Isolated Grauer's gorilla populations differ in diet and gut microbiome

Short running title: Gut microbiome and diet of Grauer's gorillas

Alice Michel[1,2], Riana Minocher[1,3], Peter-Philip Niehoff[1], Yuhong Li[1,4], Kevin Nota[5], Maya A. Gadhvi[1], Jiancheng Su[1], Neetha Iyer[2], Amy Porter[2], Urbain Ngobobo-As-Ibungu[6], Escobar Binyinyi[6], Radar Nishuli Pekeyake[7], Laura Parducci[3,8], Damien Caillaud[2], Katerina Guschanski[1,9]

[1]Animal Ecology, Department of Ecology and Genetics, Evolutionary Biology Centrum, Uppsala University, Sweden

[2]Department of Anthropology, University of California, Davis, CA, USA

[3]Department of Human behavior, Ecology and Culture, Max-Planck Institute for Evolutionary Anthropology, Leipzig, Germany

[4]Conservation Ecology Group, Groningen Institute for Evolutionary Life Sciences, University of Groningen, The Netherlands

[5]Plant Ecology, Department of Ecology and Genetics, Evolutionary Biology Centrum, Uppsala University, Sweden

[6]The Dian Fossey Gorilla Fund International, Kinshasa, DRC

[7]Institut Congolais pour la Conservation de la Nature, Kinshasa, DRC

[8]Department of Environmental Biology, Sapienza University of Rome, 00185 Rome, Italy

[9]Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, UK

Corresponding authors:

Alice Michel: aljmichel@ucdavis.edu

Katerina Guschanski: katerina.guschanski@ed.ac.uk

Abstract

The animal gut microbiome has been implicated in a number of key biological processes, ranging from digestion to behavior, and has also been suggested to facilitate local adaptation. However, studies in wild animals rarely compare multiple populations that differ ecologically, which is the level at which local adaptation may occur. Further, few studies simultaneously characterize diet and the gut microbiome from the same sample, despite the likely presence of co-dependencies. Here, we investigate the interplay between diet and gut microbiome in three geographically isolated populations of the critically endangered Grauer's gorilla, which we show to be genetically differentiated. We find population- and social group-specific dietary and gut microbial profiles and co-variation between diet and gut microbiome, despite the presence of core microbial taxa. There was no detectable effect of age, sex, or genetic relatedness on the microbiome. Diet differed considerably across populations, with the high-altitude population consuming a lower diversity of plants compared to low-altitude populations, consistent with food plant availability constraining diet. The observed pattern of covariation between diet and gut microbiome is likely a result of long-term social and ecological factors. Our study suggests that the gut microbiome is sufficiently plastic to support flexible food selection and hence contribute to local adaptation.

Keywords: Metabarcoding, 16s rRNA, *trn*L, critically endangered, genetic diversity, fecal DNA

Introduction

The ranges of many species span ecologically diverse habitats that differ in abiotic and biotic factors, leading to some degree of adaptation to the predominant local condition. Our view of how organisms adapt has recently expanded beyond natural selection acting on morphological, physiological, and behavioral traits, to also include the contribution of associated microorganisms, the microbiome (Rosenberg & Zilber-Rosenberg, 2016). In animals, the microbiome plays a critical role in key biological processes such as digestion, health, behavior (Agranyoni et al., 2021; Colston & Jackson, 2016; Davidson et al., 2020; Ley et al., 2008; Moran et al., 2019), and has even been implicated in influencing host genomic evolution (Rudman et al., 2019).

The gut microbiome is shaped by numerous factors including host evolutionary relationships, social interactions, habitat, and diet (Archie & Tung, 2015; Rojas et al., 2021; Youngblut et al., 2019). In wild animals, distinct populations living under different ecological conditions have frequently been shown to possess unique gut microbiomes (Bueno de Mesquita et al., 2021; Couch et al., 2020; Uren Webster et al., 2018). Along with spatial differences, studies often show shifts in the gut microbiome concordant with seasonal dietary changes (Baniel et al., 2021; Bergmann et al., 2015; Guo et al., 2021; Hicks et al., 2018). Such differences are expected, as microorganisms, with their large population sizes, rapid evolution, and flexible community structure, are able to react quickly to changes in environmental conditions (Koskella et al., 2017), supporting their role in local adaptation of the host (Alberdi et al., 2016). Experimental studies have used dietary and gut microbial manipulations to dissect the directionality of the diet-microbiome link. They suggest a two-way connection. On the one hand, dietary manipulations alter the composition of the gut microbiome, permitting hosts to rapidly utilize new dietary sources (Reese et al., 2021). On the other hand, the gut microbiome itself can drive dietary choice (Trevelline & Kohl, 2022). In the wild, it is possible that the microbiome may impact dietary choices by modulating host behavior, for example, by constraining the selection to similar foods even in different habitats or by promoting dispersal decisions that reduce environmental change (‘natal habitat-biased dispersal’).

73 Here, we investigate spatial variation of the gut microbiome and its potential role in local
74 dietary adaptation by jointly analyzing dietary and gut microbial diversity and composition in several
75 isolated populations of the critically endangered Grauer's gorilla (*Gorilla beringei graueri*) (Plumptre
76 et al., 2016). This gorilla subspecies is endemic to the eastern Democratic Republic of Congo (DRC).
77 Our study populations occupy the ecological extremes of the species' range, approximated here by
78 altitude (600 m above sea level [asl] and 2500 m asl). Grauer's gorillas are herbivores, consuming a
79 large diversity of plants and plant parts (Yamagiwa et al., 2005). However, due to the political
80 instability throughout their range, very little is known about ecology and diet of different populations
81 (but see van der Hoek, Binyinyi, et al., 2021; van der Hoek, Pazo, et al., 2021).
82 Using fecal DNA metabarcoding combined with host genotyping, we first investigated
83 whether isolated and genetically differentiated gorilla populations show dietary similarities. As plant
84 communities differ considerably by altitude throughout the region (Imani et al., 2016), the presence of
85 shared food taxa across populations would be indicative of restrictive dietary selection (a core
86 Grauer's gorilla diet). If such a pattern of food selection occurs at least in part via gut microbial
87 influence over host foraging, we also expect to find a conserved set of gut microbial taxa (a core
88 microbiome). In contrast, if plasticity of the gut microbiome confers dietary flexibility, potentially
89 facilitating local adaptation, we expect diet and the microbiome to differ significantly among
90 populations, with strong covariation between them and little evidence for conserved dietary and
91 microbial taxa.
92

Materials & Methods

Ethics Statement

This study was conducted in compliance with legal requirements of the DRC and the animal use policies of UC Davis. Data collection protocols were approved by Institut Congolais pour la Conservation de la Nature. Samples were collected non-invasively, without disturbing the animals.

Sample collection

Fecal samples (n=220) were opportunistically collected from Grauer's gorillas in eastern DRC between 2015 and 2018 at three sites: Kahuzi-Biega National Park (KBNP, 2.32˚S, 28.72˚E; KBNP; 2500 m asl), Nkuba Conservation Area in Walikale territory, North Kivu (NCA, 1.38˚S, 27.47˚E; NCA; 600 m asl), and Maiko National Park (MNP, 0.87˚S, 27.35˚E; MNP; 830 m asl; **Figure 1**). In KBNP, gorillas in the Chimanuka group were habituated to human presence and samples were collected from identified individuals after observing defecation. All other samples were collected from night nests without knowledge of individual identity following the two-step collection method (Nsubuga et al., 2004). Geographic location and altitude were recorded using handheld GPS for all sampling sites except for the Mankoto group in KBNP, for which this information is missing. We assigned age classes in the field based on dung diameter, as follows: infant <4cm, sharing a nest with an adult; juvenile/subadult <5cm, own nest; and adults >5cm (McNeilage et al., 2006; Schaller, 1963). For the Chimanuka group, age classes of identified individuals were known from observations.

DNA extraction

Fecal samples were exported to Uppsala University, Sweden, for molecular analysis. DNA was extracted from 50 mg of dried material using the DNeasy PowerSoil DNA Extraction Kit (Qiagen) in a dedicated primate fecal extraction laboratory. We implemented the following modifications to the manufacturer's protocol: fecal samples were incubated under shaking (500 RPM) in the C1 solution overnight at 23°C. They were then transferred into a heating block and incubated at

120 65°C for 10 minutes, followed by bead beating on a vortex at maximum speed for 1 hour at room
121 temperature. Incubation in C2 and C3 solution was on ice. We incubated the samples in C6 solution at
122 room temperature for 5 minutes before elution.
123
124 *Gorilla genotyping, individual identification, relatedness and population differentiation analyses*
125 We genotyped all 220 samples at 12 microsatellite loci (vWF, D1s550, D4s1627, D5s1457,
126 D5s1470, D6s474, D6s1056, D7s817, D8s1106, D10s1432, D14s306, and D16s2624) following the
127 two-step multiplex protocol (Arandjelovic et al., 2009) and sexed them with the amelogenin assay
128 (Bradley et al., 2001). PCR products were run on an agarose gel to confirm amplification success and
129 absence of contamination in blanks. Up to four loci were pooled, based on fluorophores and product
130 sizes, and run on the ABI GeneAnalyzer (ThermoFisher Scientific). We scored genotypes manually in
131 GeneMapper v5.0 (Chatterji & Pachter, 2006) and used Cervus v3.0.7 (Kalinowski et al., 2007) to
132 identify individuals. Samples were considered to originate from the same individual if their genotypes
133 matched at five or more loci without mismatches, with the probability of identity assuming full-sibling
134 relationship (PIDsib) less than 0.05. We manually generated consensus individual genotypes from
135 matching samples, taking into account the time and place of sample collection, and evidence about the
136 presence of other individuals from the same group.
137 We tested for deviations from Hardy-Weinberg equilibrium, heterozygote deficiency, and
138 linkage disequilibrium at each locus in GenePop v4.7.5 (Raymond & Rousset, 1995; Rousset, 2008).
139 Genetic population structure was assessed using STRUCTURE v2.3.4 (Porras-Hurtado et al., 2013)
140 with 20 independent runs for K = 1-11 (corresponding to the eleven social groups), an 100,000-
141 iteration burn-in, and data collection for 1,000,000 runs, assuming population admixture and
142 correlated allele frequencies (Falush et al., 2003). Results from different runs of K were merged in
143 CLUMPP (Jakobsson & Rosenberg, 2007; Kopelman et al., 2015), and analyzed and visualized in
144 'pophelper' in R (Francis, 2017; R Core Team, 2021). The most likely value of K was determined
145 using ΔK (Evanno et al., 2005). We used the 'adegenet' R package for Principle Component Analysis
146 (PCA) based on individual genotypes (Jombart, 2008). Population differentiation statistics F_{ST} and F'_{ST}

147 (Meirmans & Hedrick, 2011) were calculated in GenoDive v3.04 (Meirmans, 2020), and significance
148 assessed with 9999 permutations. We compared genetic relatedness between populations and social
149 groups using an AMOVA in the R package 'poppr' (Kamvar et al., 2014) and calculated pairwise
150 relatedness (*r*) between all individuals within KBNP and NCA separately in ML-Relate (Kalinowski et
151 al., 2006).
152
153 *Characterization of gorilla diet*
154 We characterized the diet of 92 unique individuals identified by genotyping (see Results),
155 from nine social groups and two lone silverbacks (**Table S1, S2**). We aimed to analyze a single nest
156 site per group, but have also included individuals from additional nest sites of the same group if they
157 were collected during the same year and season to maximize the number of studied individuals (**Table**
158 **S2**). A single sample per individual was studied. The majority of our samples were collected during
159 the dry season, but we also included some samples, social groups (Chimanuka) and one population
160 (MNP) that were collected during the rainy season (**Table S2**). We present our analyses with and
161 without these samples.
162 We amplified the P6 loop of the *trn*L chloroplast intron (Taberlet et al., 2007), a locus that has
163 been successfully used for dietary metabarcoding in primates, and for which a large database of
164 tropical plants is available (Mallott et al., 2018). We used the standard *trn*L g and h primers (**Table**
165 **S3**), tagged with 96 eight-base-pair (bp) barcodes. Each barcode differed from all others at a minimum
166 of three positions. DNA amplifications were carried out in 20 µl reactions containing 2 µl fecal DNA
167 extract, 1 U Platinum II *Taq* Hot-Start DNA polymerase, 1x Platinum II Buffer, 0.2 mM each dNTP, 2
168 mM $MgCl_2$, and 1 µM each primer. Each DNA sample was amplified twice. The duplicates were
169 placed randomly on different PCR plates to avoid potential batch effects and biases due to cross-
170 contamination of sample and/or barcoded primer (**Table S1**). We included one PCR negative and two
171 to three empty wells per plate, to check for contamination during PCR (Taberlet et al., 2018). In
172 addition, we included five DNA extraction blanks. PCR conditions consisted of 2 minutes
173 denaturation at 94˚C followed by 35 cycles of 94˚C for 30 seconds, 51˚C for 30 seconds, and 68˚C for

174 15 seconds, without final extension. PCR products were checked on a 2% agarose gel to confirm
175 amplification without contamination.

176 The barcoded PCR products were pooled column-wise (16 µl for each sample, duplicates in
177 separate pools), mixed with 640 µl PB Buffer, and purified using MinElute columns (Qiagen, The
178 Netherlands), eluting in 50 µl EB buffer. Double-indexed next-generation sequencing libraries
179 (Kircher et al., 2012) were prepared as detailed (Brealey et al., 2020; Rohland et al., 2015) but using
180 not-barcoded incomplete adapters after blunt-end repair. Two library preparation blanks were carried
181 through all steps. Each pool was quantified using qPCR with PreHyb primers (**Table S3**; Rohland et
182 al., 2015) and amplification settings as in Brealey et al. (2020).

183 Each sample pool and both library blanks received a unique combination of indices (**Table**
184 **S1**). For indexing PCR, we used the same reaction mixture and cycling conditions as Brealey et al.
185 (2020). The number of cycles ranged from 8 to 10, depending on the copy number estimated from
186 qPCR (**Table S1**). Library preparation blanks were amplified for 10 cycles to maximize capture of
187 potential contaminants. We performed MinElute purification and quantified indexed pools with qPCR,
188 as above, using i7 and i5 primers (Rohland et al., 2015, **Table S3**). Indexed sample pools were
189 combined in equimolar amounts, except for library preparation blanks, of which we added 0.5 µl each
190 into the final pool, corresponding to the lowest amount added for any sample. The final sequencing
191 pool was cleaned using AmPure XP beads (Beckman Coulter, USA) with two elutions (0.5x followed
192 by 1.8x), which remove very large fragments and fragments <100bp, respectively. This size selection
193 is optimized for the retention of *trn*L amplicons (~10-150bp in length + 148 bp of barcoded and
194 indexed adapters). Elution was performed in 30 µl of EB buffer. The cleaned library pool was
195 quantified using both a Qubit High Sensitivity fluorometer and 2200 TapeStation and sequenced at the
196 Uppsala Science for Life Laboratory on a single MiSeq lane with 150 bp paired-end sequencing with
197 version 2 chemistry.

198 Sequence processing and analysis was done in OBITools v1.2.13 (Boyer et al., 2016). Paired
199 reads with quality scores >40 and overlap >10 bp were retained and merged. Sample of origin for each
200 read was established through its index and barcode, requiring an exact sequence match. Sequences

201 were clustered into molecular operational taxonomic units (MOTUs), each representing a unique plant
202 taxon (Valentini et al., 2009). A large number of MOTUs had fewer than 10 sequences across all
203 samples and were removed as recommended (*e.g.*, Shehzad et al., 2012). We also removed sequences
204 that differed by exactly one nucleotide from a more abundant sequence and had a total count less than
205 5% of the more abundant sequence, following Boyer et al. (2016).

206 Finally, taxonomic assignment used a custom-made reference database (below). Based on a
207 frequency plot of identity to the reference database (**Figure S1**) and similar *trn*L-based studies of
208 tropical primate diet (*e.g.,* Quéméré et al., 2013), we removed sequences below an identity threshold
209 of 0.90. Below this, sequences were regarded as likely chimeric, enriched in sequencing or PCR
210 errors. No singletons were present after this filtering step.

211

212 *Compiling plant* trn*L reference database*

213 We built a local DNA barcoding reference library by downloading all 324,502 available
214 sequences from NCBI GenBank using the search query: "(trnL[All Fields] OR complete genome[All
215 Fields]) AND (plants[filter] AND (chloroplast[filter] OR plastid[filter]))" (last accessed 2 December
216 2021). In OBITools v1.2.13, the sequence list was annotated with taxonomy information downloaded
217 from NCBI (ftp://ftp.ncbi.nih.gov/pub/taxonomy/taxdump.tar.gz, last accessed 3 December 2021). To
218 complete the database of *trn*L genes, we followed established protocol (Boyer et al., 2016), using the
219 same *trn*L g-h primers as in the wet laboratory to extract *trn*L variants *in silico* in the program ecoPCR
220 v2.1 (Ficetola et al., 2010). We kept sequences that were between 10 to 230 base pairs long with at
221 most three primer mismatches total (Taberlet et al., 2018). The final database contained 21,308 *trn*L *in*
222 *silico* amplicons, in 608 families and 5,662 genera.

223 To evaluate the resolution of our reference database with respect to local plant diversity, we
224 compared plant taxa present in our database to a list of plants known to occur in the Kahuzi and
225 Itebero regions of KBNP (Yumoto et al., 1994). To enable this comparison, we updated the taxonomic
226 classification of the KBNP plant list (Yumoto et al., 1994) by searching for species names in the

227 Global Biodiversity Information Facility (GBIF). The updated list contained 328 taxa, in 81 unique
228 families and 234 genera. Of these, all families and 77.4% of genera were present in our *trn*L database.
229
230 *Characterization of gorilla gut microbiome*
231 We characterized gut microbial composition in 70 individuals from KBNP and NCA
232 populations using a single sample per individual (**Table S2**). We selected the same sample that was
233 used for dietary analyses and only dry season samples from the Bansamba group in NCA. To quantify
234 possible contamination, we also carried nine random extraction blanks through the entire data
235 generation process.
236 The V4 region of the *16S rRNA* gene was amplified with primers 515F/806R (**Table S3**) for
237 each sample in duplicate. The PCR reaction contained 2 µl of extracted DNA, 5 µM each of the
238 forward and reverse primer, 1x Phusion High-Fidelity Buffer, 0.02 units Phusion HF DNA polymerase
239 (2U/µl), 0.012 mg DMSO and 0.05 µM (each) dNTPs, with the volume made up to 20 µl with
240 Ultrapure H2O. Thermal cycling conditions were as follows: 30 seconds at 98°C, 25 cycles of 98°C
241 for 10 seconds, 52°C for 20 seconds and 72°C for 20 seconds, and 10 minutes at 72°C. PCR cycles
242 were limited to 25 to minimize the risk of unspecified products and chimeras. Duplicate reactions were
243 pooled and cleaned with AmPure beads (Qiagen).
244 Next-generation sequencing libraries were prepared from PCR products following the double-
245 barcoding, double-indexing strategy (Kircher et al., 2012; Meyer & Kircher, 2010; Rohland et al.,
246 2015; van der Valk et al., 2017). As a result, each sample had a unique combination of two barcodes
247 and two indices, which enabled bioinformatic filtering of potential chimeric molecules and
248 misassigned reads resulting from index hopping (van der Valk et al., 2017, 2020). For indexing, we
249 determined the suitable number of PCR cycles (8-11) based on qPCR of barcoded libraries, as above.
250 Indexed libraries were quantified by qPCR and pooled in equimolar amounts for sequencing on a
251 single MiSeq lane, using version 2 chemistry and 250 bp paired end sequencing at the Uppsala
252 Science for Life Laboratory sequencing facility.

253 Sequencing reads were demultiplexed and adapters removed using a Python script (Brealey et
254 al., 2021). We followed established protocol to estimate microbial amplicon sequence variants (ASVs)
255 using DADA2 (Callahan et al., 2016), rather than clustering sequences, which avoids biases due to
256 arbitrary similarity thresholds (Edgar, 2018). Forward and reverse reads were truncated to 200 and 150
257 bp, respectively, at which point read quality scores dropped below 35. We merged paired-end reads,
258 requiring an overlap of at least 12 bp, and removed sequences outside the 250-256 bp range and those
259 with any barcode mismatch, as recommended (Callahan et al., 2016).
260 Taxonomy was assigned using the SILVA 132 reference database, released in December 2017
261 (Quast et al., 2012). Species-level assignment required a strict 100% match (Edgar, 2018). We
262 removed singletons and ASVs labeled 'Unassigned', 'Eukaryota', "mitochondria", or "chloroplast".
263 We retained Archaea, although archaeal amplification from the V4 region of the *16S rRNA* is limited
264 (Raymann et al., 2017), because within-dataset comparisons are nonetheless informative. We built a
265 bacterial phylogenetic tree by aligning sequences to the Greengenes 13_5 mega-phylogeny (203,452
266 99% OTUs; DeSantis et al., 2006) in SEPP using default parameters (Mirarab et al., 2012).
267
268 *Statistical analyses of* trn*L and* 16S *datasets*
269 To examine dietary and microbiome diversity, we analyzed the *trn*L and *16S rRNA*
270 metabarcoding datasets, after first filtering out rare sequence variants below 0.5% relative abundance
271 in at least one sample, as suggested (Deagle et al., 2019). We evaluated sampling effort and
272 sequencing depth accumulation curves in the R packages 'vegan' (Oksanen et al., 2020) and
273 'ranacapa' (Kandlikar et al., 2018), respectively. We checked whether the predicted number of taxa
274 (asymptote of the sequencing accumulation curve) minus actual number of taxa (richness) related to
275 any of the considered biological variables or sequencing depth (read count) using a generalized linear
276 model (GLM) with quasi-Poisson error distribution in the R package 'lme4' (Bates et al., 2015).
277 We calculated two alpha diversity metrics for each dataset: richness, or the number of taxa,
278 and Shannon's diversity index, or evenness (Chao et al., 2014). As recommended by McMurdie &
279 Holmes (2013), we did not rarefy to minimum sequencing depth. To test the effects of population,

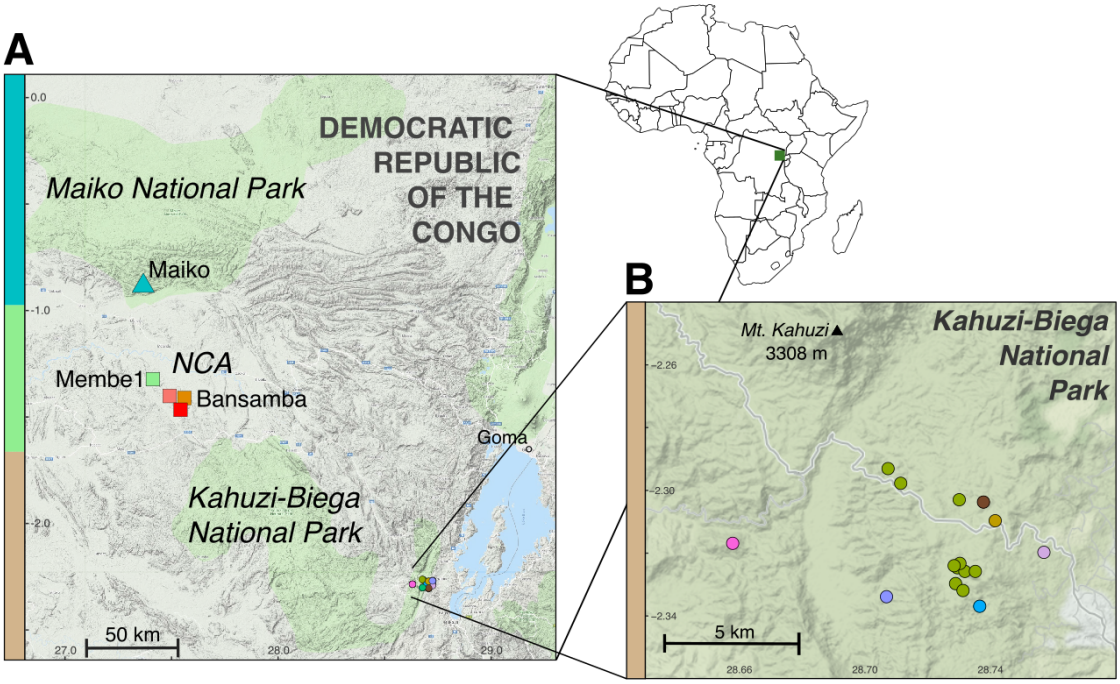
social group, altitude, sex, and age class on diversity metrics, we fitted a GLM with quasi-Poisson (for richness) or gamma (for evenness) error distribution with logit link function, followed by Tukey honestly significant difference (HSD) *post-hoc* comparisons between levels of categorical variables that were overall significant (χ^2 test with Bonferroni correction) (Lenth et al., 2021).

To assess trends in diet and microbiome beta diversity, or composition, we followed a strategy designed for the compositional nature of metabarcoding data (Gloor et al., 2017; Weiss et al., 2017). We used Bayesian multiplicative zero replacement and then centered and log-ratio (CLR) transformed each dataset using the R packages 'zcompositions' (Palarea-Albaladejo & Martín-Fernández, 2015) and 'compositions' (van den Boogaart & Tolosana-Delgado, 2008). For the microbiome dataset, we secondarily used Phylogenetic Isometric Log-Ratio Transform (phILR) to compute compositional abundance at phylogenetic balances (Silverman et al., 2017). To evaluate variation in composition of diet and microbiome, we computed Aitchison's dissimilarity (Euclidean distance between CLR values) (Aitchison et al., 2000). To quantitatively estimate which factors best predict variation in diet and gut microbiome, we modeled the composition in CLR (or phILR) transformed space as a function of ecological and biological variables using PERMANOVA, via function *adonis2* in 'vegan' (Anderson & Walsh, 2013). The predictor variables were population, social group, sex, age class, and altitude. Sequencing read count was kept as the first predictor, even if $p > 0.05$. *Post-hoc* comparisons between levels of overall significant variables were done with Bonferroni correction using 'pairwiseAdonis' (Arbizu, 2020). The influence of genetic distance (*1* - genetic relatedness) was modeled separately within each population using Mantel and partial Mantel tests (controlling for social group identity).

We estimated the covariance between diet and microbiome using a co-inertia analysis between the two matrices in the package 'omicade4' and calculated the RV coefficient (Escoufier, 1973; Robert & Escoufier, 1976) and its significance using a Monte Carlo test with 999 permutations (Meng et al., 2014). To compare the effects of diet and other variables on the gut microbiome, we fit a Multiple Regression on Matrices (MRM) model (Lichstein, 2007), an extension of the partial Mantel test, in 'ecodist' (Goslee & Urban, 2007). The explanatory variables were straight-line geographic

distance, altitude difference, diet composition (Aitchison distance), and social group and population as binary (same, 0, or different, 1). Significance was assessed using 999 permutations of the response variable, the Aitchison distance matrix of gut microbiome composition.

Differences in beta diversity can be due to differential abundance of a few key organisms, or subtle differences across an entire community. To identify dietary and microbial taxa that may have driven compositional differences, we used the R package 'ALDEx2' and focused on significant differences (Wilcoxon rank sum test with correction for false discovery rate (FDR) $p < 0.05$) with effect sizes >1, as recommended (Gloor et al., 2017).



C

	Social group		N (f,m)	Year	Season	Altitude (m)	$N_{genotypes}$
●	Bonane	KBNP	1 (0,1)	2016	Dry	2328	1
●	Chimanuka	KBNP	14 (4,9)	2016	Rainy	2300	44
●	Mankoto	KBNP	12 (1,11)	2016	Dry	NA	13
●	Mpungwe	KBNP	8 (6,2)	2016	Dry	2200	21
●	Mufanzala 2	KBNP	4 (3,1)	2016	Dry	2443	5
●	Mugaruka	KBNP	1 (0,1)	2016	Dry	2123	2
●	Namadiriri	KBNP	13 (8,5)	2016	Dry	2174	23
●	Nouvelle Famille	KBNP	6 (5,1)	2016	Dry	2282	16
■	Bansamba	NCA	11 (9,2)	2015	Dry	619	46
■	Bansamba	NCA	3 (1,2)	2016	Rainy	630	
■	Bansamba	NCA	3 (0,3)	2018	Dry	675	
■	Membe 1	NCA	11 (5,6)	2017	Dry	650	33
▲	Maiko	MNP	5 (3,2)	2016	Rainy	835	6

Figure 1. **(A)** Map of Grauer's gorilla fecal sampling locations from Maiko National Park (MNP; designated with cyan on the left-hand side of the map), Nkuba Conservation Area (NCA; green) and Kahuzi-Biega National Park (KBNP; brown), with **(B)** inset zooming in on different social groups in KBNP. Circle colors designate social groups, coded as in **(C)**. Note that multiple circles are present for the Chimanuka group, consistent with opportunistic sampling of identified individuals. Geographic coordinates were not available for the Mankoto group. The table in **(C)** shows the sample size (N=number of unique individuals, f=number of females, m=number of males) used for dietary and gut microbiome characterization of each social group. Only diet but not gut microbiome data is available for samples shaded in gray. Bansamba group (NCA) was sampled repeatedly, but only few samples were included in dietary analyses in later years (three from 2016 and three from 2018). Also shown for each social group are: collection year and season, altitude, and $N_{genotypes}$, the total number of successfully genotyped samples.

329 **Results**

330 ***Study populations of Grauer's gorillas are genetically differentiated***

331 We identified 92 unique individuals in the three study populations by microsatellite
332 genotyping: 59 in KBNP, 28 in NCA, and 5 in MNP (**Figure 1, Table S2**). Individuals belonged to six
333 different social groups and two solitary adult males (lone silverbacks) in KBNP, two social groups in
334 NCA, and one group in MNP. Genotyping revealed that each individual was sampled 1-13 times, with
335 4-17 individuals per social group.

336 None of the 12 microsatellite loci deviated from Hardy-Weinberg equilibrium after Bonferroni
337 correction for multiple testing ($p > 0.1$). On average, there were 6.1 alleles per locus (**Table S2**). The
338 average observed and expected heterozygosities were 0.66 and 0.68, respectively. The test for global
339 heterozygote deficiency was not significant overall ($p = 0.6$) or in any population ($p > 0.4$). The test
340 for genotypic linkage disequilibrium using log likelihood ratio statistic with 66 pairwise comparisons
341 between the 12 loci was not significant for any pair ($p > 0.1$). Thus, we assumed linkage equilibrium
342 and considered all loci in further analyses.

343 Analysis of the three populations using STRUCTURE (Porras-Hurtado et al., 2013) revealed
344 two distinct genetic groups (optimal K=2 according to ΔK; Evanno et al., 2005; **Figure S2**). The
345 clusters differentiated gorillas in high-altitude KBNP (2500 m asl) from those in low-altitude NCA
346 and MNP (600-830 m asl) (**Figure S3**), consistent with the PCA (**Figure 2A**). All three populations
347 were significantly differentiated from one another ($F'_{ST} = 0.26\text{-}0.45$; $p < 0.001$; **Table S4A**), with
348 largest differences between MNP and KBNP, which are furthest apart geographically (215km).
349 Individuals within social groups were more closely related than individuals in different groups in the
350 same population (AMOVA $\phi = 0.12$, $p < 0.001$; **Table S4B**), consistent with gorilla social structure
351 (Harcourt & Stewart, 2013).

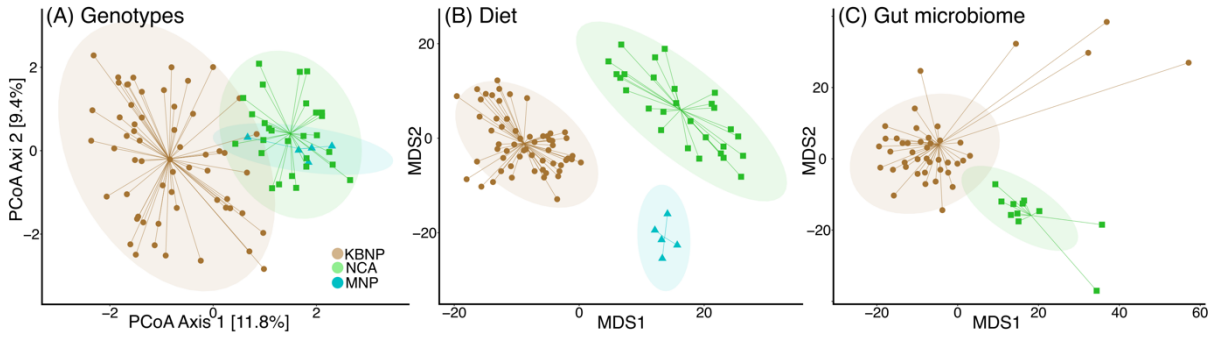


Figure 2. **(A)** PCA of genetic distances among individuals based on microsatellites. NMDS of **(B)** dietary composition and **(C)** gut microbiome composition, both in Aitchison distances. Individual samples are coloured by population of origin, with 95% confidence interval ellipses for each population (brown = KBNP, green = NCA, cyan = MNP, as in Figure 1).

Negative controls in trn*L and* 16S rRNA *metabarcoding*

To quantify contamination in the diet (*trn*L) and the gut microbiome (*16S rRNA*) dataset, we analyzed DNA extraction blanks, PCR negative controls, unused barcode combinations and library preparation negative controls (for diet) (**Table S2**; **Table S5; Table S6**). In the diet dataset, the extraction and PCR negative controls contained 16 *trn*L reads in total, identified to 12 different plant taxa. Each taxon had one to three reads summed across all negative controls, yet up to 3,620-154,357 reads per sample (**Table S5**). There were no reads with unused barcode combinations, suggesting that cross-contamination of barcodes during PCR and library preparation was negligible. In the microbiome dataset, four extraction blanks had 90 reads in total, whereas the remaining five had none (**Table S6**). These mapped to eight different *16S* taxa, with three to 26 reads each. As with the diet data, these taxa were among the most abundant in the samples (up to 2,244-14,307 reads per sample). This pattern is consistent with low-level cross-contamination from high quantity into low quantity samples typical for large-scale sequencing studies (Eisenhofer et al., 2019).

Diet of Grauer's gorillas

We characterized the diet of 92 Grauer's gorilla individuals (**Table 1C**) using the chloroplast *trn*L *P6 loop* locus. After data filtering, we retained 5,367,160 *trn*L sequencing reads (corresponding to 45% of raw reads) belonging to 120 unique taxa (**Table S7A**). PCR replicates were more similar to

377 each other than to other samples in alpha and beta diversity ($p < 0.001$, **Figure S4**), and hence their
378 sequencing data were pooled. Sample size and sequencing depth were sufficient to capture dietary
379 diversity in KBNP and NCA, but not in MNP, where only five individuals were sampled
380 (**Supplemental Text**, **Figure S5**).

381 Of the 120 detected dietary plant taxa, 115 could be identified to at least the order level (in 29
382 different orders), 110 to family (in 49 families), and 44 to genus (in 35 genera) level (**Table S8**). All
383 but 21 taxa have previously been recorded in the Grauer's gorilla diet in KBNP, NCA, and Mt.
384 Tshiaberimu (Kambale, 2018; van der Hoek, Pazo, et al., 2021; Yamagiwa et al., 1994, 2005; Yumoto
385 et al., 1994; **Table S8, columns S-T; Figure S6**). These 21 taxa are, however, present in the region
386 (Spira et al., 2018).

387 Each Grauer's gorilla fecal sample contained 36 – 80 *trn*L taxa (mean 58.52 ± 10.83) (**Figure**
388 **3A**), with each population showing a different set of most abundant and prevalent plants (**Table 1**;
389 **Table S8).** Five plant taxa were found in each sample collected during the dry season in KBNP and
390 NCA, even though they showed very low abundance in some samples (0.1%). Only two plant taxa had
391 abundances over 1% in all three populations **(Table 1**).
392

393 **Table 1.** Top three most abundant plant taxa by population and taxa shared across populations.

ID	NCBI- based finest taxonomic identity	Distribution-refined probable identity	Mean abundance in KBNP	Mean abund in NCA	Mean abund in MNP	KBNP Rank§	NCA Rank§	MNP Rank§
1	*Urera sp.*	*Urera hypselodendron*	35.1%	0.2%	0.1%	1	14	19
2	Apocynaceae sp.	*Taccazea apiculata*	21.0%	0.2%	0.2%	2	16	17
6	Urticaceae sp.	Urticaceae sp.	6.0%	12.9%	0.06%	3	13	22
8	Myristicaceae sp.	*Pycnanthus*, *Staudtia*, or *Afradisia sp.*	0.05%	14.5%	8.1%	13	1	5
5†	Apocynoideae sp.	*Baissea*, *Funtumia*, or *Motandra sp.*	4.6%	8.9%	14.7%	4	2	3
25	*Megaphrynium macrostachyum*	*Megaphrynium macrostachyum*	0.01%	3.4%	0.05%	25	3	26
31	Phyllanthaceae sp.	Phyllanthaceae sp.	0.01%	0.01%	18.8%	46	63	1
32	Alafinae sp.	*Strophanthus sp.*	0.02%	0.2%	12.9%	29	26	2
14†	*Ficus sp.*	*Ficus sp.*	2.7%	5.6%	1.9%	8	6	14
13‡	Moraceae sp.	Moraceae sp.	1.8%	3.9%	0.07%	10	4	21

394 †Taxon greater than 1% relative abundance in all three populations (identifiable also by similar, high rank).
395 Shaded rows highlight taxa present in every sample collected during the dry season. With the exception of Moraceae sp. (ID
396 13‡), these taxa were also present in each rainy season sample and hence the abundances are shown including samples
397 collected during the rainy season. Moraceae sp. (ID 13‡) was missing from one individual in the Chimanuka group (KBNP)
398 collected during the rainy season.
399 §Rank is calculated by ranking each taxon by its relative abundance in a sample and calculating its mean rank across all
400 samples in a population. It thus reflects the average abundance rank of a given taxon across all samples in a population.
401

402 ***Geography, altitude, and social group identity influence dietary diversity and composition in***
403 ***Grauer's gorillas***

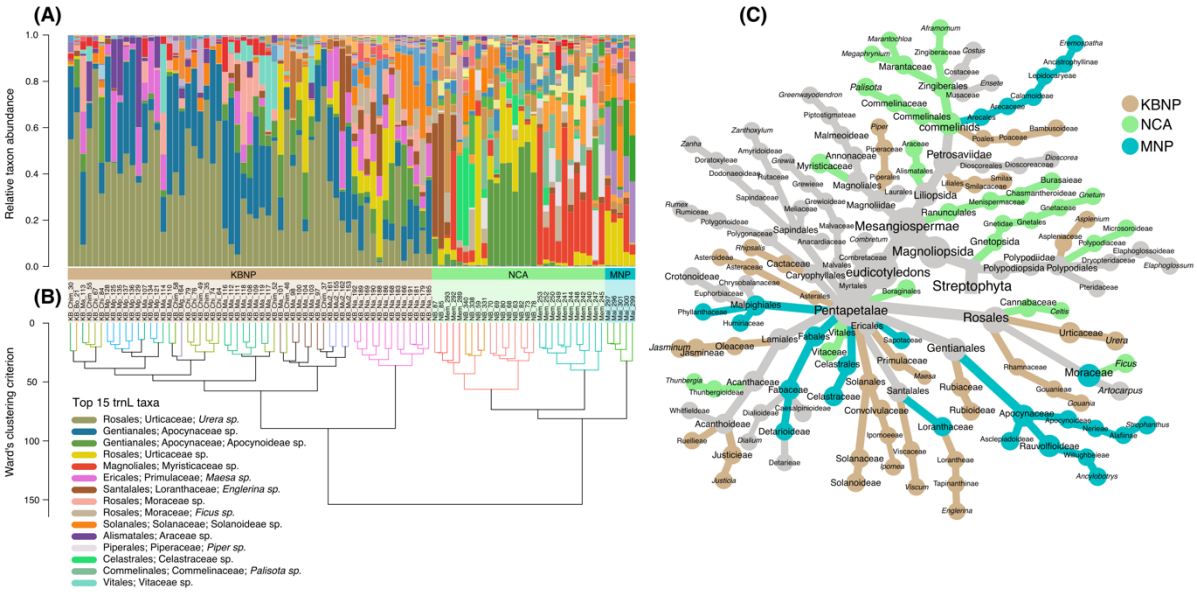
404 Dietary richness and evenness differed significantly by population and social group identity (p
405 < 0.001, **Table S9**). Both richness and evenness were significantly higher in low altitude populations
406 (NCA and MNP) than in high-altitude KBNP (mean richness: 66.8±7.5 taxa in MNP, 65.6±6.1 in
407 NCA vs. 54.5±10.4 in KBNP, $p < 0.001$; evenness: 10.0±1.3 in MNP, 8.4±2.5 in NCA, vs. 5.4±2.6 in
408 KBNP, $p < 0.001$; **Figure S7**). Altitude was also a significant predictor of dietary richness and
409 evenness in KBNP ($p < 0.001$; **Figure S8**). In contrast, neither individual's sex nor age (age class
410 available for 70 individuals) had an effect on dietary richness or evenness ($p > 0.3$; **Table S9**). We
411 obtained qualitatively similar results when analyzing only samples collected during the dry season

412 (excluding Chimanuka group, three individuals from Bansamba group, and MNP; **Table S9**), with the
413 exception that dietary richness did not significantly change with altitude in KBNP ($p = 0.2$).
414 Hierarchical clustering of dietary composition first separated high altitude (KBNP) from low
415 altitude (NCA and MNP) locations (**Figure 3B**), even though MNP samples were collected during the
416 rainy season. Within populations, individuals clustered by social group. NMDS ordination showed a
417 similar pattern (**Figure 2B**). After accounting for sequencing depth, dietary composition was
418 significantly influenced by population ($p < 0.001$, explaining 27.9% of the variance) and social group
419 ($p < 0.001$, explaining an additional 21.6%) but not by sex ($p = 0.7$) or age ($p = 0.2$; **Table 2**). All
420 social groups differed significantly from each other ($p < 0.05$; **Table 2**), except for some comparisons
421 involving the Mufanzala2 group, which had a small sample size (n=4). Restricting the analysis to two
422 similarly sized social groups in NCA and KBNP collected during the dry season, we confirmed the
423 presence of significant between-group and between-population diet differences (**Table S10**),
424 supporting the notion that populations and social groups have distinct diets and that our results are not
425 driven by differences in sample size or season.
426 Using ALDEx2, we identified differentially abundant dietary items across populations. The
427 drivers of the observed population dietary differences were among the most abundant taxa in each
428 population (**Table 1**), most of which were absent or present at very low abundance in other
429 populations (**Figure 3C**; **Table S11**). Out of the 21 previously undescribed food items (see above), 13
430 were significantly more abundant in low-altitude populations compared to the high-altitude population
431 KBNP (**Table S8, S9; Figure S6**). Within populations, each social group consumed between two and
432 32 differentially abundant taxa (mean = 11.3 ± 11.6).
433
434

435 **Table 2.** PERMANOVA model of factors influencing dietary composition

Variable	Df	R^2	F	*p*	***Post-hoc* tests**	
					Significant pairwise comparisons	$p_{Bonferroni}$
Read Count	1	0.01	1.73	0.05	–	
Population	2	0.279	17.80	<0.001	NCA – KBNP	< 0.001
					KBNP – MNP‡	< 0.001
					MNP – NCA	< 0.001
Social Group	6	0.216	4.56	<0.001	***KBNP***	
					Chimanuka‡ – Nouvelle Famille	0.02
					Chimanuka‡ – Mankoto	0.005
					Chimanuka‡ – Mpungwe	0.005
					Chimanuka‡ - Mufanzala2	0.02
					Chimanuka‡ – Namadiriri	0.005
					Nouvelle Famille – Mankoto	0.02
					Nouvelle Famille – Mpungwe	0.02
					Nouvelle Famille – Namadiriri	0.005
					Mankoto – Mpungwe	0.005
					Mankoto - Mufanzala2	0.03
					Mankoto – Namadiriri	0.005
					Mpungwe – Namadiriri	0.005
					Mufanzala2 – Namadiriri	0.02
					NCA	
					Membe1 – Bansamba(‡)	0.005
Sex	1	0.005	0.82	0.6	–	
Age class†	2	0.022	1.28	0.1	–	

436 †Age class (Infants (N=7), Juveniles/subadults (N=21), Adults (N=42)) was modeled separately using a reduced dataset, since only 70 of the
437 92 samples had age estimates. In this model the other predictor variables had estimates similar to those of the complete dataset.
438 ‡Samples from MNP, Chimanuka group in KBNP, and three out of 17 individuals from Bansamba group in NCA were collected during the
439 rainy season, whereas all other samples were collected during the dry season. Removal of these individuals did not affect results (**Table**
440 **S10**).
441


(A)
Relative taxon abundance
KBNP
NCA
MNP
(B)
Ward's clustering criterion
Top 15 trnL taxa
Rosales; Urticaceae; Urera sp.
Gentianales; Apocynaceae sp.
Gentianales; Apocynaceae; Apocynoideae sp.
Rosales; Urticaceae sp.
Magnoliales; Myristicaceae sp.
Ericales; Primulaceae; Maesa sp.
Santalales; Loranthaceae; Englerina sp.
Rosales; Moraceae sp.
Rosales; Moraceae; Ficus sp.
Solanales; Solanaceae; Solanoideae sp.
Alismatales; Araceae sp.
Piperales; Piperaceae; Piper sp.
Celastrales; Celastraceae sp.
Commelinales; Commelinaceae; Palisota sp.
Vitales; Vitaceae sp.
(C)
KBNP
NCA
MNP
Mesangiospermae
Magnoliopsida
eudicotyledons
Streptophyta
Pentapetalae
Rosales
Gentianales
Petrosaviidae
Liliopsida
Commelinids
Commelinales
Commelinaceae
Palisota
Zingiberales
Marantaceae
Magnoliidae
Magnoliales
Myristicaceae
Annonaceae
Malmeoideae
Sapindales
Malpighiales
Lamiales
Oleaceae
Jasminum
Fabales
Fabaceae
Celastrales
Celastraceae
Acanthaceae
Thunbergia
Solanales
Santalales
Primulaceae
Maesa
Rubiaceae
Rubioideae
Apocynaceae
Rauvolfioideae
Loranthaceae
Moraceae
Ficus
Artocarpus
Urticaceae
Urera
Cannabaceae
Cactaceae
Caryophyllales
Vitales
Vitaceae
Ericales
Saxifragales
Myrtales
Crotonoideae
Euphorbiaceae
Chrysobalanaceae
Phyllanthaceae
Humiriaceae
Asterales
Asteraceae
Rhipsalis
Polypodiopsida
Polypodiales
Polypodiaceae
Pteridaceae
Aspleniaceae
Gnetopsida
Gnetales
Gnetaceae
Gnetum
Ranunculales
Lauralles
Piperales
Piperaceae
Piper
Aristolochiales
Aristolochiaceae
Alismatales
Araceae
Dioscoreales
Dioscoreaceae
Dioscorea
Liliales
Smilacaceae
Smilax
Burmanniales
Burmanniaceae
Asparagales
Orchidaceae
Poales
Poaceae
Bambusoideae
Zingiberales
Zingiberaceae
Costaceae
Costus
Musaceae
Ensete
Marantochloa
Megaphrynium
Aframeomum
Marantaceae
Greenwayodendron
Zanha
Zanthoxylum
Rutaceae
Sapindaceae
Melaceae
Malvaceae
Polygonaceae
Rumicaceae
Rumex
Polygonoideae
Amaranthaceae
Combretaceae
Combretum
Myrtales
Beraginales
Lamiales
Jasminum
Oleaceae
Jasmineae
Thunbergioideae
Acanthaceae
Acanthoideae
Whitfieldeae
Dialioideae
Dialium
Detarioideae
Detarieae
Justicieae
Justicia
Solanaceae
Solanoideae
Solanum
Ipomoeae
Ipomea
Viscaceae
Viscum
Tapinanthinae
Loranthaceae
Loranthus
Englerina
Rubiaceae
Gouanieae
Gouania
Rhamnaceae
Moraceae
Ficus
Artocarpus
Urticaceae
Urera
Cannabaceae
Celtis
Rosales
Gentianales
Apocynaceae
Apocynoideae
Asclepiadoideae
Rauvolfioideae
Wrightioeae
Alstonia
Nerium
Stephanotis
Ancylobotrys
Pteridaceae
Polypodiaceae
Elaphoglossoideae
Elaphoglossum
Dryopteridaceae
Aspleniaceae
Asplenium
Microsoroideae
Polypodiales
Polypodiales
Polypodiaceae
Cyclopeltis
Cyclopeltidoideae
Lomariopsidaceae
Lomariopsis
Tectariaceae
Tectaria
Dryopteridaceae
Dryopteris
Thelypteridaceae
Thelypteris
Aspleniaceae
Asplenium
Microsoroideae
Polypodiaceae
Elaphoglossoideae
Elaphoglossum
Polypodiaceae
Polypodium
Polypodiaceae
Polypodium
Polypodiaceae
Polypodium

465 microorganisms (Delgado-Baquerizo et al., 2018). There were seven Archaea in our dataset, belonging
466 to the Methanomethylophilaceae and Methanobacteriaceae families. Each fecal sample contained on
467 average 200.29 ± 19.6 taxa (min = 160, max = 237), each with average abundance of 0.2% ± 0.4%.
468 Eleven taxa were present in every individual gorilla fecal sample from both populations (the core gut
469 microbiome), however, populations differed in the most abundant taxa (**Table 3**).
470

471 **Table 3.** Top three most abundant gut microbiome taxa by population and taxa shared across
472 populations.

ASV	NCBI-based finest taxonomic identity	Mean abundance in KBNP	Mean abund NCA	Rank KBNP§	Rank NCA§
3†	Bacteria; Firmicutes; Clostridia; Clostridiales; Family XIII; *AD3011 group*	2.40%	2.49%	1	3
6†	Bacteria; Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae; *UCG-004*	2.32%	1.16%	2	17
4	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Faecalibacterium*	2.78%	0.84%	3	39
5†	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; *RC9 gut group*	1.61%	6.36%	7	1
2†	Bacteria; Firmicutes; Clostridia; Clostridiales; Christensenellaceae; *R-7 group*	2.90%	3.97%	4	2
1†	Bacteria; Chloroflexi; Anaerolineae; Anaerolineales; Anaerolineaceae; *Flexilinea*	2.72%	4.27%	6	4
22	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; *Prevotella 7*	1.09%	0.18%	10	85
21	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *UCG-005*	0.84%	0.99%	13	18
31	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Oribacterium*	0.96%	0.27%	19	61
30	Bacteria; Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; *Sutterella*	0.95%	0.14%	16	86
33	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Ruminiclostridium 9*	0.73%	0.76%	17	35
59	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *UCG-002*	0.39%	0.51%	43	31
70	Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales; Eggerthellaceae; *Senegalimassilia*	0.32%	0.48%	67	40
152	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae	0.15%	0.11%	100	123
128‡	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; *Flavonifractor*	0.18%	0.22%	82	67
51‡	Bacteria; Firmicutes; Erysipelotrichia; Erysipelotrichales; Erysipelotrichaceae; *Solobacterium*	0.46%	0.16%	48	98
8‡	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; *Oribacterium*	2.11%	0.49%	14	37

473 † Taxon greater than 1% relative abundance in both populations (identifiable also by similar, high rank).
474 Shaded rows highlight taxa present in every sample collected during the dry season. With the exceptions of *Flavonifractor*
475 sp. (ASV128‡), *Solobacterium sp.*, (ASV51‡), and *Oribacterium sp.* (ASV8‡), these taxa were also present in each rainy
476 season sample and hence the abundances are shown including samples collected during the rainy season. *Flavonifractor* sp.
477 (ASV128‡), *Solobacterium sp.*, (ASV51‡), and *Oribacterium sp.* (ASV8‡) were missing from one, one, and two individual(s),
478 respectively, in the Chimanuka group (KBNP) collected during the rainy season.
479 § Rank is calculated by ranking each taxon by its relative abundance in a sample and calculating its mean rank across all
480 samples in a population. It thus reflects the average abundance rank of a given taxon across all samples in a population.
481

In accordance with previous studies on great apes (Campbell et al., 2020; Gomez et al., 2016b; Hicks et al., 2018; Nishida & Ochman, 2019), Grauer's gorilla gut microbiome in both populations was dominated by the phyla Firmicutes (65.6% in KBNP, 60.0% in NCA%), Bacteroidetes (20.7% in KBNP, 23.1% in NCA.0%), Spirochaetes (3.5% in KBNP, 5.4% in NCA), Chloroflexi (2.7% in KBNP, 4.3% in NCA), Proteobacteria (2.8% in KBNP, 3.4% in NCA), and Actinobacteria (2.0% in KBNP, 1.8% in NCA) (**Figure S10),** representing a diversity of microbial families (**Figure 4A**).

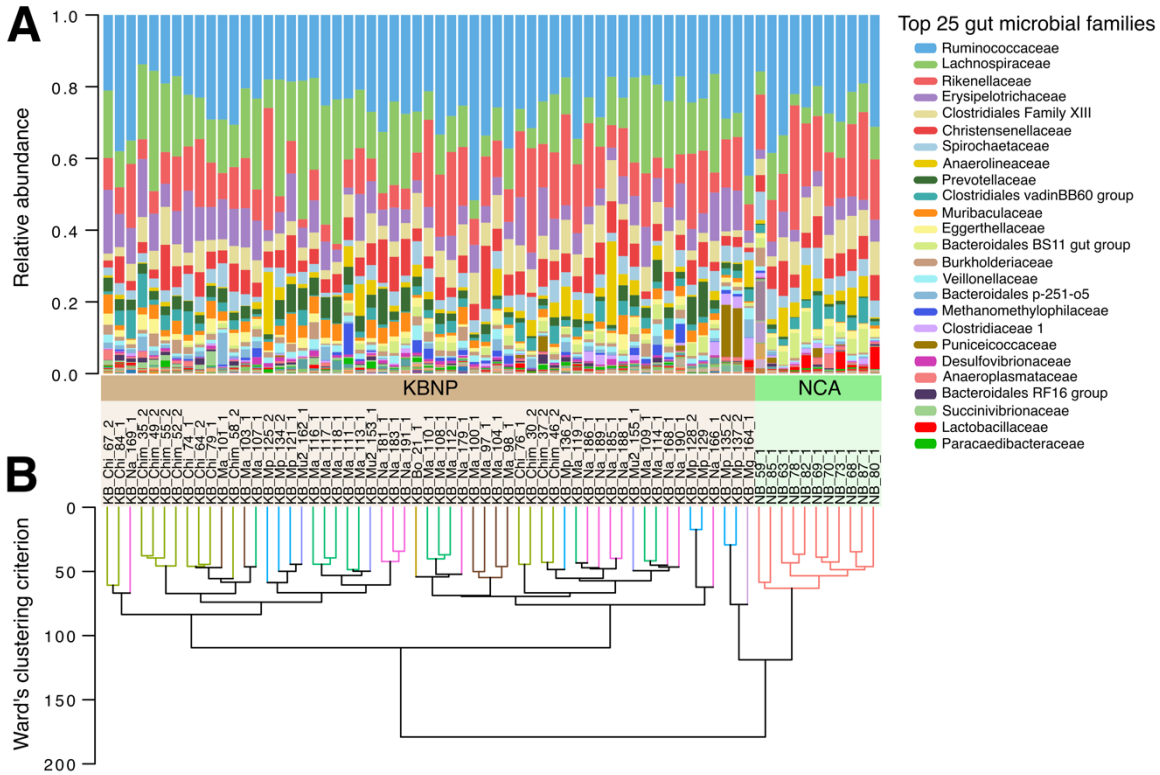


Figure 4. Gut microbiome composition **(A)** at the family level and **(B)** showing population clustering in composition, using CLR Aitchison distances dendrogram based on Ward's clustering criterion (Murtagh and Legendre 2014).

Diversity and composition of the gut microbiome in Grauer's gorillas correlates with population and social group identity

Gut microbiome richness and evenness were significantly higher in the high-altitude population (richness: mean KBNP = 202.2±20.0 taxa vs. NCA = 190.2±14.0; p = 0.02; evenness:

498 83.7±17.8 vs. 74.2±12.8, p = 0.01), the opposite trend to diet, although neither microbiome richness
499 nor evenness were related to altitude within KBNP (p = 0.07, 0.9; **Table S13**; **Figure S11**). While
500 richness of the microbiome did not differ by sex (p = 0.2), females had more even microbiomes than
501 males (85.7 vs. 78.5, p = 0.002). There were no differences by age (richness p = 0.3; evenness p =
502 0.1). The gut microbiome alpha diversity differed significantly by population even after removing
503 rainy season samples (excluding Chimanuka group; richness p = 0.001, evenness p = 0.008; **Table**
504 **S13**).

505 Gut microbiome composition differed between the two populations (KBNP and NCA)
506 (**Figure 2C**) and among social groups **(Figure 4A-B**), with population explaining 10.5% of the total
507 variance, and social group in KBNP explaining an additional 17.8% (p < 0.001; **Table 4**). Intergroup
508 differences were significant, including among groups collected during the dry season (**Table 4**).
509 Overall, gut microbiome dissimilarity was largest between individuals of different populations,
510 followed by individuals from different social groups, and smallest between individuals from the same
511 social group (**Figure 5C**). While altitude explained 12.7% of the variance across populations (N=56, p
512 < 0.001) it only accounted for 3.8% in KBNP (N=45, p = 0.01). Genetic distance among gorillas was
513 not a significant predictor of gut microbiome composition in NCA (N=11, ρ = -0.080, p = 0.7) or
514 KBNP when social group was also considered (ρ = 0.015, p = 0.3). Microbiome composition did not
515 differ by sex or age (p > 0.05, **Table 4**). Results using only dry season samples (**Table S14A**) and
516 phylogeny-informed (phILR) distances were qualitatively similar (**Table S15**).

517 We identified 42 taxa that significantly differed in abundance between NCA and KBNP (p <
518 0.05, effect size > 1) (**Table S16**). At the family-level, gorilla gut microbiomes in KBNP had a higher
519 abundance of Muribaculaceae and Erysipelotrichaceae, whereas the gut microbiomes in NCA had
520 more Spirochaetaceae and Christensenellaceae. At a finer phylogenetic level, populations differed in
521 abundance of specific ASVs belonging to common, shared families like Rikenellaceae,
522 Lachnospiraceae, and Ruminococcaceae.

523

524 **Table 4.** PERMANOVA model of factors influencing microbiome composition

Variable	Df	R^2	F	*p*	***Post-hoc* tests**	
					Significant pairwise comparisons	$p_{Bonferroni}$
Read Count	1	0.019	1.68	0.05	–	
Population	1	0.105	7.97	<0.001	KBNP – NCA	< 0.001
Social Group	5	0.178	2.18	<0.001	Chimanuka‡ – Nouvelle Famille Chimanuka‡ – Mankoto Chimanuka‡ – Mpungwe Chimanuka‡ – Namadiriri Nouvelle Famille – Mankoto Nouvelle Famille – Namadiriri Mankoto – Mpungwe Mankoto – Namadiriri	0.004 0.01 0.007 0.004 0.02 0.01 0.007 0.004
Sex	1	0.014	1.18	0.2	–	
Age class†	2	0.031	1.22	0.2	–	

525 †Age class (Infants (N=3), Juveniles/subadults (N=21), Adults (N=38)) was modeled separately using a reduced dataset, since only 62 of the
526 68 samples had age estimates. In this model the other predictor variables had similar estimates as in the complete dataset.
527 ‡Samples from Chimanuka group in KBNP were collected during the rainy season, whereas all other samples were collected during the dry
528 season. Results were similar when removing Chimanuka (**Table S14A**).
529
530

531 ***Diet and gut microbiome co-vary across studied populations***

532 Compositional differences in dietary and gut microbial profiles showed significant co-inertia
533 (RV = 0.557, $p < 0.001$; **Figure 5B**) and were correlated ($\rho = 0.32$, $p < 0.001$; **Figure 5C**), even after
534 removing the rainy season samples from Chimanuka (RV = 0.599, $p < 0.001$; $\rho = 0.35$, $p < 0.001$). We
535 detected no correspondence between dietary and gut microbial richness ($p = 0.2$; **Figure 5A**) or
536 evenness ($p = 0.1$). Microbiome composition is known to change with diet in individuals and also
537 differs between populations with different dietary preferences (*e.g.,* Reese et al., 2021; Youngblut et
538 al., 2019). However, in our dataset, only population and social group were significantly correlated
539 with gut microbiome composition, whereas dietary composition, geography, and genetic relatedness
540 had no effect after accounting for social group and population of origin (**Table 5; Table S14B**). As
541 with other analyses, dataset subsampling indicated that results were robust to sample size differences
542 between populations (**Table S17**).
543

Table 5. MRM model comparing the effects of geography, diet, and sociodemographic factors on Grauer's gorilla gut microbiome composition‡

MRM Model of Gut Microbiome Composition			MRM Model Statistics		
Explanatory Variable	**Spearman's ρ**	***p***	**N**	**R^2**	**F-statistic**
Geographic distance	0.07	0.4	56	0.278	98.35
Altitudinal difference	-0.08	0.6			
Diet composition†	-0.19	0.07			
Population	**0.64**	**< 0.001**			
Social group	**0.45**	**< 0.001**			

† Microbiome and diet composition in Aitchison distances.
‡Model results without Chimanuka are shown in Table S14B.

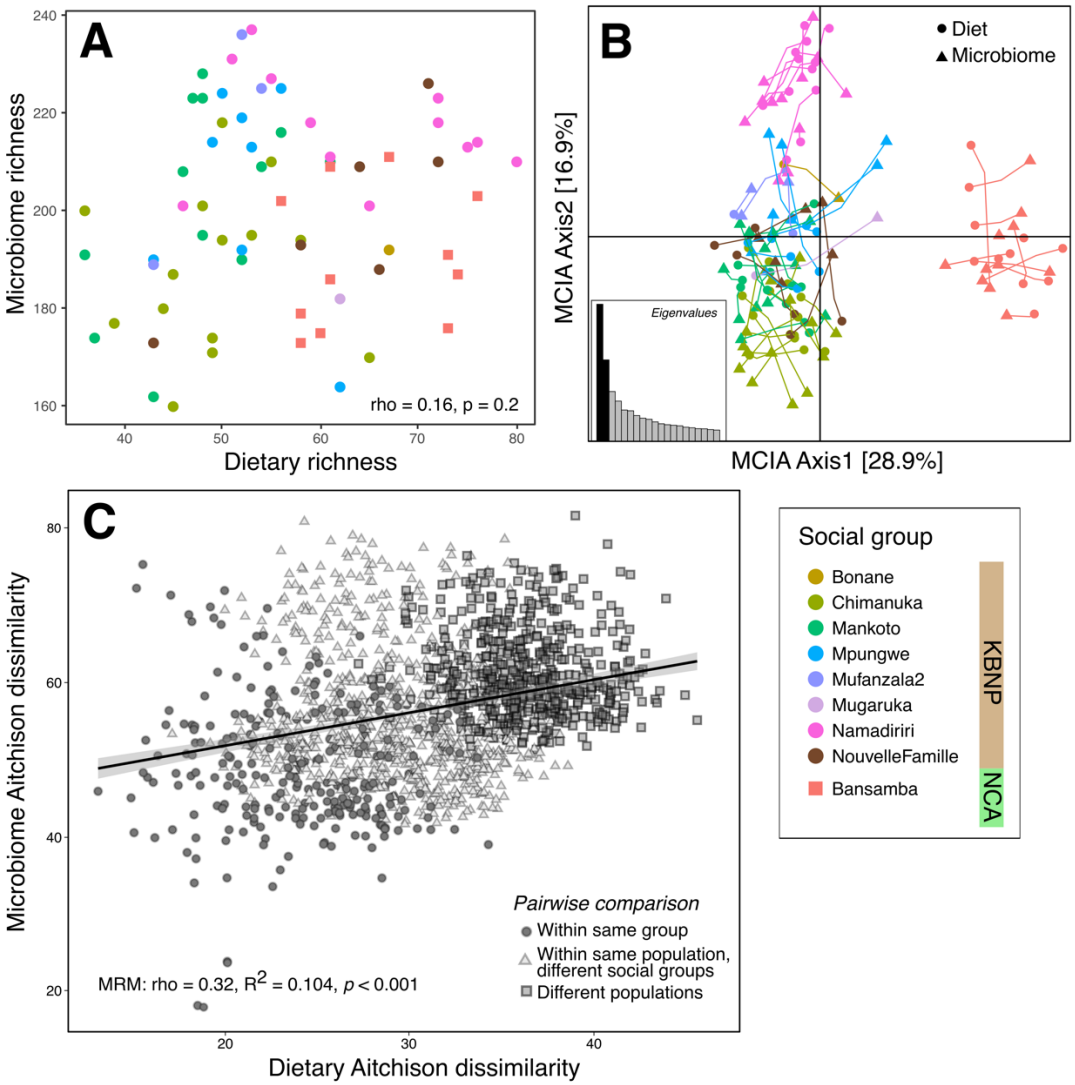


Figure 5. Relationship between diet and gut microbiome. **(A)** Microbiome and dietary richness, assessed as per-sample total sequence count, are not correlated (p = 0.2). **(B)** High multiple co-inertia (MCIA) between microbiome and diet composition in CLR-transformed space with Aitchison distance (RV = 55.7%, MC $p < 0.001$ based on 999 permutations). **(C)** Compositional differences (Aitchison distances) in diet and microbiome between samples (i.e., individual gorillas) are correlated in matrix regression.

555 **Discussion**

556 In this study, we applied fecal genotyping and DNA metabarcoding to identify Grauer's
557 gorilla individuals and characterize their diet and gut microbiome in three populations from across the
558 species' range. We were able to include a so far unstudied population from MNP and show that it is
559 genetically distinct from two previously assessed populations KBNP and NCA (Baas et al., 2018).
560 Grauer's gorillas occur across the greatest altitudinal range of all gorilla taxa (Plumptre et al., 2016)
561 and our study sites include the low and high altitude extremes. This provided us with the opportunity
562 to test for dietary and gut microbial co-differentiation among the isolated populations of this critically
563 endangered great ape. In particular, we set out to investigate if the gut microbiome may facilitate local
564 adaptation by supporting digestion of diverse foods. Alternatively, the presence of conserved dietary
565 patterns across populations along with a core gut microbiome would be indicative of a stabilizing role
566 of gut microorganisms, which may limit ecological adaptation. Gorillas consume a wide variety of
567 herbaceous vegetation, and fruits, when available, and their diet shows seasonal variation (Harcourt &
568 Stewart, 2013; Rogers et al., 2004; Rothman et al., 2008; Yamagiwa et al., 1994). In Grauer's gorillas,
569 studies that rely on different methodologies suggested some differences in diet between populations
570 (van der Hoek, Pazo, et al., 2021; Yamagiwa et al., 2005). However, previous studies did not assess
571 the gut microbiome, and hence could not characterize its contribution to these differences.

572 Our joint diet and gut microbiome analyses provide little evidence for dietary conservation
573 across populations but uncover a stable set of gut microorganisms that are shared among
574 geographically, genetically, and ecologically distinct populations of Grauer's gorillas. We detect co-
575 variation in diet and microbiome, likely as a result of habitat differences and social factors among
576 populations and social groups. Our results are thus consistent with the notion that the gut microbiome,
577 although being conserved to some degree, provides sufficient flexibility to allow exploitation of
578 diverse dietary resources and hence could contribute to local adaptation. In addition, we obtain
579 evidence that dietary choice in Grauer's gorillas is at least partially determined by plant availability,
580 with a larger dietary repertoire at lower elevations.

581

582 *New insights into Grauer's gorilla diet and feeding behavior*

583 Grauer's gorillas in the three study populations consumed 120 different plant taxa (**Table S8**),
584 which is similar to the dietary composition and diversity reported in observational studies (116 and
585 100 different plants; van der Hoek, Pazo, et al., 2021; Yamagiwa et al., 2005; respectively). Low
586 altitude populations consumed a greater diversity of plants than high altitude populations (**Figure 3**;
587 **Figure S7**; **Table S9**), consistent with higher biodiversity (including plant diversity) at lower altitudes
588 (Imani et al., 2016; Rahbek, 1995). We documented an average of 54-66 different plant taxa in each
589 fecal sample, which is considerably more than reported daily diversity of consumed plants per
590 individual based on behavioral observations (17 plant taxa per day on average in KBNP; Yamagiwa et
591 al., 2005). In captivity, gorilla gut retention time was estimated to be 24 to 60 hours (Remis, 2000).
592 Therefore, each fecal sample could represent plants consumed over the period of up to three days, with
593 some items digested faster than others. Alternatively, our method could capture taxa that are missed in
594 observational studies because they are consumed infrequently or in small quantities, at times of the
595 day that are rarely observed (early in the morning or late in the evening), or which may be
596 contaminants, parts of nest building material or involved in play or display and unrelated to diet. We
597 discuss other potential limitations of molecular dietary analyses in detail below.

598 We detected 21 plant taxa that have, to our knowledge, not been reported as Grauer's gorilla
599 foods (**Table S8**; **Figure S6**). Some of these plants grow in KBNP (Spira et al., 2018) and are
600 consumed by mountain gorillas (*e.g.,* Solanoideae; Rothman et al., 2014; Watts, 1984) or western
601 lowland gorillas (*e.g.,* Laruales; Remis et al., 2001). Other plants, such as *Gnetum* and Humiriaceae,
602 have not been documented in KBNP but are western lowland gorilla foods (Rogers et al., 2004;
603 Takenoshita & Yamagiwa, 2008), which is consistent with their significantly higher abundance in the
604 low-altitude sites of MNP and NCA.

605 Grauer's gorillas in different populations consumed distinct diets (**Figure 2, 3**; **Table 1, 2**),
606 with only two taxa shared across all three populations at an average abundance >1% per sample: *Ficus*
607 *sp*. and Apocynoideae sp. (likely *Baissea sp., Funtumia sp.*, or *Motandra sp.* based on plant
608 distribution; Spira et al., 2018). At broader taxonomic scales, all individuals consumed four plant

609 families (Urticaceae, Apocynaceae, Moraceae, and Vitaceae), but the relative abundances varied
610 considerably across populations, from less than 1% to up to 42%. The detection of shared taxa
611 suggests that the same plants or their close relatives are present in the habitat of all three populations.
612 However, the pronounced differences in their relative abundance suggest either that (1) their
613 availability differs across study sites, and gorilla dietary choice is essentially passive and primarily
614 based on food availability, or (2) that gorilla dietary choice is strongly determined by social factors,
615 and food selection is a result of variation in culturally-transmitted feeding preference that differ across
616 populations and social groups. Higher dietary diversity of low-altitude populations supports the first
617 notion of rather opportunistic consumption of available plants. However, we also uncover distinct
618 dietary signatures of social groups from the same population, which is consistent with social factors
619 playing a role. Since gorilla groups show extensive range overlap, they would be well-suited for future
620 investigations into the role of cultural versus ecological factors affecting dietary choices by evaluating
621 if group-specific dietary patterns persist even when different social groups use the same habitat.
622
623 *The role of Grauer's gorilla gut microbiome in ecological adaptation*
624 In accordance with previous studies (Amato et al., 2019; Campbell et al., 2020; Gomez et al.,
625 2016b; Moeller et al., 2014), we detect evidence for the presence of a Grauer's gorilla core gut
626 microbiome (**Table 3**, **Figure 4**). We identified eleven taxa belonging to cellulose- and other
627 carbohydrate-degrading clades that were present in all study samples. Many of the microbial phyla,
628 families, genera that are conserved across Grauer's gorilla samples are also common in other great ape
629 gut microbiomes, including western lowland gorillas, chimpanzees, and humans (Campbell et al.,
630 2020; Fontsere et al., 2021; Gomez et al., 2015, 2016b; Hicks et al., 2018; Nishida & Ochman, 2019).
631 Despite shared taxa, the gut microbiome composition in Grauer's gorillas differed by population and
632 to a lesser extent by social group, but considerably less so than dietary composition. This could be
633 explained by the functional constraints placed on the gut microbiome, with key taxa required to
634 perform essential functions in digestion. Other taxa may be allowed to vary and co-diversify with the
635 host. Indeed, inter-species studies in primates find a strong effect of host evolutionary relationships on

636 gut microbiome structure and composition (Amato et al., 2019) and we expect to detect similar, albeit
637 less pronounced differences across isolated populations.

638 Our results indicate that dietary choice is not constrained by the gut microbiome. This is in
639 line with many studies showing that animals, including all subspecies of gorillas (Harcourt & Stewart,
640 2013), experience seasonal dietary changes, which are also accompanied by gut microbiome changes
641 (Baniel et al., 2021; Gomez et al., 2016a; Hicks et al., 2018; Orkin et al., 2019; Sharma et al., 2020).
642 Here we detect differences between isolated populations, sampled during the same season, which are
643 likely the joint result of gut microbiome-host co-diversification and plasticity of the gut microbial
644 community that may facilitate local adaptation to different environmental conditions. Inter-population
645 differences tended to derive from differential abundance of specific taxa within common bacterial
646 families, like Lachnospiraceae and Rikellenaceae, which is consistent with the presence of the core
647 microbiome in our study populations. There were, however, several differences at the family level that
648 exemplify microbiome plasticity. For example, compared to KBNP, *Treponema* (ASV322,
649 Spirochaetaceae) was significantly more abundant in NCA, where the plant taxa Marantaceae and
650 Zingiberaceae were more abundant. Hicks et al. (2018) found the same correspondence between these
651 gut microbial and dietary taxa in western lowland gorillas and suggested that it was due to the high
652 fiber content of these fallback foods, which are also important for Grauer's gorillas at low-elevation
653 (van der Hoek, Pazo, et al., 2021).

654 We detect no effects of genetic relatedness or geographic distance on gut microbiome
655 composition, despite clear group-specific microbiome patterns. Our findings thus support previous
656 studies that show the influence of sociality on gut microbiome composition in primates (chimpanzees,
657 Degnan et al., 2012; Moeller et al., 2016; baboons, Tung et al., 2015; colobus monkeys, Wikberg et
658 al., 2020; black howler monkeys, Amato et al., 2017; sifakas, Perofsky et al., 2017, 2021; Rudolph et
659 al., 2022; humans, Dill-McFarland et al., 2019) and other group-living animals (*e.g.* bighorn sheep,
660 Couch et al., 2020). Members of the same social group travel together and experience the same
661 environments over extended periods of time, which could synchronize their diet and also their
662 microbiome. The gut microbiome may in addition be directly influenced by social interactions, such as

663 grooming and coprophagy (Amato et al., 2016; Archie & Tung, 2015; Graczyk & Cranfield, 2003).
664 However, this does not mean that host genetics are unimportant, as longitudinal studies in Amboseli
665 baboons have shown that the primate gut microbiome is highly heritable, which cannot easily be
666 detected in shorter-term studies (Grieneisen et al., 2021).

667 The plasticity of the gut microbiome supports its potential role in facilitating adaptation to
668 different ecological conditions, which has important consequences for species evolution, dispersal and
669 conservation. Adaptation to changes in ecological conditions as a result of climate change, range
670 expansion, or population dispersal into novel habitats may be supported by the ability to digest diverse
671 foods. Several studies have reported habitat-biased dispersal in mammals, including in mountain
672 gorillas (Guschanski et al., 2008), where individual dispersal decisions appear to be driven by the
673 availability of familiar foods. If the microbiome was implicated in restricting dietary choice, we would
674 expect much greater conservation of dietary items across populations than what we observe here,
675 particularly as similar food plants appear to be available in different regions. This means that gut
676 microbiome flexibility may provide the necessary support for translocations of individuals or
677 populations into different habitats, which is an open question in conservation management (West et
678 al., 2019). Nevertheless, the gut microbiome may impose constraints on the diet by driving selection
679 of foods of similar nutrient content, even if they differ taxonomically. For example, giant pandas have
680 typical carnivore gut microbiomes despite being bamboo specialists because the nutritional value of
681 consumed bamboo is similar to that of meat (Nie et al., 2019). Similarly, the gut microbiome of wild
682 rhesus macaques is strongly correlated to seasonal patterns of macronutrient intake, but not food type
683 (*e.g.*, fruit, leaves, etc.) (Cui et al., 2021). Metabolic analyses of gorilla diet, as performed for different
684 social groups and seasons in other gorilla species (*e.g.,* Gomez et al., 2015; Rothman et al., 2008), will
685 enable investigating whether nutritional values are conserved in different populations.

686

687 *Understanding diet and ecology of wild animals requires a combination of approaches*

688 As every method, the metabarcoding approach to diet and microbiome faces limitations,
689 specifically in the form of marker gene selection, reference database bias, threshold decisions, and

690 interpretation of abundance. While Grauer's gorillas predominantly feed on vegetative plants, they
691 occasionally consume insects and fungi (van der Hoek, Pazo, et al., 2021; Yamagiwa et al., 1991). By
692 choosing a chloroplast gene, *trn*L, we restricted dietary characterization in this study to plants only.
693 For dietary analysis of species with omnivorous diets, expanding to multiple loci that are able to
694 characterize the diversity of consumed foods would be necessary (Taberlet et al., 2018). Further,
695 metabarcoding relies on a reference database for taxonomic identification, making it limited by the
696 content of these databases, which may be incomplete for biodiversity-rich or extreme habitats and
697 unstudied microbiomes (Hird, 2017; Taberlet et al., 2018). Similarly, chosen thresholds for sequence
698 identity and relative abundance could remove genuine dietary or microbial constituents. We used
699 conservative sequence identity and relative abundance thresholds similar to those employed in other
700 studies of diet and gut microbiome (Deagle et al., 2019; Hibert et al., 2013; Quéméré et al., 2013;
701 Srivathsan et al., 2016). However, this does not completely guard against removal of genuine taxa,
702 particularly for dietary characterization, due to the small size and high variability of the *trn*L locus.
703 Additionally, estimated abundances of the different plant and microbial taxa may not accurately reflect
704 their abundances (Deagle et al., 2019; Gloor et al., 2017). DNA copy number can be biased by plant
705 tissue type (*i.e.,* fruit, pith, leaves, the latter of which contain more chloroplasts; Egea et al., 2010), the
706 copy number of the rRNA locus, relative digestibility (*i.e.,* amount of fiber), and PCR amplification
707 success (reviewed by Deagle et al., 2019). However, other methods for dietary characterization also
708 face biases. For example, accuracy of macroscopic fecal analysis depends on the types of tissues
709 consumed and the extent of digestion (King & Schoenecker, 2019). Observational studies can
710 overestimate the dietary importance of foods with longer handling times (Matthews et al., 2020) and
711 require habituating study animals, which may make them more vulnerable to poaching and increase
712 exposure to human-transmitted diseases (Green & Gabriel, 2020). Hence, understanding ecological
713 and particularly dietary diversity of different animal species would benefit from a combination of
714 approaches. Molecular methods are particularly suited for the study of unhabituated animals, in
715 regions where tracking over a long time period is not feasible or desirable.

716 The use of shotgun metagenomics will ameliorate many of the limitations described above and
717 allow for more complete interpretation by also enabling functional characterization of gut microbial
718 communities. It would thus be feasible to test if the gut microbiome differs in functional profiles as a
719 result of dietary differences across populations, or if functions remain conserved, suggesting that
720 nutritional values of different diets are indeed similar. With the decrease in sequencing costs and
721 massive growth of whole genome reference databases that become available as a result of genome
722 sequencing initiatives (Formenti et al., 2022; Lewin et al., 2018), the use of shotgun metagenomics
723 will increase in the coming years, fueling the application of the hologenomic framework to wild
724 animal populations.
725
726 *Conclusions*
727 Our results suggest that the animal gut microbiome may contribute to adaptation to new
728 environments, while retaining a core set of potentially essential constituents. We provide evidence that
729 this microbial plasticity is associated with dietary flexibility, and as such the gut microbiome may
730 enable the host to exploit new resources, a precursor to local adaptation. If so, the microbiome may
731 indirectly encourage subsequent cultural adaptation to feeding on new dietary items. We emphasize
732 the utility of fecal sampling for minimally-invasive population monitoring of different aspects of
733 endangered species biology, from genetics to ecology and foraging behavior. Despite its limitations, a
734 molecular approach can reveal otherwise clandestine insights into the biology of elusive animals and
735 is particularly powerful when combined with traditional observational methods. Our results highlight
736 the importance of incorporating multiple axes of population differentiation into studies of endangered
737 animals, since safeguarding ecological and genetic biodiversity is the primary objective of species
738 conservation.
739

Acknowledgements

We thank L'Institut Congolais pour la Conservation de la Nature (ICCN) and local landowners and community members for permitting us to work in the Kahuzi-Biega National Park and the Nkuba Conservation Area. We are indebted to numerous rangers and field assistants in Kahuzi-Biega National Park as well as field assistants, gorilla trackers, and local community members in Nkuba Conservation Area who, through their effort and commitment, support this project and ensure the survival of the Grauer's gorillas. This research was supported by the Royal Physiographic Society of Lund Jan Löfqvist and Nilsson-Ehle Endowments to KG, the Erasmus Mundus Master Programme in Evolutionary Biology Consortium Scholarship to AM, and the Swedish Phytogeographical Society and the Extensus Foundation Grants to LP. The Dian Fossey Gorilla Fund's work in DRC was supported by individual donations and grants from the Great Ape Conservation Fund of the US Fish and Wildlife Service, the Arcus Foundation, the Daniel L. Thorne Foundation, and the Turner Foundation. Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala. The SNP&SEQ Technology Platform is part of the National Genomics Infrastructure Sweden and Science of Life Laboratory. The SNP&SEQ Platform is also supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. We also acknowledge the National Bioinformatics Infrastructure for providing computational resources to this project.

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1159 **Data Accessibility & Benefit-Sharing Statement**

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1161 Data Accessibility

1162 Sequences and associated metadata for the gut microbiome and diet generated in this project have
1163 been uploaded to the European Nucleotide Archive (ENA) under Accession no.: PRJEB49814.

1164

1165 Benefit-Sharing

1166 This research addresses a priority concern, the conservation of a critically endangered species. This
1167 was made possible by maintaining long-term collaborations with scientists in the DRC, and all
1168 collaborators are included as co-authors. Benefits of this research include the sharing of our data
1169 (above) and results with the broader scientific community as well as with conservation practitioners.

1170

1171 **Author Contributions**

1172 AM, RM and KG planned the study design. AM generated dietary data and performed all analyses.
1173 RM generated gut microbial data. PN, YL, MAG, and JS generated gorilla genotyping data. KN and
1174 LP provided reagents and expertise for dietary analyses. NI, AP, UN, EB, RNP, DC, and KG collected
1175 fecal samples and provided support in the field. DC, LP and KG provided project supervision. KG
1176 supervised the experiments and data analyses. AM and KG wrote the manuscript, with contribution
1177 from all authors. All authors reviewed and approved of the final manuscript.