ley *et al*., 2008).

Previous studies have found that mammalian gut microbial composition is strongly associated with host diet and phylogeny (Ley *et al*., 2008; Yildirim *et al*., 2010; Hong *et al*., 2011; Degnan *et al*., 2012; Bolnick *et al*., 2014; Delsuc *et al*., 2014). Therefore, we compared the gut microbiota of our baboon samples to those of other mammals. As expected, the 13 of 14 fecal samples (one from each adult baboon) from our study clustered with other primates, especially those with omnivorous diets (Fig. 2). Samples from within the same order or diet group were significantly more similar than samples from different orders or diet groups (Supporting Information Table S2). Hence, host phylogeny and diet both seem to play domi-

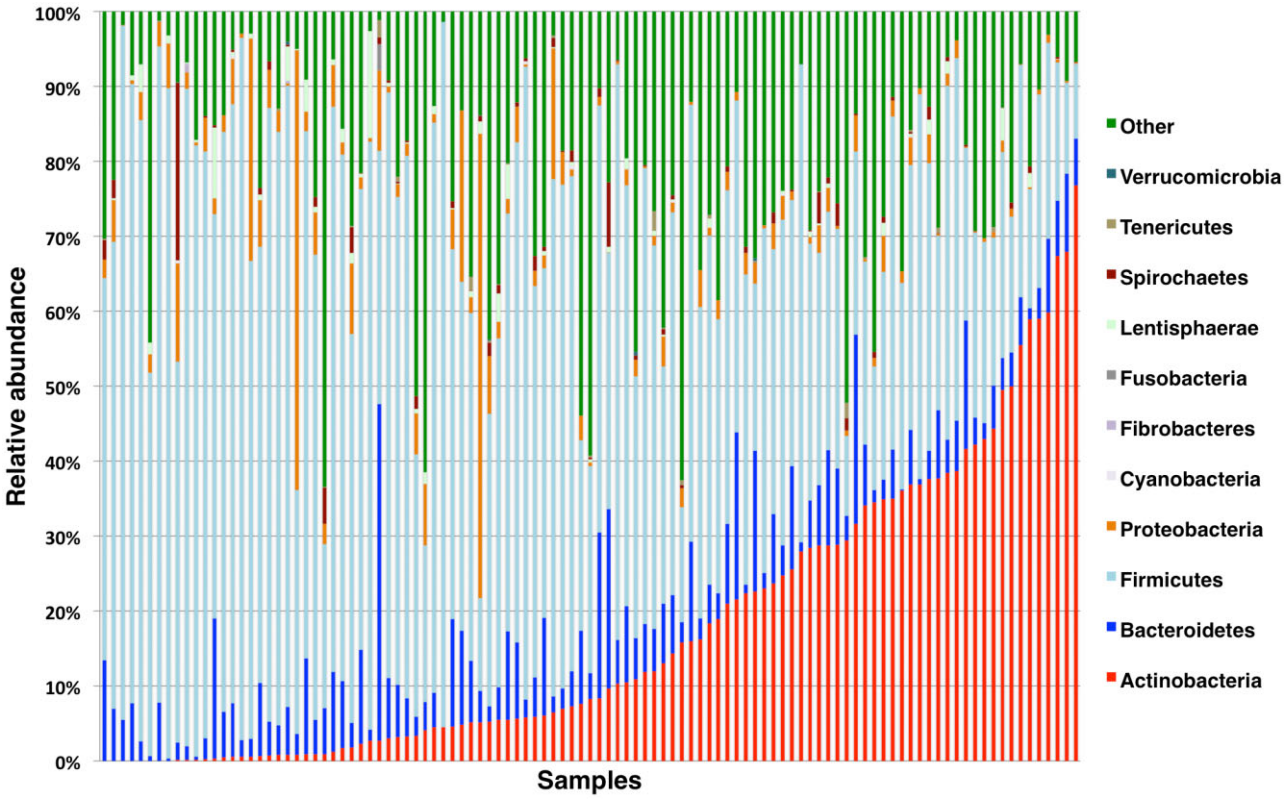

Fig. 1. Phylum level bacterial composition across 107 samples from 24 individual baboons. Each column represents one fecal sample. Y-axis values represent the relative abundance of each phylum classified by RDP classifier. Samples are sorted by the relative abundance of Actinobacteria in the sample.

nant roles in determining variation in gut microbial composition between host species. We note that these patterns were based on the most abundant bacteria in each sample because of the small number of reads in the mammal dataset (Ley *et al.*, 2008).

Juvenile baboons exhibited lower bacterial alpha diversity, but higher variance than adults

Alpha diversity is an important component of microbial diversity in the gut, especially in the context of microbiota development and pathogen resistance (Dillon *et al.*, 2005; McKenna *et al.*, 2008; Degnan *et al.*, 2012; Flores *et al.*, 2012; Yatsunenko *et al.*, 2012; Ahn *et al.*, 2013). Furthermore, given that some of our samples were collected more than 15 years prior to analysis (Table 1), we were concerned that DNA degradation might affect microbial alpha diversity in our samples, and hence our ability to characterize gut microbial composition. However, we found no evidence that older samples exhibited lower alpha diversity than younger samples (linear mixed models with sample age in years as a fixed effect and host identity as a random effect: species richness: $\beta = -1.37$, $P = 0.76$; Shannon's H: $\beta = 0.004$, $P = 0.89$; Chao1: $\beta = -4.14$, $P = 0.63$; Faith's phylogenetic diversity: $\beta = -0.19$, $P = 0.28$).

In addition to sample age, we tested several other predictors of gut microbial alpha diversity, including host age, sex, rainfall in the 30 days prior to sample collection, current social group, natal social group, current social group size, host diet composition, host diet alpha diversity, adult social rank, and for adult females only, reproductive state (as pregnant, lactating or ovarian cycling). Because host identity significantly predicted variation in alpha diversity for two of the four measures (ANOVA; species richness: $F_{(12,83)} = 2.03$, $P = 0.031$; Shannon's H: $F_{(12,83)} = 2.78$, $P = 0.003$; Chao1: $F_{(12,83)} = 1.39$, $P = 0.187$; Faith's phylogenetic diversity: $F_{(12,83)} = 1.72$, $P = 0.078$), individual identity was included as a random effect in all linear mixed models. In prior studies on humans and chimpanzees, age was a primary predictor of gut microbial alpha diversity (Degnan *et al.*, 2012; Yatsunenko *et al.*, 2012). However, in our dataset, neither age nor any other fixed effects predicted any of the four measures of alpha diversity in linear mixed models. However, when we divided samples into juveniles and adults rather than testing age as a continuous variable, we found that adults had greater species richness (Wilcoxon rank-sum test;

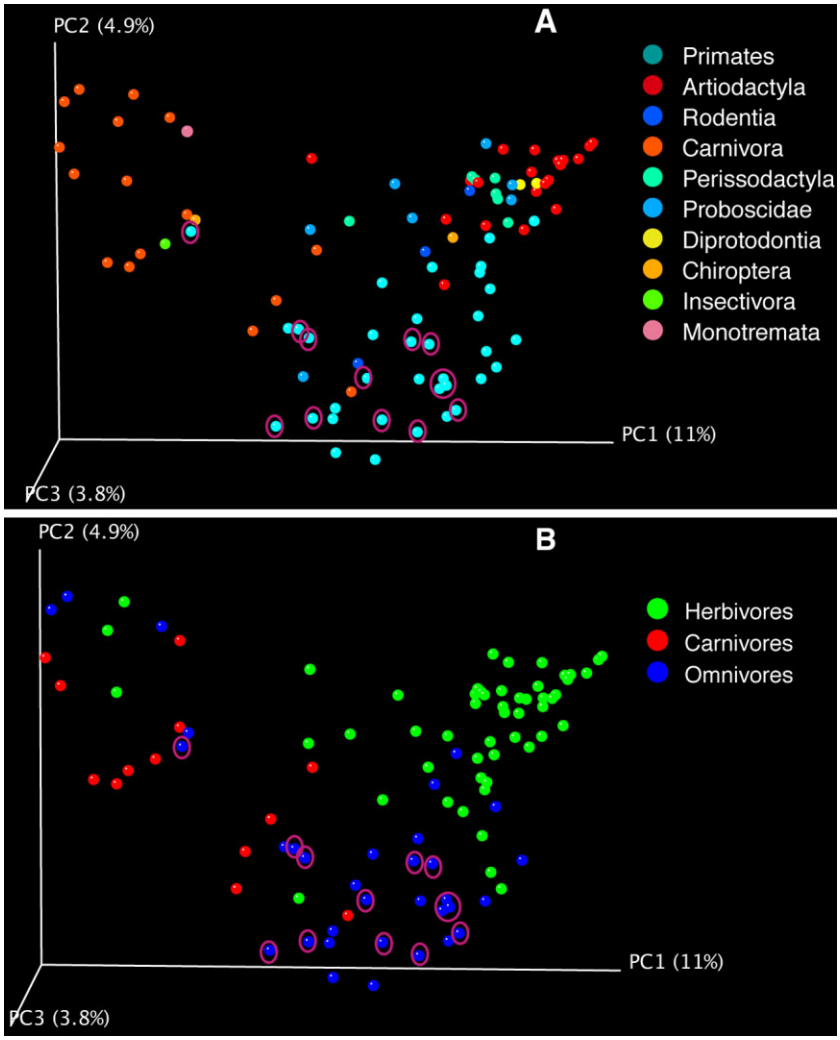

Fig. 2. PCoA analysis of the weighted UniFrac dissimilarities comparing gut microbiota of baboons to other mammals. Each point corresponds to a sample coloured by (A) host taxonomy and (B) host diet type. Baboon samples are circled in red; the 14 baboon samples were drawn at random representing one each from the 14 adult individuals (AMIGO, BEAM, DRONGO, ECHO, GOLON, HEKO, LADHA, LAZA, LARK, LEBANON, PLATO, VANGA, VIXEN, VORTEX) included in our dataset. The percentage of the variation explained by the plotted principal coordinates is indicated on the axes.

W = 1723.5, $P = 0.049$) and Shannon's H (Wilcoxon rank-sum test; W = 1783, $P = 0.019$) than juveniles. Additionally, similar to one prior study in humans (Yatsunenko *et al*., 2012), we found that infants and juveniles exhibited significantly higher variance in Shannon's H than adult baboons (Fig. 3; Brown–Forsythe test; $F_{(1,\ 104.876)} = 6.988$, $P = 0.009$). Taken together, these results indicate that gut microbiota may be less diverse and less stable in young baboons as compared with adults, suggesting that the transition to adulthood marks a developmental milestone in the microbiota.

Variation in microbial composition was best explained by host age, diet and rainfall

We tested whether several host traits and environmental factors were associated with variation in baboon gut microbial composition, including host identity, host age, host sex, rainfall in the 30 days prior to sample collection, current social group, natal social group and group size. To

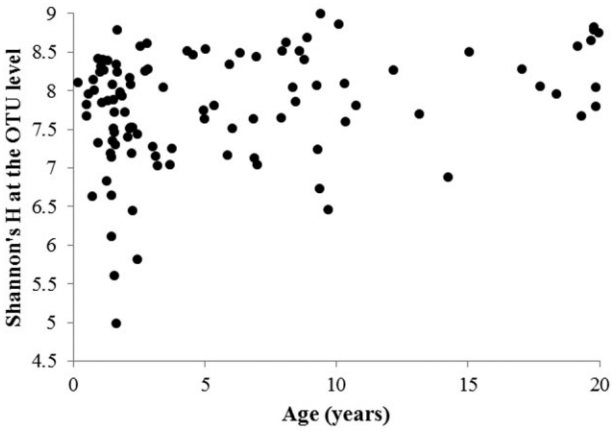

Fig. 3. The relationship between baboon age (in years) and gut microbiota alpha diversity as measured by OTU Shannon's H. Infant and juvenile baboons had higher variance in Shannon's H than adults (Brown–Forsyth test $F_{(1,\ 104.876)} = 6.988$, $P = 0.009$).

test these factors, we first performed an exploratory principal coordinates analysis (PCoA) on weighted UniFrac dissimilarities. Overall, 37.5% of the global variation was explained by the first three principal coordinates (PC1 = 21%, PC2 = 9.3%, PC3 = 7.2%). Visual inspection revealed no obvious clustering patterns by any of our predictor variables (Fig. S2A–E).

To further test which factors best explained variation in baboon gut microbial composition, we carried out canonical correspondence analysis (CCA) (Palmer, 1993) at the phylum, genus and OTU levels (Table 2). For the main dataset ($n = 107$ samples), we again tested the effects of host identity, host age, sex, rainfall and aspects of social group membership. Interestingly, no factors explained significant variation at the OTU level, perhaps because closely related species are often ecologically interchangeable (Harvey and Pagel, 1991), and ecological patterns in bacterial communities may be more apparent at higher taxonomic levels. In support, some recent studies have found ecological coherence among higher bacterial taxonomic ranks (Fierer *et al.*, 2007; Lozupone and Knight, 2007; von Mering *et al.*, 2007; Fulthorpe *et al.*, 2008; Pointing *et al.*, 2009; Philippot *et al.*, 2010; Koeppel and Wu, 2012). Indeed, we were able to explain significant shifts in high-level taxa associated with changes in environment. Specifically, rainfall and age predicted significant variation at both the phylum level (Table 2: rainfall, $P = 0.02$; age, $P = 0.02$) and the genus level (rainfall, $P = 0.01$; age, $P = 0.02$). Interestingly, while age predicted beta diversity, when baboon infants, juveniles and adults were considered separately, age was no longer significant, suggesting that either the subsets of samples do not have enough statistical power or developmental transitions are more important than age *per se* (Table 2). Host sex was significant when we considered infants alone, perhaps due to differences in maternal care as a function of infant sex (Nguyen *et al.*, 2012). These sex differences seem to disappear in adulthood, however.

While some prior studies have found evidence for social group membership on gut microbial composition (Degnan *et al.*, 2012; Yatsunenko *et al.*, 2012), including in our own population (Tung *et al.*, in press), the wide temporal distribution of samples in our dataset probably made it difficult to detect such effects. We found no other physiological or social effects on gut microbial composition, including female reproductive state, social group size, or male or female dominance rank.

The effects of rainfall on microbiota may be linked to seasonal changes in either diet or drinking water availability. To test the specific effects of diet, we conducted a second CCA using only the subset of 76 individuals (excluding infants) for which we had data on the time spent foraging on different food types. In this new model, we found several effects of diet (Table 2; Table S4). First,

Table 2. CCA analysis of environment and host traits that predicted variation in gut microbial community composition rarefied to a level of 1500 reads.

Dataset	Number of samples	Factors we attempted to include in the model	Significant factors at		
			Phylum level	Genus level	OTU level
Main dataset	107	Age, rainfall, sex, individual ID, social group, natal social group, group size	Rainfall ($P = 0.02$), age ($P = 0.01$)	Rainfall ($P = 0.02$), age ($P = 0.02$)	None
Subset with diet information	76	Age, rainfall, sex, diet diversity (richness, Shannon's H or PCoA axis), individual ID	Rainfall ($P = 0.02$), age ($P = 0.04$), diet Shannon's H ($P = 0.05$) or diet PC1 ($P = 0.02$)	Rainfall ($P = 0.03$), age ($P = 0.05$), diet Shannon's H ($P = 0.02$) or diet PC1 ($P = 0.02$)	None
Infant	22	Age, rainfall, sex, individual ID	Sex ($P = 0.04$)	Sex ($P = 0.03$)	None
Juvenile	38	Age, rainfall, sex, individual ID	None	None	None
Infant/juvenile	60	Age, rainfall, sex, suckle status, individual ID	None	None	None
Adult male with rank information	21	Age, rainfall, adult male rank, natal social group, individual ID	Rainfall ($P = 0.05$)	Rainfall ($P = 0.03$)	None
Adult female with rank information	24	Age, rainfall, adult female rank, reproductive status, individual ID	Rainfall ($P = 0.14$)	Rainfall ($P = 0.02$)	None

Table 3. Best-supported generalized linear mixed models (Poisson link) explaining variation in abundance of the four most common bacteria phyla for the main dataset ($n = 107$ samples). Host identity is modelled as a random effect.

Bacteria phylum	Fixed effects	Estimate	SE	Z	*P*-value
Actinobacteria	Age	−0.024	0.004	−6.599	<0.001
	Rainfall	−0.003	0.0001	−17.897	<0.001
Bacteroidetes	Age	−0.027	0.005	−4.918	<0.001
Firmicutes	Age	0.008	0.002	4.44	<0.001
	Rainfall	0.003	<0.0001	37.21	<0.001
Proteobacteria	Age	−0.0299	0.008	−3.69	<0.001

diet alpha diversity (Shannon's H for diet components) explained significant variation at both the phylum ($P = 0.02$) and genus levels ($P = 0.05$), while diet richness (the number of distinct food types; Table S3) did not, suggesting that dietary evenness rather than a high number of dietary components is important to the gut microbial composition. This pattern runs counter to that seen in Bolnick *et al*. (2014), which found that dietary richness rather than evenness predicted gut microbial diversity in fish. Second, the dietary tradeoff between the proportion of time spent consuming grass versus fruit in the diet (diet PC1; see Experimental Procedures; Fig. S3) was significantly associated with microbial composition at both phylum level ($P = 0.02$) and genus level ($P = 0.02$).

To assess the influence of sequencing depth on our results, we repeated the CCA using the smaller dataset rarefied to 3000 reads ($n = 54$ samples; diet information on $n = 38$ samples). We obtained best models similar to those of 1500-read dataset, although none of the factors were significant, probably as a result of a loss of statistical power (Table S4).

Finally, to identify which of the four most common bacterial phyla were associated with differences in host age, rainfall and diet, we performed generalized linear mixed models with a Poisson link and host identity as a random effect. We found that samples collected in rainier periods harboured a higher proportion of Firmicutes, but less Actinobacteria than samples from drier months (Table 3). In terms of host age (measured as a continuous variable), younger animals harboured relatively more Actinobacteria, Bacteroidetes and Proteobacteria but less Firmicutes than older animals, perhaps due to differences in milk consumption or disease susceptibility in animals of different ages. For the subset of samples with diet information, gut microbiota from groups that consumed relatively more fruits and less grass harboured higher levels of Actinobacteria and Proteobacteria and lower levels of Firmicutes and Bacteroidetes than groups consuming low fruits (diet PC1, Table S5). Furthermore, the addition of rainfall significantly improved models with diet factors, indicating that the effects of rainfall are not solely driven by seasonal changes in diet. During the dry season, the baboons drink from small, highly concentrated and qualitatively dirty water holes whereas during rainy months they obtain most of their water from seemingly cleaner, transient rain puddles, which may have consequences for gut microbiota.

Longitudinal sampling reveals that baboon gut microbiota are highly dynamic

Prior research on humans and chimpanzees has found that individuals contain distinct gut microbiota, and that samples from the same individual, even those collected over a year apart, are more similar to each other than they are to samples collected from different hosts over the same time period (Turnbaugh *et al*., 2009; Caporaso *et al*., 2011; Degnan *et al*., 2012; David *et al*., 2014). However, we found no evidence for such effects in our study subjects. For instance, in the CCA analyses described above, we never observed a significant effect of individual identity at any taxonomic level. Similarly, samples from the same individual were as different from each other as they were from samples collected from different individuals in the same developmental stage (mean ± SE weighted UniFrac dissimilarity: between samples from the same individual = 0.342 ± 0.008; between samples from different individuals at the same stage = 0.345 ± 0.002, $P = 0.35$). However, we note that the sequence depth in our study is lower than those of the previous studies.

This high degree of dynamism in baboon gut microbiota can be visualized by plotting pairwise weighted UniFrac dissimilarities between samples of the same individual as a function of time between sampling points (Fig. 4). Microbial communities sampled from the same individual a few days apart were almost as different from each other as samples collected several years apart. Only 1 of 13 individuals with >3 samples displayed a significant relationship between sampling time interval and microbiota dissimilarity (Table S6). It is unclear why baboon gut microbiota appeared to be so dynamic. One possible explanation is that seasonal variation in the baboons' diets selects for different gut microbial compositions at different times of year, as the availability of fruits, seeds and vegetation fluctuates with seasonal patterns in plant reproduction. However, such seasonal variation is unlikely to explain turnover on the scale of days or weeks during which baboon diets are more consistent. Another expla-

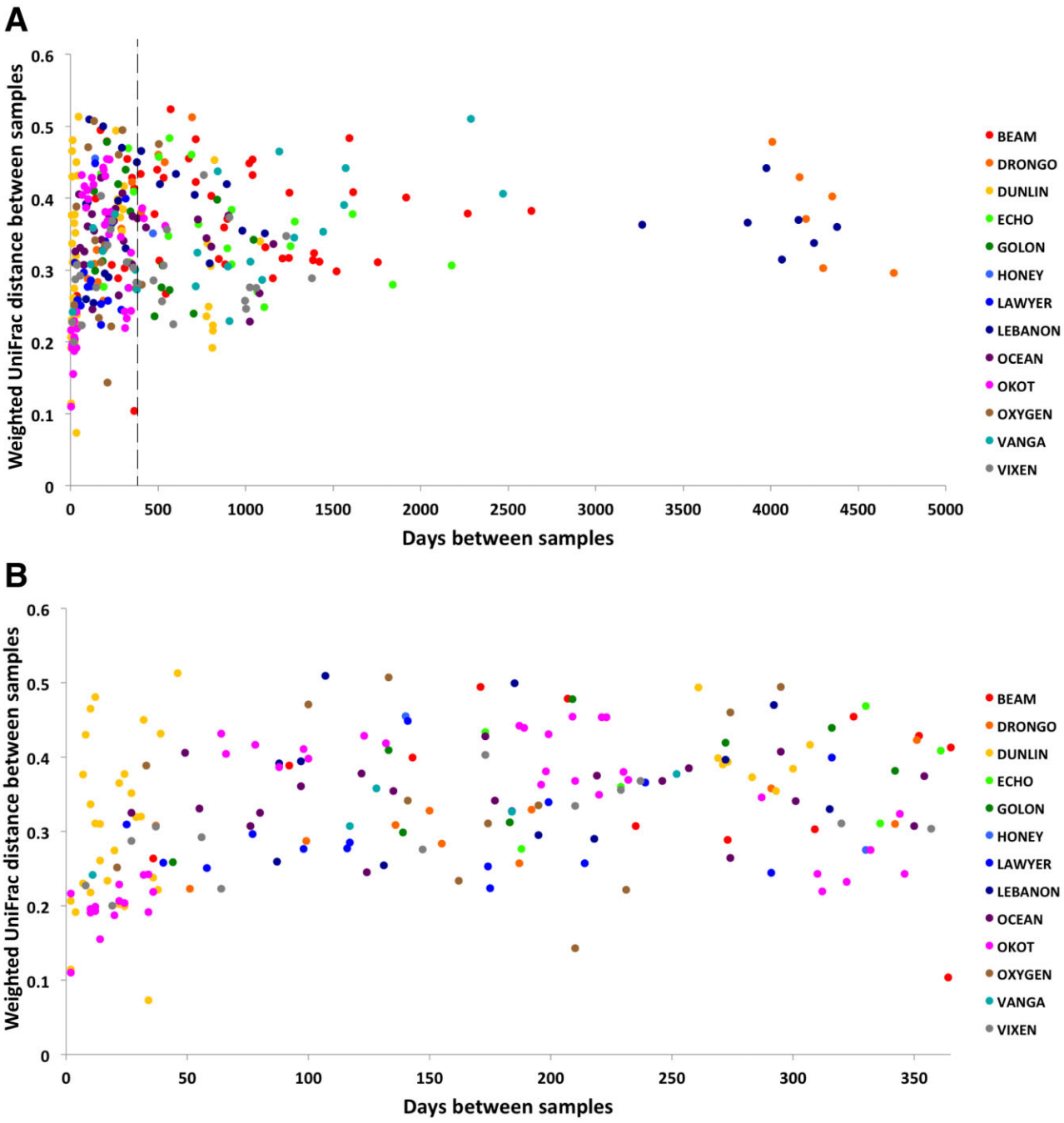

Fig. 4. A time-decay plot of gut microbiota dissimilarity. Each dot represents a comparison between two samples of the same baboon collected at different time points, with different marker colours representing different baboons. X-axis represents the time span (in days) between the sample collection times. Y-axis represents the weighted UniFrac dissimilarity. (A) 5000 days. (B) 365 days. The dotted line in panel A marks 365 days. Correlation between sampling time span and microbiota weighted UniFrac dissimilarity for each individual is summarized in Table S6.

nation is that wild baboons live in microbially heterogeneous environments, regularly walking through fecal deposits of other species, drinking from waterholes that contain fecal material from livestock and wild mammals, and pulling plants from the ground with their mouths. This could lead to higher turnover in gut microbial species.

Core gut microbiota

The high degree of inter- and intra-individual variations in baboon microbiota raises the question of whether baboon gut microbiota contain a set of core microbial taxa, as is observed in humans (Tap *et al.*, 2009; Martínez *et al.*,

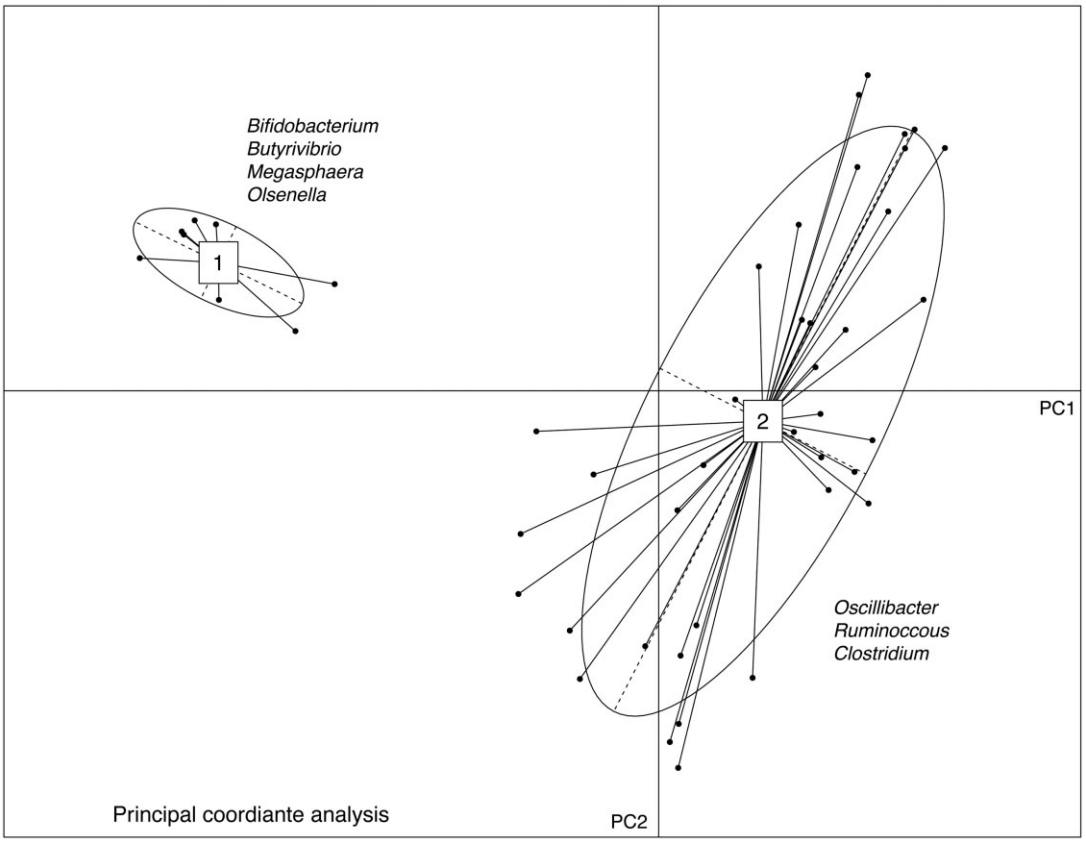

Fig. 5. PCoA visualization of baboon enterotypes (ellipses) identified by PAM clustering. Black dots represent abundance distributions of bacterial genera from an individual host and numbered white rectangles mark the centre of each enterotype. Bacterial genera that mainly contribute to each enterotype are listed.

2013). We defined core taxa as taxa present in more than 90% of our 107 samples, assigned at the lowest possible taxonomic level. Despite the dynamic nature of baboon gut microbiota, we found evidence for some core taxa: three at the family level (Lachnospiraceae, Peptostreptococcaceae and Veillonellaceae) and four at the genus level (*Faecalibacterium*, *Prevotella*, *Bifidobacterium* and *Oscillibacter*).

To investigate how core microbiota have changed with host phylogeny, we attempted to identify core gut microbial members of the 57 mammalian species (89 individuals) used in Ley *et al*. (2008). Given the large variation in mammalian genomes, diets and lifestyles, it is not surprising that we did not find any core taxon below the phylum level that is shared by all mammals. However, when we limited our scope to primates alone, we found two family-level (Ruminococcaceae and Lachnospiraceae) and one genus-level (*Prevotella*) core taxa. Since these taxa are present in most primates surveyed, these core taxa were most likely present in the last common ancestor of primates, suggesting they might be important in the codiversification of the gut microbiota and the primate hosts.

Enterotypes in baboons

Previous studies reported that humans and chimpanzees harbour compositionally similar gut enterotypes (Arumugam *et al*., 2011; Moeller *et al*., 2012). To test for the presence of enterotypes in our subjects, we clustered gut microbiota for the 47 (of 107) samples collected from sexually mature, adult baboons by applying the partitioning around means clustering method on the Bray–Curtis dissimilarities calculated using genus level abundances (Arumugam *et al*., 2011). Our analysis revealed an optimum of two clusters (Fig. 5, CH index: 39; average silhouette coefficient: 0.265; prediction strength: 0.79). Although the silhouette coefficient is comparable to those reported in earlier enterotype studies (Arumugam *et al*., 2011; Moeller *et al*., 2012), it would be considered low according to the thresholds proposed more recently (Koren *et al*., 2013). Therefore, the enterotypes identified in this study are tentative. The genera that contributed most significantly to each cluster were *Bifidobacterium*, *Butyrivibrio*, *Megasphaera* and *Olsenella* in enterotype 1 ($n = 9$) and *Oscillibacter* and *Ruminococcus* in enterotype 2 ($n = 38$). The relative abundances of genera in the adult

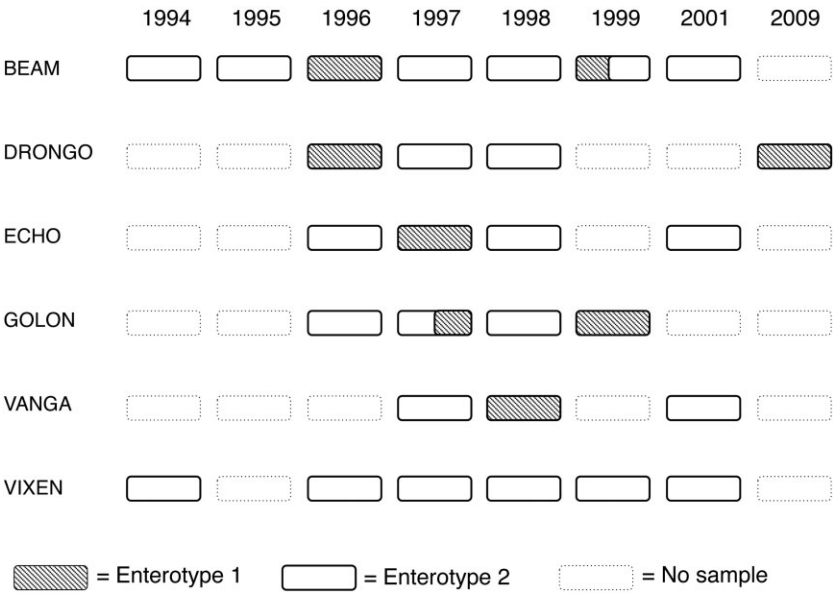

Fig. 6. Baboon enterotypes switched over time. Samples were collected from 1994 to 1999, and in 2001 and 2009, eight (of 14) adults only had one sample and therefore were not shown here. Filled rectangles: enterotype 1; unfilled rectangles: enterotype 2; rectangles with dashed line: sample missing. When an individual switched enterotypes during the middle of the year, it is represented by a hybrid rectangle (half filled and half unfilled).

samples are listed in Table S7. These two enterotypes differ from those of humans and chimpanzees. One parsimonious explanation is that enterotypes in humans and chimpanzees may have evolved since the split between apes and old world monkeys ~30 million years ago.

Previous studies found that enterotypes can be replaced within 1 year in chimpanzees (Moeller *et al*., 2012) and within 1 week in wild mice housed in captivity (Wang *et al*., 2014). We observed enterotype replacements for most baboons when we assessed the samples of the same individual at multiple time points (Fig. 6). Enterotypes changed rapidly in baboons, sometimes switching in as little as 45 days. Past studies have suggested that proportion of protein versus carbohydrates in host diet is linked to the host's enterotype (Wu *et al*., 2011; Wang *et al*., 2014). However, the baboon enterotypes that we found were not significantly associated with any factors tested, including diet diversity (richness, Shannon's H and PCoA axis), age, rainfall, host identity, season, sex or social group (Wilcoxon rank-sum test or Fisher's exact test). Consistent with the finding of Wang *et al*. (2014), we found no enterotypes at the OTU level.

Experimental procedures

Study subjects and predictors of microbiota structure

Study subjects were wild baboons living in the Amboseli Ecosystem in Kenya, a semi-arid savannah located northeast of Mt. Kilimanjaro (2°40′S, 37°15′E, 1100 m altitude). Since 1971, the baboons in this area have been studied by the ABRP (Alberts and Altmann, 2012). Several types of data are collected throughout the year on known individuals by full-time, experienced observers, two to three times per week per group. Here we describe the data collection on the specific predictor variables we tested; sample sizes vary somewhat for each predictor variable because some data were only available for or relevant to some individuals and samples.

Sex, age and developmental stage. Baboons are sexually dimorphic, and sex is known from conspicuous external genital morphology. Ages were known to within a few days for 20 of 24 animals in our main dataset ($n = 107$). The remaining four individuals immigrated into the population after birth and their ages were estimated using well-defined metrics and comparison to known-age animals (Alberts and Altmann, 1995). These four animals had birth dates estimated to be accurate within 1 year ($n = 1$), 2 years ($n = 2$) or 3 years ($n = 1$). As baboons mature, they pass through several developmental stages that may also influence the gut microbiota, including: (i) infancy, during which diet includes both milk and foods from the environment (from birth to 1.5 years; $n = 22$ fecal samples from nine individuals), (ii) the juvenile period, which begins post weaning (~1.5 years) and ends at sexual maturity (~4.5 years for females; ~5.4 years for males; 38 samples from 10 individuals; Onyango *et al*., 2013) and (iii) adulthood, defined by the onset of sexual maturity ($n = 47$ samples from 14 individuals).

Diet. In addition to the dietary changes associated with the transition from the infant to the juvenile stage, we tested the effect of diet composition on gut microbiota. Specifically, for a subset of subjects ($n = 76$ fecal samples from 10 juveniles and 14 adults), we estimated diet composition using behavioural sampling on all the juvenile and adult female members of the social group in the 30 days prior to sample collection. Social group members consume similar foods in roughly similar proportions; hence, group-level diets provide suitable estimates of the composition of individual diets. The baboons' diets included 11 food categories: (i) grass, including corms, blades and grass seed heads, (ii) gum from the bark of *Acacia xanthophloea*, (iii) leaves from herbaceous plants or

trees, (iv) fruits, (v) blossoms, (vi) bark from *A. xanthophloea*, (vii) fresh, green seed pods from *Acacia* spp., (viii) dried seeds from *Acacia* spp., (ix) invertebrates, (x) liquid from or items in or under dung and (xi) unknown unidentifiable items (Table S3).

We used these data to characterize diet alpha and beta diversity, noting that time spent feeding is not always proportional to the amount of food ingested. Diet alpha diversity was measured as both the total number of foods (diet richness) and dietary Shannon's H using the vegan package in R (Oksanen *et al*., 2013). Diet beta diversity was estimated via PCoA on a Bray–Curtis dissimilarity matrix of diet composition using vegan. The first three axes of the PCoA explained 80% of the variation in the diet (PC1 = 46%, PC2 = 23%, PC3 = 11%); PC1 was associated with a tradeoff in relative proportions of grass (–) versus fruit (+); PC2 was associated with the proportion of invertebrates (–) versus fruit (+); and PC3 was associated with the proportion of the diet attributed to the 'unknown' category (–) (Figs S3–S5).

Rainfall. Semi-arid savannah ecosystems are characterized by highly seasonal patterns of rain that may affect diet as well as bacterial exposures through sources of drinking water. Each year, Amboseli experiences a 5 month dry season (June–October) during which no rain falls. In the remaining 7 months (November–May), the ecosystem receives highly variable amounts of rain (yearly average = 350 mm; range = 141–757 mm) (Alberts *et al*., 2005). The effects of rainfall were assessed by summing the total amount of rain that fell in the 30 days prior to sample collection.

Social relationships. One prior study has linked aspects of primate social group membership to microbial composition (Degnan *et al*., 2012). We tested three aspects of social group: (i) the identity of the animal's social group on the day of sample collection, (ii) the size of the animal's social group on the day of sample collection, as the number of members and (iii) the identity of the animal's natal social group, if known (in baboons, males are the dispersing sex and the current group of an adult male invariably differs from his natal group).

In addition, in baboons, dominance rank has been linked to physiology and health (Sapolsky and Altmann, 1991; Alberts *et al*., 1992; Gesquiere *et al*., 2011); hence, we also tested for associations between microbial composition and dominance rank. Rank was assigned monthly by observing dyadic agonistic interactions and assigning winners and losers based on the outcome. These wins and losses were used to construct dominance matrices, resulting in an ordinal rank for each member of the group (Hausfater, 1975).

Adult female reproductive status. Prior research has shown that reproductive cycle changes in human women can influence gut microbial composition (Koren *et al*., 2012). To test this idea, samples from adult females (24 samples from nine individuals) were assigned to one of three reproductive states (ovarian cycling, pregnant or lactating) using previously published and well-defined criteria (Altmann, 1973; Wildt *et al*., 1977; Shaikh *et al*., 1982; Beehner *et al*., 2006; Gesquiere *et al*., 2007).

Sample collection, DNA extraction and 16S rRNA sequencing

Gut microbiota were characterized from fecal samples. Samples for this analysis spanned from 1994 to 2009 and included 144 samples from 32 individuals. Samples were chosen to provide both cross-sectional and longitudinal information, including multiple samples from a subset of 13 individuals. All fecal samples were collected within a few minutes of defecation, after which the sample was mixed and preserved in 95% ethanol. Samples were stored in an evaporative cooling structure (approximate daily maximum temperature of 25°C) until shipment to the US, where they were stored at −80°C. DNA was extracted from each sample by bead beating and phenol-chloroform extraction. For each DNA extract, the V1–V3 hypervariable regions of the 16S rRNA gene were PCR amplified and pyrosequenced as described previously (Ren *et al*., 2013) on a 454 Life Science Genome Sequencer FLX platform (University of Virginia Department of Biology Genome Core Facility).

Sequence processing, quality control and OTU classification

Sequencing reads were processed using the QIIME pipeline (Caporaso *et al*., 2010). Each read was assigned to a sample by a bar code and then filtered to remove reads with: (i) lengths less than 200 base pairs or greater than 550 base pairs, (ii) average Phred equivalent quality scores less than 25, (iii) improper primer or bar code sequences or (iv) the presence of ambiguous base calls. Eukaryotic, mitochondrial sequences were removed by BLAST search against the SILVA database (Quast *et al*., 2013). Chloroplast sequences were removed using the Ribosomal Database Project (RDP) classifier (Wang *et al*., 2007). Chimeric sequences were identified using UCHIME with the de novo detection algorithm and default parameters (Edgar *et al*., 2011). Read filtering removed 17.8% of the total reads, with the majority removed as chimeras. The remaining reads were clustered to 97% OTUs by cdhit (Fu *et al*., 2012). To further remove potential sequencing artefacts, we excluded any OTU with ≤5 reads across all samples. The most abundant sequence of each OTU was chosen as the representative sequence and classified using the RDP classifier.

Statistical analyses

Of our initial set of 144 samples, three were excluded as outliers at two or more of four measures of OTU alpha diversity (these outliers had alpha diversity values more than three times the interquartile range below the lower 25% percentile). Three additional samples were removed as outliers during initial beta diversity analyses. During rarefaction, 31 and 84 samples were removed due to insufficient reads, leaving 107 samples (Table 1) and 54 samples (Table S1) for 1500- and 3000-read datasets respectively. Up to 1500- and 3000-read datasets were compared using the Pearson correlation coefficient and Mantel tests implemented in QIIME pipeline. The two datasets were found to be highly similar (see *Results and discussion*); hence, in the main text we present the results of the 1500-read dataset.

Comparison to other mammals. To understand how gut microbiota from the Amboseli baboons are compared with other primates and mammals, we conducted PCoA on unweighted UniFrac matrix to compare one randomly selected sample from each adult baboon ($n = 14$) to 89 individual mammals of 57 species surveyed by Ley *et al*. (2008). Only the V1–V3 regions of the 16S rRNA gene were compared. Samples were rarefied to 140 reads due to the small number of reads in the mammal dataset (Ley *et al*., 2008).

Testing predictors of gut microbial alpha diversity. To test which factors best predicted microbial alpha diversity, we constructed linear mixed models of four measures of OTU alpha diversity: OTU richness (i.e. the number of distinct OTUs in a sample), Shannon's H, Chao1 (log transformed) and Faith's phylogenetic diversity. All models included host identity as a random factor; the best-fitting models were identified using the log likelihood criterion.

Testing predictors of gut microbial beta diversity. To investigate the predictors of gut microbial composition, we first performed exploratory PCoA, followed by hypothesis testing via CCA (Palmer, 1993). PCoA was performed on unweighted and weighted UniFrac dissimilarities calculated from the relative abundance of OTUs in each sample (Lozupone and Knight, 2005). CCA was performed on the relative abundance of taxa at the phylum, genus, and OTU level and host associated metadata using the vegan package in R (Table 2 and Table S4). For each test, the best model was selected using the log likelihood criterion, and the significance of each predictor was assessed by permutation tests. We did not correct for multiple comparisons in our CCAs because of the nested nature of these analyses. It would be overly conservative to account for multiple comparisons because tests of many of the factors (e.g. age, rainfall) are not independent across models. To test which factors predicted the relative abundance of the four most common bacteria phyla, we constructed generalized linear mixed models with host identity as a random factor, and a Poisson-distributed error structure. The best-fitting models were chosen using the log likelihood criterion.

Core microbiota. Since closely related bacterial taxa are sometimes ecologically interchangeable (Harvey and Pagel, 1991), it may be useful to consider phylogenetic relationships when identifying core taxa. Core OTUs were identified using a tree-based algorithm and were defined as those OTUs that belonged to the same lineage and occurred in more than 90% of the samples. We identified core OTUs in both baboon and other mammalian (Ley *et al*., 2008) gut microbiota.

Enterotype analyses. We performed the enterotype analysis of the adult baboon gut microbiota as described in Arumugam *et al*. (2011), which used Calinski–Harabasz index as an indicator of optimal clustering. In addition, we calculated the silhouette coefficient and prediction strength using the methods suggested by Koren *et al*. (2013) in R (packages: cluster, clusterSim, fpc). Genera that mainly contribute to each enterotype were identified with Randomforest implemented in QIIME. We consider a genus as a main contributor if its removal increases >15% overall estimated generalization error (an estimate of how much error the classifier would have on a novel dataset). Associations between enterotype and age, rainfall or diet PC axis were tested with Wilcoxon rank-sum test. Association between enterotype and host identity, season, sex or social group was tested with Fisher's exact test.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. The distribution patterns of the unclassified and classified OTU at the phylum level across the samples. OTUs have been sorted into bins based on their prevalence in the samples (X-axis). Y-axis is the count of OTUs in each bin.

Fig. S2. PCoA analysis of the weighted UniFrac dissimilarities comparing baboon gut microbiota. Each point corresponds to a sample coloured by (A) individual identity, (B) sex, (C) age class and (D) season, (E) diet group. Baboons with diet composition information ($n = 76$) were divided into three diet groups by the relative abundance of grass, fruit and invertebrate in their diet guided by the PCoA plot of diet Bray–Curtis dissimilarity: 1. Fruit, if fruit percentage is >=20%; 2. Invertebrate, if there is invertebrate in diet; 3. Grass, if grass percentage is >=70%.

Fig. S3. The first principal coordinate of variation in diet composition (diet PC1) as a function of the 11 primary diet components (Table S3). Blue lines represent lowess regression fits. PC1 explained 46% of the variation in diet composition and is associated with a tradeoff in the proportion of grass (–) versus fruit (+) in the baboons' diets.

Fig. S4. The second principal coordinate of variation in diet composition (diet PC2) as a function of the 11 primary diet components (Table S3). Blue lines represent lowess regression fits. PC1 explained 23% of the variation in diet composition and is associated with a tradeoff in proportion of insects (–) versus fruit (+) in the baboons' diets.

Fig. S5. The third principal coordinate of variation in diet composition (diet PC3) as a function of the 11 primary diet components (Table S3). Blue lines represent lowess regression fits. PC1 explained 11% of the variation in diet composition and is associated with the proportion of the diet attributed to 'unknown' categories (–).

Table S1. Sample size information, including the number of individuals and fecal samples used in analyses of the dataset rarefied to 3000 reads.

Table S2. Unweighted UniFrac dissimilarity comparison within and between mammalian orders or diet types.

Table S3. Diet items included in each diet category.

Table S4. CCA analysis of environment and host factors for the 3000-read dataset.

Table S5. Best-supported generalized linear mixed model (Poisson link) explaining variation in abundance of the four most common bacteria phyla for the subset of 76 samples with diet data. Individual identity is a random effect in all models.

Table S6. Mantel test of correlation between sampling time interval and microbiota weighted UniFrac dissimilarity between samples that were collected from the same individual.

Table S7. Genus level abundance table of 47 adult baboon samples used in the enterotype analysis.